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The GacA global regulator of *Vibrio fischeri* is required for normal host tissue responses that limit subsequent bacterial colonization

Cheryl A. Whistler,[1,2]* Tanya A. Koropatnick,[2] Amber Pollack,[3] Margaret J. McFall-Ngai[2,3] and Edward G. Ruby[2,3]

[1]*Department of Microbiology, University of New Hampshire, Durham, NH 03824-3587, USA.*
[2]*Pacific Biomedical Research Center, University of Hawaii, Honolulu, HI 96813, USA.*
[3]*Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706-1532, USA.*

Summary

Harmful and beneficial bacterium–host interactions induce similar host-tissue changes that lead to contrasting outcomes of association. A life-long association between *Vibrio fischeri* and the light organ of its host *Euprymna scolopes* begins when the squid collects bacteria from the surrounding seawater using mucus secreted from ciliated epithelial appendages. Following colonization, the bacterium causes changes in host tissue including cessation of mucus shedding, and apoptosis and regression of the appendages that may limit additional bacterial interactions. We evaluated whether delivery of morphogenic signals is influenced by GacA, a virulence regulator in pathogens, which also influences squid-colonization by *V. fischeri*. Low-level colonization by a GacA mutant led to regression of the ciliated appendages. However, the GacA mutant did not induce cessation of mucus shedding, nor did it trigger apoptosis in the appendages, a phenotype that normally correlates with their regression. Because apoptosis is triggered by lipopolysaccharide, we examined the GacA mutant and determined that it had an altered lipopolysaccharide profile as well as an increased sensitivity to detergents. GacA-mutant-colonized animals were highly susceptible to invasion by secondary colonizers, suggesting that the GacA mutant's inability to signal the full programme of light-organ responses permitted the prolonged recruitment of additional symbionts.

Received 19 June, 2006; revised 4 September, 2006; accepted 5 September, 2006. *For correspondence. E-mail cheryl.whistler@unh.edu; Tel. (+1) 603 862 2359; Fax (+1) 603 862 2621.

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Introduction

Early in their life cycle, animals interact with a myriad of bacterial species to which they must respond appropriately. Because of protective epithelial barriers, microbial encounters are limited in most tissues to infection or injury. In contrast, the epithelium is in constant contact with large numbers of bacteria, most of which are harmless. Secretion of mucus from specialized epithelium can entrap bacteria, which are subsequently cleared by cilia, as occurs in the respiratory airways; alternatively, it can stabilize interactions by separating the bacteria from direct contact with the underlying host tissue allowing homeostasis as is the case with the native gut microbiota (Tesfaigzi *et al*., 2000; Deplancke and Gaskins, 2001). Here, the native microbiota provides an important barrier to infection and modulates host immunity (Hooper, 2004; Ismail and Hooper, 2005). Host defences must effectively identify the rarely encountered pathogen among the background of commensals and prevent it from gaining access (Miller *et al*., 2005). Our progress towards understanding how hosts accomplish this is hindered by the complexity of most host-associated microbial communities; therefore, simpler model systems can provide important insights (Hooper, 2004).

The binary symbiosis between the bioluminescent bacterium *Vibrio fischeri* and its squid host *Euprymna scolopes* is an example of an association leading to accommodation and homeostasis. As with essentially all animals, these squid have a defensive innate immunity that presumably protects it from pathogenic infection, while at the same time allows beneficial association (Goodson *et al*., 2005). Upon hatching, juvenile squid respond to bacteria in the surrounding seawater by secreting mucus from ciliated epithelial appendages (Fig. 1A) (Nyholm *et al*., 2000). Bacteria become trapped in the mucus near pores that lead to crypt spaces of a specialized organ used for culturing the symbiont (Nyholm *et al*., 2000). *V. fischeri* is especially adept at aggregating in this mucus (Fig. 1B), and only *V. fischeri* migrates into and survives within the light organ (Nyholm *et al*., 2002).

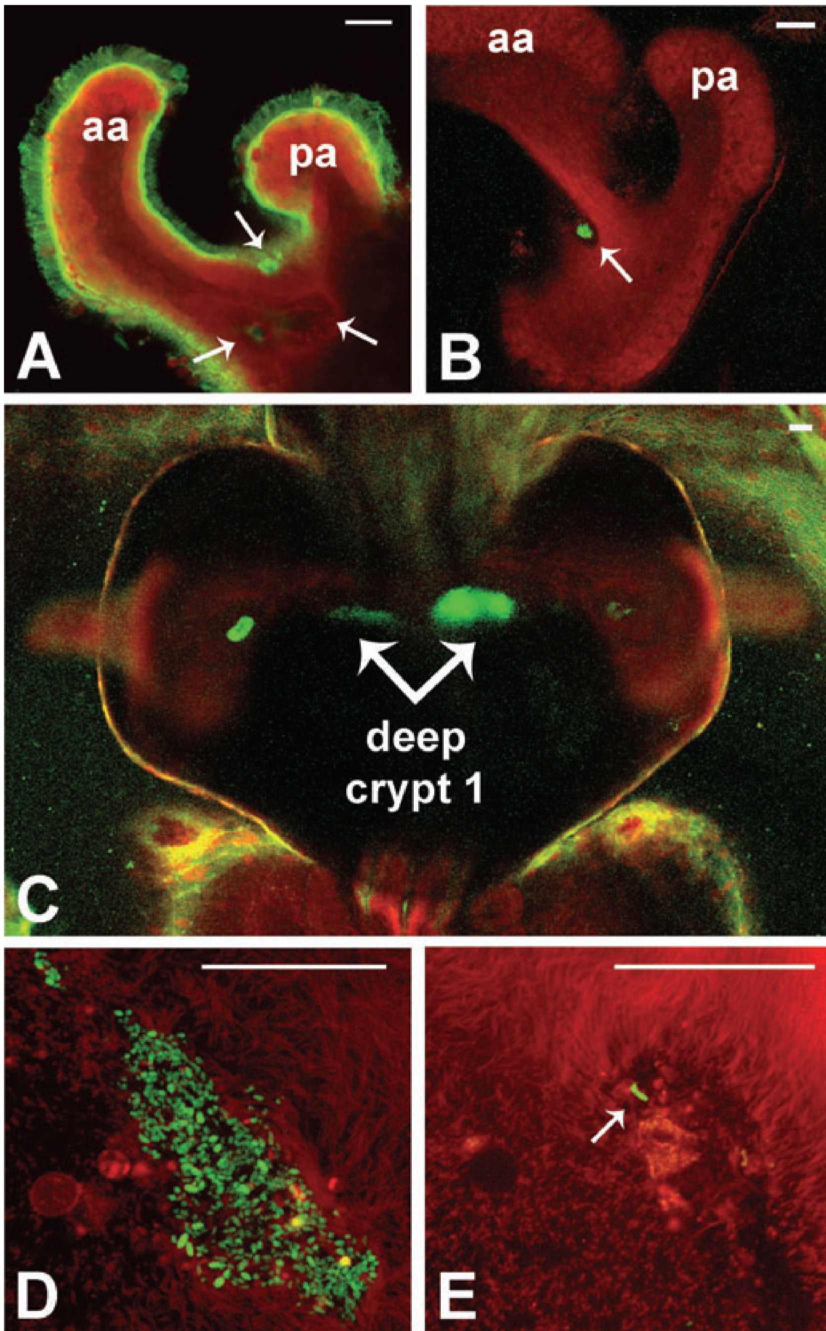

Fig. 1. Interactions of *V. fischeri* and mutant derivatives with host-mucus during colonization.
A. Under LSM, fluorescently labelled squid mucus (green) is seen to be secreted by the anterior (aa) and posterior (pa) ciliated appendages at the base of which are three pores (white arrows) that lead to the light organ.
B. A large aggregate of GFP-labelled wild-type bacteria is viewed entering the pore that leads to crypt 1 (white arrow); the other two pores leading to crypt 2 and crypt 3 do not have associated aggregates in this image.
C. An overview of a squid light organ colonized by GFP-labelled GacA mutant-bacteria shows bacteria in the deep regions of crypt 1.
D. A large wild-type-aggregate associated with unlabelled squid mucus.
E. A GacA mutant cell entering the pore that leads to crypt 1 without aggregating. Size bars are 35 μm.

Upon reaching the epithelium-lined light organ crypt spaces, *V. fischeri* grows on host-provided nutrients (Graf and Ruby, 1998), populates the light organ (Fig. 1C) and produces light. Even though the entrance to the light organ remains open to the environment for the life of the squid, only *V. fischeri* maintains a long-term albeit dynamic symbiosis: the squid vents 95% of the bacterial contents of the light organ at dawn and grows a fresh bacterial culture daily (Lee and Ruby, 1994). Within the crypt spaces, these luminescent bacteria are assured a nutrient-rich environment essentially free of competitors.

Following the early stages of recruitment and successful colonization, the bacterium induces host responses and tissue changes as part of the normal maturation of the light organ. Once they have colonized the interior of the light organ, the bacteria trigger apoptosis, or programmed cell death, in the external mucus-secreting appendages (Foster *et al.*, 2000). The bacterium also induces a cessation of mucus shedding from these appendages that

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becomes apparent 48 h after colonization (Nyholm *et al*., 2002). After even a transient, 12 h bacterial colonization, the ciliated field of the appendages deteriorates and the appendages fully regress over a 5-day period (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995). These changes are the hallmarks of successful symbiosis, as uncolonized (aposymbiotic) animals do not undergo this programmed epithelial regression (Doino and McFall-Ngai, 1995), although regression of the appendages occurs during extended incubation of juvenile squid in the absence of *V. fischeri* (Claes and Dunlap, 2000). Because mutants of *V. fischeri* that aggregate in the mucus but cannot enter the crypt spaces do not trigger apoptosis, regression, or cessation of mucus shedding (Doino and McFall-Ngai, 1995; Foster *et al*., 2000; Nyholm *et al*., 2002), perception of at least some morphogenic signals must occur within the epithelial lined ducts or crypts.

Previous studies indicate that bacterial cell wall components contribute to bacterium-induced host morphogenesis. In the absence of any bacteria, lipopolysaccharide (LPS) will trigger apoptosis in the appendages (Foster *et al*., 2000). In contrast, peptidoglycan (PGN), and more effectively the disaccharide-tetrapeptide monomer of PGN secreted by *V. fischeri* induces regression (Koropatnick *et al*., 2004). LPS and PGN synergistically induce appendage regression; therefore, together these two compounds are sufficient to induce normal morphogenesis in the absence of symbionts (Koropatnick *et al*., 2004). Although PGN induces mucus secretion (Nyholm *et al*., 2000), the bacterial signals involved in cessation of mucus shedding have not been investigated.

Although biochemical studies implicate PGN and LPS as key morphogenic factors (Foster *et al*., 2000; Koropatnick *et al*., 2004; Nyholm and McFall-Ngai, 2004), no symbiotically competent bacterial mutants characterized thus far fail to trigger apoptosis or regression. Furthermore, little is known about whether or how this signalling is regulated by the bacterium during host-colonization. Because of the similarities between this intimate symbiosis and pathogenic infections we reasoned that induction of squid morphogenesis could be regulated similarly to virulence. The GacA global activator of a number of bacteria including *V. fischeri* regulate host association and for many important pathogens they direct the delivery of virulence factors (Heeb and Haas, 2001). Previously, we learned that for *V. fischeri*, GacA mutants initiate symbiosis poorly and grow within light organs generally to between 0.5% and 20% of wild-type levels; however, they maintain a symbiotic relationship with the host, suggesting that some recognition and accommodation between the partners has occurred (Whistler and Ruby, 2003). In this study, we evaluated the ability of a GacA mutant of *V. fischeri* to induce external light organ morphogensis. Here, we demonstrate that a mutant of *V. fischeri* defective in the GacA global activator is impaired at triggering several important host responses. The defects observed in the GacA mutant were consistent with altered LPS, a conclusion that was supported by *in vitro* LPS analysis. These findings indicate that delivery of this signal may be regulated along with other colonization traits. The resulting symbiosis with the GacA mutant left the host light organ highly susceptible to subsequent invasion by bacterial competitors, suggesting that apoptosis and cessation of mucus secretion may provide an important bacterial induced barrier to subsequent colonization once the correct bacterium has established a productive symbiotic relationship.

Results

The GacA mutant does not aggregate in host-derived mucus during initiation of symbiosis

We began our evaluation of the role of GacA during the development of symbiosis by assessing whether the mutant had an altered aggregation behaviour in host mucus. Wild-type bacteria formed large aggregates in the mucus outside of the light organ pores (Fig. 1D). When added at the same concentration, the GacA mutant did not exhibit this aggregation behaviour, although individual cells entered the pores (Fig. 1E). The aggregation defect may result from the slight hyperflagellation of the GacA mutant (Whistler and Ruby, 2003); other hyperflagellated mutants are also impaired at aggregation, and additionally are delayed in colonization (Millikan and Ruby, 2002). To determine whether the presence of the wild-type strain influenced the GacA mutant's ability to aggregate, we combined equal amounts of unlabelled wild-type cells with green fluorescent protein (GFP)-labelled GacA cells. Only the wild-type aggregated when hatchlings were exposed to this mixed inoculum, suggesting that the wild-type could not complement the mutant's aggregation defect.

GacA and LysA mutants obtain similar, low populations in host light organs

A primary objective of this study was to evaluate the GacA mutant's ability to trigger host responses during development. GacA mutants have an apparent auxotrophy when cultured on minimal medium with sugars as sole carbon sources, although they utilize amino acids as growth substrates and have a similar growth rate when compared with the wild type (Whistler and Ruby, 2003). Additionally, these mutants achieve low populations within light organs as do several amino acid auxotrophs with undefined mutations (Graf and Ruby, 1998; Whistler and

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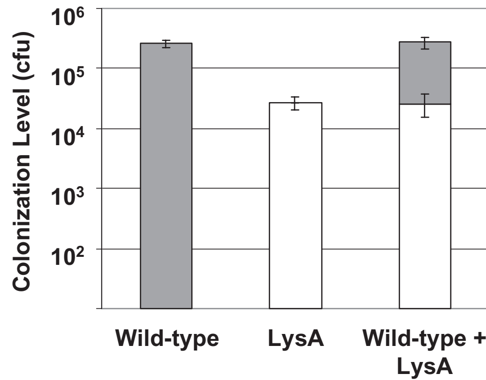

Fig. 2. Colonization of squid light organs by *V. fischeri* strains. Light organ populations of LysA bacteria (white) and wild-type bacteria (grey) were determined for squid exposed to either single or mixed (1:1) inocula. Error bars are SE, $n = 9$ animals per treatment.

Ruby, 2003). To discriminate the possible effect of having a lower than normal number of bacteria in the light organ (e.g. a reduced signal level due to fewer bacteria) from a differential effect of the GacA mutation on signal production (e.g. reduced signal level per bacterial cell) we constructed an insertion in *lysA*, which encodes the biosynthetic enzyme diaminopimelate decarboxylase (EC:4.1.1.20) (http://www.genome.ad.jp/kegg/pathway.html), creating a lysine auxotroph with a predicted colonization level of only 4% of normal due to the limitation of available lysine in the light organ (Graf and Ruby, 1998). Such a colonization level would be similar to that found for the GacA mutant (Whistler and Ruby, 2003).

The LysA mutant had no defects in colonization initiation (data not shown); however, as expected, it grew to only between 4% and 30% of the wild-type level (Fig. 2). When introduced together, wild-type and LysA bacteria co-resided in light organs, and the wild-type did not interfere with the LysA mutant's ability to achieve this same population density (Fig. 2), as has been seen for the GacA mutant (Whistler and Ruby, 2003), providing further evidence that amino acids are not the primary growth substrate in the light organ. Therefore, we reasoned that the LysA mutant could be used to assess the effect of a low-level of colonization, by itself, on host-developmental changes.

The GacA mutant fails to trigger apoptosis in the ciliated epithelial appendages

The ability of wild-type, LysA auxotroph and GacA mutant bacteria to trigger normal apoptosis following colonization was assessed. As expected from their growth-yield defect in the light organ, the crypt populations of GacA and LysA bacteria observed by confocal microscopy appeared less dense than those of wild-type. However, all three strains were indistinguishable in their spatial colonization pattern, and were similarly observed in the deepest portions of crypt 1 (Fig. 1C). Normal apoptosis occurred in the anterior and posterior appendages of both wild-type colonized animals (on average 50 ± 4 pycnotic nuclei) and LysA-colonized animals (54 ± 7 pycnotic nuclei) at 20 h post hatch. In contrast, normal levels of apoptosis did not occur in GacA-colonized animals (3 ± 2 pycnotic nuclei), and instead these animals appeared similar to aposymbiotic animals that were not exposed to bacteria (9 ± 8 pycnotic nuclei) (Fig. 3). Even 46 h after hatching, GacA mutant-colonized animals showed little apoptosis compared with those colonized by LysA auxotrophs and wild-type, and appeared very similar to aposymbiotic animals in tissue conformation and structure (data not shown).

The GacA mutant has an altered LPS profile and is more sensitive to membrane disruption

The discovery that bacterial LPS triggers apoptosis in juvenile squid (Foster *et al.*, 2000; Koropatnick *et al.*, 2004) suggested that the inability of the GacA mutant to trigger apoptosis could be due to a qualitative and/or quantitative alteration in its LPS. Indeed, it had been noted previously that the GacA mutant has a translucent

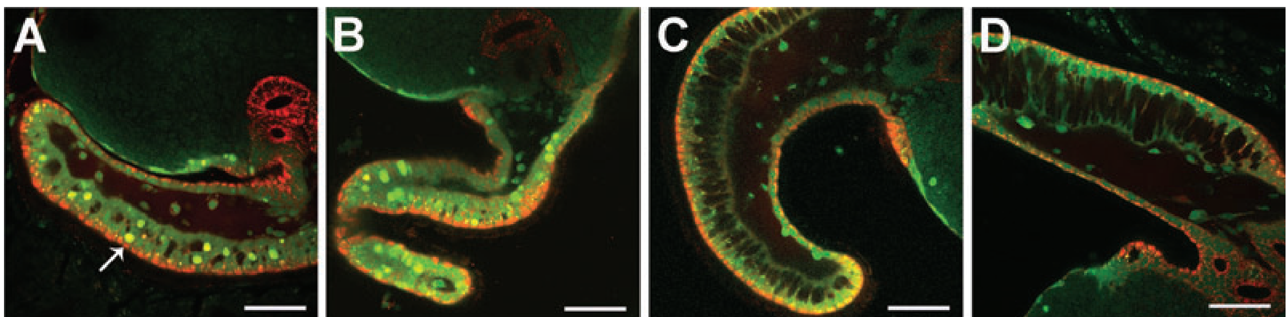

Fig. 3. Apoptosis during appendage regression. Evidence of apoptotic nuclei can be seen by LSM as the presence of bright yellow-green spots of condensed chromatin (white arrow) in squid colonized with wild-type (A), LysA mutant (B), or GacA mutant (C) bacteria, or in aposymbiotic squid not exposed to bacteria (D) at 20 h post hatching. Size bars are 35 μm.

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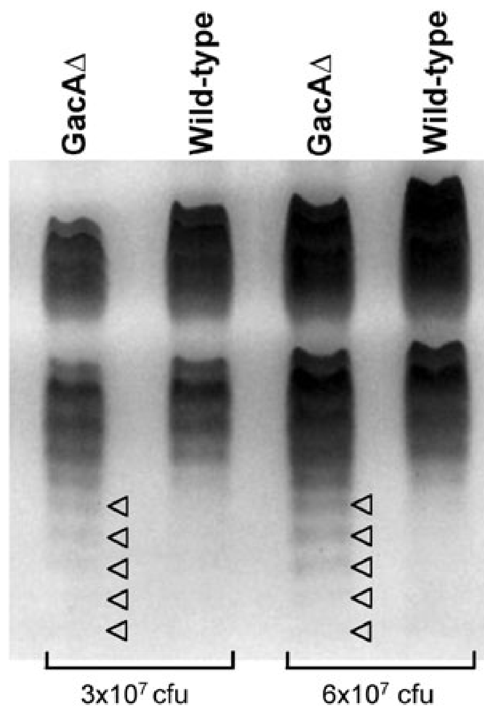

Fig. 4. DOC-PAGE analysis of LPS extracts. The same LPS extracts from exponentially growing cultures of wild-type and *gacA* mutant *V. fischeri* are loaded at two concentrations. At the lower concentration, both strains show 10 distinct LPS species, whereas the *gacA* mutant has five additional low-molecular-weight species (open triangles) that are not visible in the wild-type LPS extract.

colony morphology on medium without divalent cations when compared with wild-type (Whistler and Ruby, 2003), as has been noted in LPS mutants of other bacterial species. Analysis by gel electrophoresis revealed that, although the GacA mutant contained the 10 LPS species observed in wild type, it produced an additional five lower-molecular-weight species that were not typically visible in wild-type even when the gel was overloaded (Fig. 4). Although the cause of this difference is not known, it is consistent with an altered pattern of LPS synthesis.

Because LPS provides a protective barrier between the cell and the external environment by limiting membrane permeability (Lerouge and Vanderleyden, 1920), we tested the GacA mutant for its relative level of resistance to detergents and the cationic antimicrobial peptide polymyxin B sulfate. The GacA mutant was notably more sensitive than the wild-type to all three agents, showing 4.0 ± 0.7-fold, 3.1 ± 0.4-fold and 1.4 ± 0.2-fold increases in sensitivity to SDS, DOC and polymyxin B sulfate, respectively (Fig. 5).

Low-level colonization by the GacA mutant causes delayed regression of the ciliated appendages

We also assessed the ability of the GacA mutant to cause regression of the light organ's ciliated epithelial appendages. At 2 days post inoculation all symbiotic squid showed significantly ($P < 0.001$) more regression than aposymbiotic animals, which showed only a small amount of regression (Fig. 6). Interestingly, there was

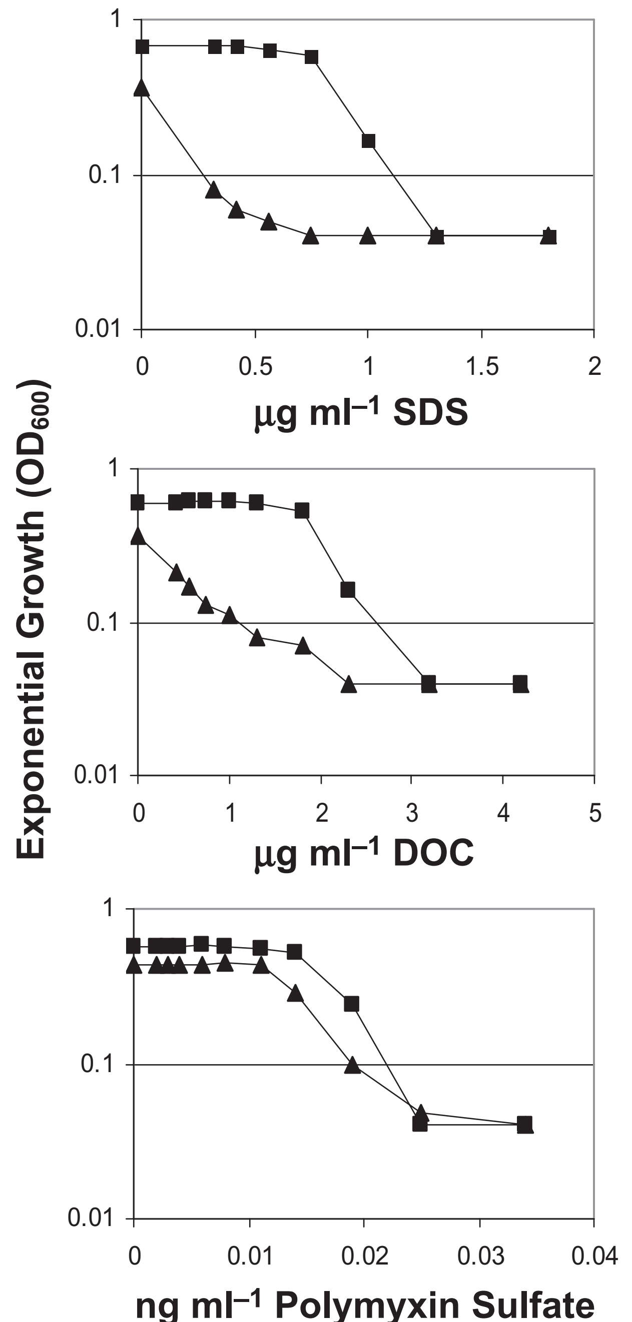

Fig. 5. Effects of detergent and polymyxin B sulfate on growth. The minimum inhibitory concentration (MIC_{50}) for each substance, defined as the amount required to reduce the exponential growth by 50% of wild-type (squares) and *gacA* mutant (triangles), was derived from each graph as follows: for SDS wild-type was 0.9 μg ml^{-1} and GacA mutant was 0.2 μg ml^{-1}; for DOC wild-type was 2.1 μg ml^{-1} and GacA mutant was 0.6 μg ml^{-1}; for polymyxin B sulfate wild-type was 22 pg ml^{-1} and GacA mutant was 15 pg ml^{-1}.

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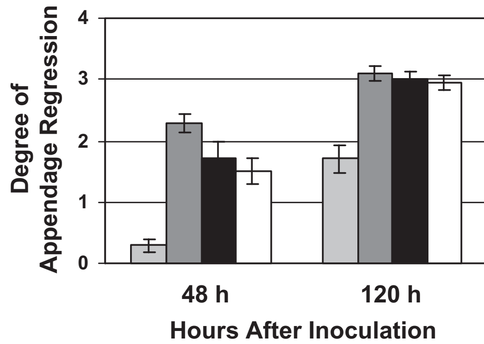

Fig. 6. Progression of appendage regression. The degree of appendage regression at 48 h ($n \geq 15$ per treatment) and 120 h ($n \geq 18$ per treatment) post inoculation in filtered artificial seawater was scored on an ordinal scale in aposymbiotic animals (light grey), wild-type colonized (dark grey), LysA mutant-colonized (black), and GacA mutant-colonized (white). The numeric values (0, 1, 2, 3 and 4) represent the amount of regression typically seen at 0, 1, 2, 3 and 4 days following colonization of squid with wild-type bacteria in natural seawater. Error bars are SE. One-way ANOVA followed by Tukey's pairwise comparisons revealed that at each time point the colonized animals showed significantly more regression than aposymbiotic animals ($P < 0.001$).

generally less regression in the squid colonized by the GacA-mutant compared with those colonized by wild-type, although the differences were not statistically significant ($P > 0.05$) (Fig. 6). However, by 5 days post colonization, appendage-regression for all symbiotic squid appeared the same (Fig. 6).

GacA-colonized animals continue to secrete mucus

Cessation of mucus secretion was also assessed for GacA and LysA mutants on the fourth day following colonization. Unlike aposymbiotic squid that continued to secrete mucus from their appendages, very little mucus was observed in wild-type and LysA-colonized squid (Fig. 7). In contrast, GacA-colonized squid appeared to have a level of mucus shedding that was intermediate to that of colonized and aposymbiotic squid, and consistently continued to secrete mucus from their regressing appendages. Interestingly, we also observed that the mucus in GacA-colonized squid continued to collect above the pores (arrow in Fig. 7) much as it does during the period of initial recruitment.

GacA-colonized squid are more susceptible to invasion by competitors

Because the light organ remains open to the environment following colonization, the absence in GacA-colonized (but not LysA-colonized) animals of certain aspects of normal development (i.e. apoptosis and cessation of mucus shedding) may lengthen the host's susceptibility to colonization by other bacterial symbionts. Because both GacA-colonized and LysA-colonized squid can attain a fully colonized light organ when these mutant bacteria co-colonize with the wild-type, nutrient levels in light organs colonized by the mutants appear to be sufficient to support additional non-mutant bacteria. However, the regression triggered by GacA may be sufficient to curtail recruitment. Thus, we asked whether colonization that does not induce subsequent apoptosis and mucus cessation will increase the likelihood of a secondary infection with wild-type symbiosis competent bacteria.

To test this hypothesis, we challenged wild-type-, LysA- and GacA-colonized squid with a secondary inoculum of an antibiotic-resistance marked but otherwise wild-type

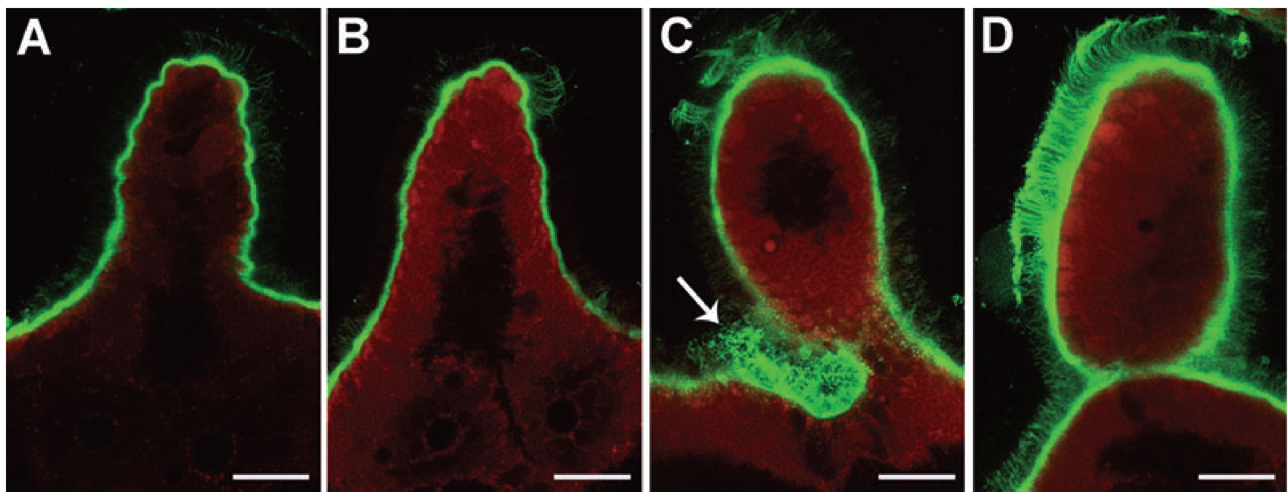

Fig. 7. Cessation of mucus secretion from ciliated appendages. Fluorescently labelled mucus (green) was visualized by LSM 96 h post hatching in squid colonized with wild-type (A), LysA mutant (B), or GacA mutant (C) bacteria, or in aposymbiotic animals not exposed to bacteria (D). Size bars are 35 μm.

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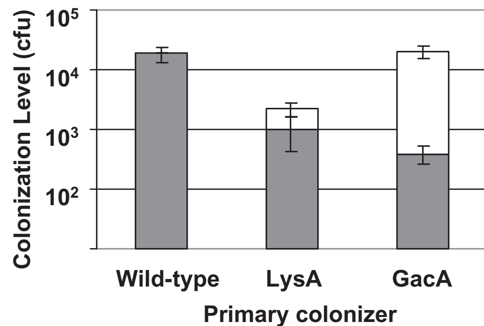

Fig. 8. Secondary invasion of light organs by *V. fischeri* strains following primary colonization. The constituents of light organ populations resulting from either the primary (grey) or secondary (white) inoculum were determined for squid primarily colonized with wild-type, LysA mutant, or GacA mutant, which were then challenged with a secondary inoculum of a chloramphenicol-resistant wild-type strain. Error bars represent SE, $n = 9$ animals per treatment. For comparison, the colonization level for wild-type (2.2×10^4), *lysA* (1.1×10^3) and *gacA* (3.9×10^2) was additionally determined from control animals that were not challenged with secondary inoculum ($n = 9$ animals per treatment).

bacterium. The timing of challenge was 60 h after hatching and primary colonization, a time that coincided with cessation of mucus shedding in animals colonized by wild-type bacteria, and the normal morning venting of the bacteria, when the light organs contain relatively low bacterium populations. We then assessed the composition of light organ populations that were present 36 h later (i.e. 96 h post hatching) to determine the degree to which a secondary inoculum colonized light organs. Wild-type-colonized squid were susceptible to invasion (Fig. 8) but the bacteria from the secondary inoculum only averaged 0.2% of the total population. This result could be due to the developmental changes that occurred or to the large population of wild-type bacteria already present, even following venting ($\cong 10^3$ cfu), or both factors. LysA-colonized squid were also susceptible to secondary colonization (Fig. 8). Although the secondary colonizers constituted on average 28% of the total population of these light organs, the total population still remained significantly lower than wild-type colonized light organs (Fig. 8), a result that is consistent with a very small founding population of secondary invaders (McCann *et al*., 2003), perhaps caused by a reduced efficiency of colonization. Finally, GacA-colonized light organs were highly susceptible to colonization and subsequent population by the secondary colonizers, as these bacteria constituted 97% of the light organ population (Fig. 8). The final population levels in GacA-colonized light organs challenged with wild-type bacteria did not differ from wild-type colonized light organs, and this level was significantly higher than in LysA-colonized squid (Fig. 8), further supporting the hypothesis that LysA-colonized squid presented a greater obstacle to subsequent colonization than GacA-colonized squid.

Discussion

Within hours after hatching, juvenile *E. scolopes* squid begin to recruit symbionts, offering nutrients in exchange for protective counter-illumination (Nyholm and McFall-Ngai, 2004). It is not known what special attributes set *V. fischeri* apart from other closely related bacteria that cannot enter into symbiosis, but phylogenetic studies indicate that coevolution has led to impressive specificity (Nishiguchi *et al*., 1998). Only bacteria that prove they are the correct partner by navigating the gauntlet of host defences (Davidson *et al*., 2004; Nyholm and McFall-Ngai, 2004), eventually reaching and growing inside the light organ, can induce host-tissue changes, some on the inside of the light organ, and most importantly for this study many on the outer surface of the light organ (Doino and McFall-Ngai, 1995; Nyholm *et al*., 2002). The external changes, including cessation of mucus shedding, and apoptosis and regression of ciliated epithelial appendages, are similar to animal tissue responses to pathogens; however, in this context the changes appear to specifically foster symbiosis by limiting access of other bacteria to the light organ once a productive association is established (Fig. 8) (Claes and Dunlap, 2000; Nyholm and McFall-Ngai, 2004). What if a defective symbiont is the first to arrive? Can the host re-negotiate the terms of association and continue to search for a better partner?

Using a GacA mutant of *V. fischeri* that is symbiotically competent but impaired in certain colonization traits (Whistler and Ruby, 2003), we establish here that the host can discriminate between different but isogenic symbiont strains and alter some of its responses accordingly, indicating the importance of both bacterial signalling and host recognition. Although it did trigger appendage regression (Fig. 6), the GacA mutant did not induce either normal apoptosis (Fig. 3) or cessation of mucus shedding (Fig. 7), demonstrating that symbiont occupation alone is not sufficient to cause all the changes associated with morphogenesis; instead, normal response requires factors under the control of GacA. Corroborating previous biochemical studies (Foster and McFall-Ngai, 1998; Koropatnick *et al*., 2004), we provide evidence that LPS (Fig. 4), a powerful inducer of innate defences during pathogenic infections of animals, is indeed a key signal compound affecting host tissue responses during the development of symbiosis. Because GacA mutant-colonized squid were more susceptible than other symbiotically colonized squid to invasion by secondary colonizers (Fig. 8), the absence of certain host responses may allow recruitment to continue even after association was established with this mutant symbiont.

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Host responses to symbiotic bacteria

Assuring association with the correct symbiont species may require bacteria-induced developmental changes be either accurate or flexible. In this symbiosis, both of these strategies appear to be used. For example, appendage regression is irreversibly triggered by the bacterium between 10 and 12 h after colonization (Doino and McFall-Ngai, 1995), suggesting that delivery of some morphogenic signal(s) above a certain threshold effectively locks the host into a programme of development. Assurance of the accuracy of the triggering appears to be dependent, in part, on the site of signal deposition: the light organ crypts (Doino and McFall-Ngai, 1995), a site that only *V. fischeri* can reach. In contrast, even after mucus shedding is curtailed, this process can be re-initiated (Nyholm *et al.*, 2000) revealing the flexibility of the host to adapt its responses to changes in other bacterial signals. The GacA mutant succeeded at delivery of the regression signal (Fig. 6), but was apparently impaired at delivering the mucus cessation (Fig. 7), and apoptosis (Fig. 3) inducing signal(s).

In the absence of bacterial symbionts, uncoupling of regression and apoptosis can be accomplished by treatment with different effectors, either LPS, which triggers apoptosis (Foster *et al.*, 2000), or TCT, which triggers regression (Koropatnick *et al.*, 2004). However, a confounding observation is that apoptosis in the ciliated fields, and these fields' subsequent regression, absolutely correlate and have previously been inseparable events during symbiotic colonization. In this study, colonization with the GacA mutant, but not the LysA mutant, uncoupled these two host responses (Figs 3, 6 and 7) in a manner similar to direct biochemical treatments with either PGN or TCT alone (Koropatnick *et al.*, 2004). The finding that the *gacA* mutation influenced synthesis of LPS (Fig. 4) supports our conclusion that the GacA mutant has a severe defect in the delivery of the LPS signal. Interestingly, apoptosis and mucus cessation remain correlated in the GacA mutant (Figs 3 and 7). Although this correlation might occur even if such events are regulated independently by GacA, the two pathways could share a common signal, such as LPS. It is also possible that apoptosis functions in part to restrain hypermucus secretion. Such a mechanism is hypothesized for the resolution of mucus cell metaplasia after LPS exposure of nasal mucosa (Tesfaigzi *et al.*, 2000). Mucus reduction and apoptosis are also associated during interaction of LPS from *Helicobacter pylori* with mucosal cells (Slomiany and Slomiany, 2003).

Apoptosis has long been associated with pathogenic infections. In mammals, apoptotic responses to LPS are tissue-specific and often highly detrimental to the host. For example, infection by *H. pylori* induces apoptosis in stomach epithelium as part of its pathogenicity (Wagner *et al.*, 1997). An even more severe apoptotic response occurs in endothelial tissue where bacterial infections can lead to sepsis and death (Bannerman and Goldblum, 2003). This response may isolate the infection, restricting its access to other tissues. In contrast, the apoptosis that occurs during the squid–*Vibrio* symbiosis is associated with normal tissue remodelling and persistent association with this bacterium. These and other studies suggest an intriguing model, whereby similar ligands of the bacterial membrane of either pathogens or beneficial bacteria are recognized by hosts and lead to different outcomes of association.

Although dramatic tissue responses occur on the exterior of the light organ, bacterium–host signalling likely occurs within the light organs where bacteria are in close contact with host epithelium (Koropatnick *et al.*, 2004). In addition to the apoptotic and mucus cessation responses on the light organ surface, there are a number responses in the tissue within the light organ including an increase in the brush border density, an increase in mucus secretion and a change in the cell morphology (Lamarcq and McFall-Ngai, 1998), which is consistent with a protective homeostatic immune response (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995; Nyholm *et al.*, 2002). The responses of squid tissue as well as the identity of the bacterial signals further suggests host innate immune receptors are likely involved in recognition and tolerance of the symbiont *V. fischeri*. *E. scolopes* is equipped with an innate immune arsenal that is highly conserved with vertebrates and includes the Toll-NF-kappaB pathway (Goodson *et al.*, 2005) and the apoptotic response appears driven by homologues of the p53 family (Goodson *et al.*, 2006). In animals, the ability of host tissue to recognize pathogens requires the Toll-like receptors (TLRs) that recognize a variety of conserved bacterial components including cell wall LPS and PGN, which subsequently initiate host innate immunity (Cario, 2005; Miller *et al.*, 2005). Recognition of a pathogen through the TLRs can trigger a pro-inflammatory response (Miller *et al.*, 2005); however, TLRs are also implicated in the recognition of and homeostasis with commensal bacteria (Neish *et al.*, 2000; Cario *et al.*, 2002; Rakoff-Nahoum *et al.*, 2004; Cario and Podolsky, 2005; Kelly and Conway, 2005; Lan *et al.*, 2005). In fact, commensal associations of the intestine are fostered by a modulated hypo-inflammatory response (Collier-Hyams *et al.*, 2005). In the case of another common bacterial ligand, flagellin, both apoptotic and pro-inflammatory (anti-apoptotic) pathways are simultaneously triggered through TLR5 and the prevailing path is determined by downstream modulation (Zeng *et al.*, 2006). Such a dual role for bacterial signalling is consistent with the observed squid host responses considering the different fates of external versus internal light organ tissues.

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Regulation of morphogenic signals by GacA

Along with the sensor kinase GacS, GacA is the response regulator in a two-component sensory transduction system that has been shown to co-ordinately regulate colonization, adaptation to host-environments, and production of host- or tissue-specific effectors [reviewed in studies by Heeb and Haas (2001) and Whistler and Ruby (2003)]. Although the influences of GacA on LPS elaboration have not been characterized for most bacterial species, GacA or GacS mutants of root-colonizing bacteria, *Pseudomonas fluorescens* F113 (Sanchez-Contreras *et al*., 2002) and *Pseudomonas* spp. (van Den Broek *et al*., 2003) as well as the pathogen, *Pseudomonas tolaasii* (Han *et al*., 1997), have translucent colony morphologies sometimes referred to as 'phase variation' (van der Woude, 2006). Morphological variation resulting from changes in LPS is a common occurrence in host-associated bacteria and can be a result of modifications to the lipid A moiety, sugar composition in the outer core, and variation in the number of O-chain repeating units as well as the sugar composition and linkages (Caroff *et al*., 2002; Trent, 2004). In addition to phase variation changes resulting from mutation, bacterial LPS modifications in response to environmental factors can also be accomplished through a number of regulatory systems, most notably PhoP-Q (Groisman, 2001; Hitchen *et al*., 2002; Miller *et al*., 2005). For many pathogens, such modifications are associated with a switch to a pathogenic lifestyle and can result in changes in host recognition or interaction (Hornef *et al*., 2002). Perhaps in the case of *V. fischeri*, GacA-regulated polysaccharide changes facilitate switching to a symbiotic lifestyle.

In the GacA mutant, analysis of the LPS extracts revealed a distinctive shift to additional lower-molecular-weight LPS-species (Fig. 4) whose exact cause is yet uncharacterized. Similarly, a phosphoglucomutase mutant of *V. fischeri*, which is proposed to have a synthesis defect due to limitation of precursors, specifically UDP-glucose (DeLoney *et al*., 2002), produced an altered LPS profile that also contained lower-molecular-weight species. Like the GacA mutant, it was more sensitive to detergents and had a symbiosis-initiation defect (DeLoney *et al*., 2002). Furthermore, a phosphoglucomutase mutant of *Stenotrophomonas maltophilia* had a modest increase in anitmicrobial susceptibility, and was impaired in an animal model of infection (McKay *et al*., 2003). If such an LPS synthesis defect also occurred in GacA mutants, it could alter the GacA mutant's ability to withstand host antimicrobial peptides or defensins during colonization (Nyholm and McFall-Ngai, 2004). Alternatively, the synthesis defect could influence the bacterium's ability to modify LPS during symbiosis, and perhaps affect proper host-recognition events. This latter possibility is particularly attractive as bacterial LPS modification can occur specifically in response to host association and is important both during pathogenic associations to evade host immune responses and during plant-symbiosis to facilitate proper interaction with the host (Lerouge and Vanderleyden, 1920; Preston and Maskell, 2002; Fraysse *et al*., 2003). Several genes involved in lipid A modification have already been identified from the *V. fischeri* genome, but those mutants' *in vitro* phenotypes differ from that of the GacA mutant (E. Stabb and D. Adin, pers. comm.). The nature of the GacA-LPS alterations remains an important part of future studies, and may provide clues into how LPS modifications lead to recognition and tolerance during animal–bacterial association.

The development of symbiosis

The developmental events we evaluated in this study all occur on the outer surface of the light organ, distal to the site of bacterium-colonization. A striking feature of these changes in organ morphology is not only that they influence symbiont-recruitment, but also that they limit the host's interaction with other bacteria in the environment [Fig. 8 and the study by Nyholm *et al*. (2002)]. Indeed, the ciliated appendages and the mucus that they produce allow the host to effectively recruit cells of *V. fischeri* (Nyholm and McFall-Ngai, 2004). However, because inappropriate and potentially harmful bacteria are also concentrated within the mantel cavity, this recruitment process may be an inherently risky event for the host. Therefore, symbiont-induced changes to the light organ that curtail active recruitment may serve both to protect the host from unwanted colonizers, and to decrease the chances that the initial symbiont population will face subsequent competitors that arrive later in the light organ. Thus, these bacterial induced changes are beneficial to both symbiont and host. Using the squid–*Vibrio* symbiosis as a model, and with the discovery of a symbiont impaired at normal host signalling, we are poised to decipher the molecular dialogue between microbe and host that leads to accommodation, long-term colonization and protection.

Experimental procedures

Bacterial strains, culture conditions and reagents

All reagents were from Sigma-Aldrich (St Louis, MO) unless otherwise noted. Wild-type *V. fischeri* ES114, isolated from an adult specimen of *E. scolopes* (Boettcher and Ruby, 1990), and its derivatives were routinely grown at 28°C in either liquid seawater-tryptone broth (SWT) containing 0.5% tryptone (Difco, Detroit, MI), 0.3% Bacto™ yeast extract (Difco), and 0.3% glycerol in 70% natural seawater (SW) (Boettcher and Ruby, 1990) with shaking at 200 r.p.m., or in LBS medium containing 1% tryptone, 0.5% yeast extract and 2% NaCl (Fisher) in a 20 mM

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Tris-HCl (Fisher) buffer (pH 7.4) (Graf *et al.*, 1994). *V. fischeri* was also grown on minimal agar plates supplemented with either ribose or lysine (Graf and Ruby, 1998), as a carbon source. *Escherichia coli* strains were routinely grown in Luria–Bertaini (LB) broth (Sambrook *et al.*, 1989). Where required, media were supplemented with antibiotics at the following concentrations: for *V. fischeri*, kanamycin (Km) at 50 μg ml^{-1}, and chloramphenicol (Ch) at 5 μg ml^{-1} for multiple copies of the resistance gene in plasmids or at 2.5 μg ml^{-1} for a single copy on the chromosome; for *E. coli*, Km at 50 μg ml^{-1}, Ch at 25 μg ml^{-1}. Plates were supplemented with 40 mg of Xgal (5-bromo-4-chloro-3-indoyl-β-galactopyranosidase) per millilitre for visualization of β-galatosidase (LacZ) activity.

Recombinant DNA techniques

Standard molecular methods were used for transformations, digestions with restriction enzymes, gel electrophoresis, Southern analysis and polymerase chain reaction (PCR) (Sambrook *et al.*, 1989). Restriction enzymes were from New England BioLabs (Beverly, MA). Gel purification of restriction-enzyme-digested DNA was performed using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Plasmids for laboratory procedures were purified using the Qiaprep Spin Miniprep Kit (Qiagen). Plasmid DNA for sequence analysis was prepared using the Perfect Prep Plasmid Mini Kit (Eppendorf Scientific, Westbury, NY). Ligations were performed by the thermal cycling method (Lund *et al.*, 1996). Genomic DNA was isolated by a cetyltrimethylammonium bromide method (Ausubel *et al.*, 1990). Digoxigenein-11-dUTP-labelled probes for Southern blotting were generated by PCR using materials and protocols supplied by the manufacturer of the Genius™ system (Boeringer Mannheim Corporation, Indianapolis, IN).

Cloning and mutagenesis of lysA

Using the genome sequence of *V. fischeri* (http://www.ergo-light.com/ERGO/) we identified a single gene encoding diaminopimelate decarboxylate (hereafter called *lysA*), the last step in the synthesis of the amino acid lysine from the substrate damiopinelate. Forward (LysAF1: AGAGCTTTACTCTCAAGGGC) and reverse (LysR1: ATCAACATCATCGACAACGG) primers were designed, generated (Integrated Technologies, Coralville, IA) and used to amplify by PCR the entire open reading frame with an additional *c.* 500 bp flanking the gene from genomic DNA isolated from *V. fischeri* ES114. The fragment was cloned using a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA) following protocols supplied by the manufacturer generating plasmid pVCW3H3 and subsequently subcloned as an EcoRI fragment into EcoRI-digested pEVS79 plasmid DNA (Stabb and Ruby, 2002) generating pVCW3C4. The identity of the insert was confirmed following sequence analysis. Plasmid pVCW3C4 was mutagenized using the EZ::TN™ <KAN-2> insertion kit following protocols supplied by the manufacturer (Epicentre, Madison, WI). Insertions in *lysA* were identified by PCR amplification and confirmed to be in the *lysA* open reading frame by sequence analysis of the clones. One random EZ::TN <KAN> insertion was identified in pVCW3D5. The mutagenized gene in pVCW3D5 was recombined with the chromosomal copy of *lysA* by marker exchange mutagenesis. Briefly, a tri-parental mating into ES114 was performed with pEVS104 as the helper as described previously (Stabb and Ruby, 2002). Following selection of a transconjugate on LBS agar with Ch, several colonies were streaked twice on LBS agar without antibiotics. Individual colonies were then screened for loss of chloramphenicol resistances (Ch^S), and maintenance of kanamycin resistance (Km^R) by replica plating. A colony, designated strain VCW3F6, hereafter called the LysA mutant, was confirmed to have the *lysA*::EZ::TN <KAN> mutation in a genomic fragment of the predicted size using Southern analysis with EZ::TN <KAN> as a probe. The LysA auxotroph did not grow on minimal medium supplemented with ribose as a sole carbon source, but the addition of lysine to the medium restored its growth.

Animal colonization

Colonization of juvenile *E. scolopes* squid with *V. fischeri* was performed as previously described (Whistler and Ruby, 2003). Bacteria used in aggregation, apoptosis, regression and mucus secretion experiments were GFP-labelled using plasmid pKV111 (Nyholm *et al.*, 2000) that requires antibiotic selection during growth in culture. Initial colonization experiments with LysA and secondary invasion experiments were performed with unlabelled bacteria grown without antibiotics. For experiments to determine apoptosis, appendage regression, mucus secretion and secondary challenge exponentially growing bacteria (OD_{600} between 0.2 and 0.4) from cultures shaking at 200 r.p.m. were suspended in filtered-sterilized seawater (FSW) at a final concentration of 2×10^4 cfu ml^{-1}. This level of inoculum ensures colonization by the initiation impaired GacA mutant (Whistler and Ruby, 2003). For comparison of lysA with the wild-type, bacteria were suspended to a final concentration of 4×10^3 cfu ml^{-1} either singly or in a 1:1 ratio. Newly hatched juvenile squid were placed collectively into bowls of inoculated FSW for overnight inocubation. The squid were then transferred into fresh FSW before placing them into individual vials containing 4 ml of FSW. Each morning, the squid were aseptically transferred to fresh vials containing 4 ml of FSW. For apoptosis and regression experiments, animals were maintained in FSW that was supplemented with Ch at 2.5 μg ml^{-1}, whereas for studies of secondary invasion and cessation of mucus secretion, the animals were maintained without antibiotic selection. GFP-labelled bacteria remained detectably fluorescent for at least 3 days even without antibiotic selection. For aggregation experiments, squid were immersed in unfiltered SW (to encourage mucus secretion) containing 5×10^5 cfu ml^{-1} *V. fischeri*, a concentration at which wild-type cells reliably form aggregates. Colonization of squid light organs was confirmed by visualizing GFP-labelled bacteria in the crypts using laser-scanning confocal microscopy (LSM) as described previously (Nyholm *et al.*, 2000). Aposymbiotic animals that were not exposed to bacterial inoculation but were treated identically to inoculated squid were also observed by LSM and plated to confirm the absence of contaminating *V. fischeri* bacteria. In each experiment, bacteria were enumerated from control animals sampling a subset of squid at 20, 48, or 120 h post inoculation, rinsing them in FSW, and then freezing the animals at −70°C, before homogenizing. The homogenates were serially diluting, and plated on LBS agar plates to determine the number of cfu of *V. fischeri* per light organ.

Secondary invasion experiments were performed using a wild-type ES114 derivative containing a Ch^R gene inserted in the genome in single copy at the Tn7att site in JRM200 (McCann

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et al., 2003), and Km^R GacA mutant containing a EZ::TN <KAN> insertion VCW2F5 (Whistler and Ruby, 2003). Following initial overnight colonization with wild-type, LysA, or GacA bacteria, the animals were maintained in FSW until 60 h after hatching, when venting was induced by removing animals from the dark into bright light. Animals were transferred to FSW containing the secondary inoculum of JRM200 at 1.2×10^3 cfu ml^{-1} for 24 h. Animals were rinsed and transferred acceptically to FSW 12 h later (96 h post hatching), frozen and subsequently plated. The identity of the light organ symbionts was assessed by plating light organ contents on LBS agar and on antibiotic-containing LBS agar selective for the three strains: 50 μg ml^{-1} Km for LysA or GacA, 2 μg ml^{-1} Ch for JRM200.

Aggregate formation

Aggregation of GFP-labelled *V. fischeri* and derivatives was performed as previously described with few modifications (Nyholm *et al*., 2002). Newly hatched juvenile squid from single clutches (sibling animals) were monitored from 1 to 4 h after inoculation with 1×10^6 cfu ml^{-1} each strain. For co-aggregation assessment, a 1:1 ratio of both strains was used, where only the *gacA* mutant was GFP-labelled and wild-type aggregates observed with light microscopy. The squid were superficially stained for 5 min in 1 μM CellTracker orange (Molecular Probes, Eugene, OR) and then anaesthetized for 2 min in 2% ethanol in FSW. The animals were carefully dissected by a lateral incision of the mantel and funnel to expose the light organ without disturbing aggregates in unlabelled mucus, which were observed by LSM. Multiple, single-clutch experiments were performed from which representative images for one experiment were selected. Co-aggregation was assessed.

Visualization of apoptosis

Pycnotic nuclei typical of apoptosis were visualized as bright spots of condensed chromatin in juveniles that were incubated for between 5 and 30 min in a solution containing acridine orange (2 μg ml^{-1}), a fluorescent dye that binds nucleic acids (Foster and McFall-Ngai, 1998; Koropatnick *et al*., 2004) and 1 μM CellTracker orange, a counterstain. After staining, the animals were rinsed twice in 2 ml of FSW and anaesthetized using 2% ethanol in FSW for 2 min. The light organ was exposed by dissection and viewed by LSM. The number of pycnotic nuclei in both the anterior and posterior appendages of one side of the bi-lobed organ was enumerated for each animal. The experiments were repeated at several time points between 12 h and 46 h post inoculation and a summary of two experiments at 20 h post inoculation is presented.

LPS preparations and analysis

Bacterial LPS from exponentially growing cultures ($OD_{600} = 0.6$) was enriched from cell lysates using a hot phenol–water extraction, followed by desalting using MicroSpin G-25 columns (Amersham). The extracts were analysed by 18% deoxycholate polyacrylamide gel electrophoresis (DOC-PAGE) as previously described (DeLoney *et al*., 2002). To enhance the separation of the notably low-complexity LPS (often called LOS for lippooligosaccharides) we used a modified bilayer stacking gel protocol (Inzana and Apicella, 1999) that included a comb gel containing 1× running buffer (DOC-TrisGlycine, pH 8), and a standard stacking gel (Tris, pH 6.8) both containing 5.3% acrylamide, followed by an 18% acrylamide separating gel (Tris, pH 8.8). The LPS was visualized by ammoniacal silver staining (Tsai and Frasch, 1982). Extractions were repeated several times during both exponential and stationary-phase growth, and the relative banding pattern differences presented were consistently observed. Only the exponentially growing gels are presented with two different concentrations of loaded extract.

Detergent and antimicrobial peptide sensitivities

Detergent and antimicrobial sensitivities were determined on LBS cultures of exponentially growing *V. fischeri* wild type and derivatives using a microtitre plate protocol previously described (Lupp *et al*., 2002). For each experiment, the results were reported as minimum inhibitory concentration (MIC_{50}) required to reduce the growth by 50% compared with no treatment. The experiments were repeated at least two times and similar relative levels of inhibition between GacA and wild-type were found. One representative experiment is presented graphically. The fold-increase in sensitivity was reported as the average of two replicate experiments ± the standard deviation.

Regression of ciliated appendages

Methods for surveillance of appendage regression were as previously described (Montgomery and McFall-Ngai, 1994; Doino and McFall-Ngai, 1995). Briefly, for each experiment, single clutches (siblings) were triple rinsed in FSW before placing in inoculum. At 48 h and 120 h post colonization juveniles were fixed in 4% formaldehyde in FSW and processed by subsequent ethanol dehydration series and a final treatment with hexamethyldisilazane. The specimens were mounted, the mantle and funnel were removed, and the degree of regression was blind scored using an ordinal scale from 0 to 5. Roughly, the scale corresponds to the amount of regression seen in a juvenile under optimal conditions from 0 to 5 days following exposure to and successful colonization by *V. fischeri*; 0 = newly hatched before or shortly after colonization; 1 = 24 h post colonization, 2 = 48 h post colonization, 3 = 72 h post colonization, 4 = 96 h post colonization, and 5 = 120 h post colonization. Experiments were analysed by one-way ANOVA followed by Tukey's pairwise comparisons (95% confidence interval).

Mucus secretion

The appearance of mucus secretion was observed as previously described (Nyholm *et al*., 2002) with few modifications. Animals were counterstained for 30 min in 1 μM CellTracker Orange in FSW and then incubated for 5 min in FSW containing (per millilitre) 10 μg of OregonGreen-labelled wheat germ agglutinin (WGA) (Molecular Probes), a lectin that specifically binds the *N*-acetylneuraminic acid present in light organ mucus. The animals were subsequently rinsed two times in FSW by careful transfer. Animals were anaesthetized in 2% ethanol in FSW, and transferred to a depression slide where their light organs were carefully exposed by lateral dissection of the mantel and funnel. The presence of mucus was visualized under LSM.

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