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NO means 'yes' in the squid-vibrio symbiosis: nitric oxide (NO) during the initial stages of a beneficial association

Seana K. Davidson,[†] Tanya A. Koropatnick, Renate Kossmehl, Laura Sycuro and Margaret J. McFall-Ngai*[‡]
Pacific Biomedical Research Center, Kewalo Marine Laboratory, 41 Ahui Street, University of Hawaii, Honolulu HI 96813, USA.

Summary

During colonization of the *Euprymna scolopes* light organ, symbiotic *Vibrio fischeri* cells aggregate in mucus secreted by a superficial ciliated host epithelium near the sites of eventual inoculation. Once aggregated, symbiont cells migrate through ducts into epithelium-lined crypts, where they form a persistent association with the host. In this study, we provide evidence that nitric oxide synthase (NOS) and its product nitric oxide (NO) are active during the colonization of host tissues by *V. fischeri.* NADPH-diaphorase staining and immunocytochemistry detected NOS, and the fluorochrome diaminofluorescein (DAF) detected its product NO in high concentrations in the epithelia of the superficial ciliated fields, ducts, and crypt antechambers. In addition, both NOS and NO were detected in vesicles within the secreted mucus where the symbionts aggregate. In the presence of NO scavengers, cells of a non-symbiotic *Vibrio* species formed unusually large aggregates outside of the light organ, but these bacteria did not colonize host tissues. In contrast, *V. fischeri* effectively colonized the crypts and irreversibly attenuated the NOS and NO signals in the ducts and crypt antechambers. These data provide evidence that NO production, a defense response of animal cells to bacterial pathogens, plays a role in the interactions between a host and its beneficial bacterial partner during the initiation of symbiotic colonization.

Received 30 March, 2004; revised 11 May, 2004; accepted 12 May, 2004. *For correspondence. E-mail mjmcfallng@wisc.edu; Tel. (+1) 608 262 5952; Fax (+1) 608 262 8418. Present address: [†]Department of Civil and Environmental Engineering, Box 352700, University of Washington, Seattle, WA 98195, USA; [‡]Department of Medical Microbiology and Immunology, University of Wisconsin, Madison, WI 53706, USA.

Introduction

Nitric oxide (NO) has been implicated in the regulation of a wide variety of processes in both plants and animals (Wendehenne *et al*., 2001). At lower concentrations, NO is involved in diverse housekeeping functions, such as neurotransmission, mucus secretion and control of certain activities of the circulatory system. At higher concentrations, NO is cytotoxic. As such, it is important in the innate immune response of both invertebrate and vertebrate animals as well as plants, acting as one component of an arsenal of reactive oxygen and nitrogen species directed against invading pathogens (Zhu *et al*., 1992; Conte and Ottaviani, 1995; Fang, 1997; Miller and Britigan, 1997; Luckhart *et al*., 1998; Bolwell, 1999; Flak and Goldman, 1999; Chan *et al*., 2001). Less is known about the role of NO in the establishment and maintenance of beneficial associations with microbes. While NO has been reported to be a critical signal molecule in the early interactions between leguminous plants and their nitrogen-fixing bacterial partners (Herouart *et al*., 2002; Ferguson and Mathesius, 2003), very few data are available on possible roles of NO in mediating beneficial animal–microbe interactions.

The binary relationship between the Hawaiian bobtail squid, *Euprymna scolopes,* and its luminous bacterial partner, *Vibrio fischeri*, provides an experimental model for the study of the associations of host animals with their bacterial symbionts (McFall-Ngai, 1999; Visick and McFall-Ngai, 2000). This symbiosis represents perhaps the most common type of animal–bacterial association (McFall-Ngai, 2002): Gram-negative bacteria, acquired postembryonically each generation, that associate extracellularly with the apical surfaces of polarized epithelial cells, as in the mammalian gut (Xu and Gordon, 2003). During *E. scolopes* embryogenesis, a nascent light organ is formed that includes the six crypt spaces that will be colonized by the symbiont (Montgomery and McFall-Ngai, 1993). The nascent light organ crypts develop into the central core of the adult light organ that houses the luminous bacteria. Upon hatching, the juvenile host must harvest *V. fischeri* cells from the mixed-species bacterial populations in the surrounding seawater. This specific recruitment of symbionts poses a series of challenges for

the host, as it must not only engage the proper symbiont, but must also promote its entry, adherence and growth within host tissues, while simultaneously avoiding infection by potential pathogens or colonization by other non-specific bacteria in the environment.

Recent studies of the initial colonization of the host light organ have revealed that these challenges are met through a complex sequence of events that result in successful inoculation by the specific symbiont within hours of the host's hatching (Fig. 1). Both the host and symbiont participate in this process, which involves a step-wise elimination of environmental bacteria that are not symbiotically competent (Nyholm *et al*., 2000, 2002; Nyholm and McFall-Ngai, 2003). Specifically, within minutes of hatching, superficial fields of epithelial cells on the light organ surface secrete mucus in response to the peptidoglycan of the environmental bacteria. Only Gram-negative cells are capable of adhering to the mucus and only *living* Gram-negative bacteria form tight aggregates of cells within the mucus matrix.

The next step in the process depends on the concentration of *V. fischeri* in the environment. At high concentrations of environmental *V. fischeri* cells characteristic of habitats with dense host populations (hundreds to thousands of cells per millilitre of seawater), the symbiont aggregates within 2–3 h and is a competitive dominant in the mucus, i.e. >90% of the cells are *V. fischeri* (Nyholm *et al*., 2000; Nyholm and McFall-Ngai, 2003). Thus, specificity of this association can be established within a mucus matrix outside of the sites of eventual colonization. The mechanism mediating *V. fischeri* dominance in the mucus is unknown; they do not outgrow or kill non-specific bacteria that adhere to the mucus (Nyholm and McFall-Ngai, 2003). Host tissues are also specifically colonized only by *V. fischeri* when it occurs at lower environmental cell numbers (tens of cells per millilitre of seawater), albeit more slowly (McCann *et al*., 2003). When *V. fischeri* is absent or in low numbers in the environment, other Gram-negative bacteria will aggregate in the mucus (Nyholm *et al*., 2000), but no other bacterial species is normally capable of completing the inoculation process by colonizing the crypts (McFall-Ngai and Ruby, 1991). Thus, other specificity determinants that are further downstream prevent colonization by non-specific bacteria when *V. fischeri* is scarce in the environment.

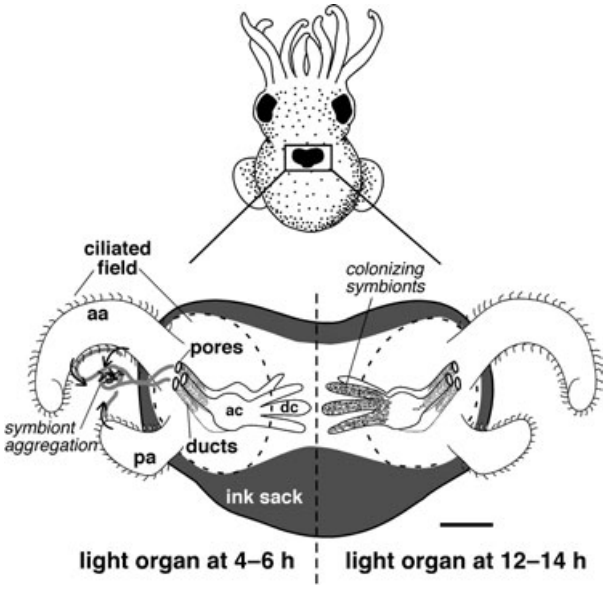

Fig. 1. The light organ of hatchling *E. scolopes* during the process of symbiont colonization. The organ (circumscribed by the rectangle, upper) is located on the ventral side of the ink sack within the mantle cavity. Beginning shortly after hatching (bottom, left), the superficial ciliated fields of cells shed mucus (curved arrows, grey lines) within which the symbionts aggregate over the first several hours. The action of the anterior (aa) and posterior (pa) appendages of the ciliated fields suspends the embedded symbiont cells above pores on the organ surface, which are the sites where the symbionts will enter host tissue. Following aggregation, the symbiont cells migrate to the pores, down the ducts, through the crypt antechambers (ac) and into the deep-crypts (dc), where they proliferate to fill the deep-crypt spaces within 12–14 h (bottom, right). In this diagram, details of crypt 1 are shown; crypts 2 and 3, which lie dorsal to crypt 1, are obscured in this view. Bar, 50 μm.

These additional protections against invasion by non-specific bacteria involve characteristics of the host tissues themselves as well as specific capabilities of the symbiont cells. The ciliary activity of the superficial field of cells suspends the symbiont-containing mucus above a series of pores on the surface of the light organ (Fig. 1). To colonize host tissues, the bacteria migrate to the pores, travel down ciliated ducts and into the blind-ended light organ crypts (Nyholm *et al*., 2000). The ducts present a biomechanical impedance. The cilia lining the ducts beat outward (McFall-Ngai and Ruby, 1998), and mutations in *V. fischeri* that affect motility render it defective in negotiating the duct and colonizing the organ (Graf *et al*., 1994; Millikan and Ruby, 2002; 2003). However, highly motile non-specific bacteria rarely enter the ducts, which suggests that these areas also present an additional, perhaps chemical, gauntlet that only *V. fischeri* can negotiate. Once through the ducts, the bacterial symbionts enter the crypt spaces. Recent confocal analyses (Sycuro *et al*., 2003) have revealed that each crypt has two distinct areas (Fig. 1), both of which are lined by polarized, non-ciliated epithelia: (i) a wider antechamber, a passageway where bacteria do not reside, and (ii) a set of narrower deep crypt diverticula, where the bacteria densely colonize throughout the life of the host.

Within 12–14 h following initial exposure to *V. fischeri*, the crypt spaces have filled with symbiont cells that induce a series of developmental events (Visick and McFall-Ngai, 2000), the most dramatic of which is the regression of the superficial ciliated epithelium that facilitates the initial inoculation of the organ with the symbiont. This process, which

occurs over the first 4–5 days following colonization, is signalled by symbionts within the crypt spaces, i.e. several cell layers away from the superficial epithelium (Doino and McFall-Ngai, 1995). In experiments using antibiotics to cure the light organ of symbionts at various time points, it was determined that the bacteria deliver an irreversible signal at 12 h following their initial exposure to the host that triggers the 4 days morphogenesis (Doino and McFall-Ngai, 1995). In addition, these fields of cells cease to shed mucus and bacterial cells no longer aggregate after about 24 h following colonization (Nyholm *et al.*, 2002). However, in contrast to the irreversible induction of epithelial field regression, the cessation of mucus shedding and bacterial aggregate formation is reversible; i.e. if the light organ is cured of its symbionts, mucus shedding from the remaining epithelial cells resumes and *V. fischeri* cells once again aggregate in this mucus (Nyholm *et al.*, 2002). This behaviour is limited to the first 4–5 days, because during this time of field regression, the number of mucus secreting cells decreases.

In this study, we investigated the role of NO production during symbiont colonization of the light organ. We sought to determine (i) whether NO and nitric oxide synthase (NOS), the enzyme that catalyses the generation of NO, are produced in the light organ and, if so, when and where; (ii) to describe any symbiosis-induced changes in NOS expression and NO production; and (iii) to investigate a possible role of NO as a specificity determinant during establishment of the association. Our findings implicate NOS and NO in the mediation of the symbiont infection of the *E. scolopes* light organ by *V. fischeri*.

Results

Evidence for the presence of NOS and NO in squid light organ tissues

NADPH-diaphorase staining. The nitro-blue tetrazolium (NBT) NADPH-diaphorase assay, in which solubilized, membrane permeable NBT is converted intracellularly to membrane-impermeable formazan crystals, was used to visualize the location of NOS in light organ tissues (Pearse, 1972; Hope and Vincent, 1989). Because the NADPH-diaphorase activity of NOSs is resistant to mild fixation, unlike most other enzymes exhibiting this type of activity, the light organ tissues were first subjected to mild fixation to detect NOS activity above the background of other enzymes reacting with NBT. Incubation of lightly fixed juvenile light organs with NBT led to the formation of dark purple formazan precipitates in the cells lining the ducts and the antechambers of the crypts (Fig. 2). This technique identified regions of NADPH-diaphorase activity, indicative of NOS activity. Positive staining occurred along each duct as it formed during embryogenesis (Fig. 2). Staining was relatively weak in the deep crypts and the superficial ciliated epithelial tissue. The ink gland, which in other cephalopods has high levels of NOS (Palumbo *et al.*, 1997; 2000), also exhibited light staining in juvenile animal light organs (data not shown). Omission of NADPH eliminated the formation of formazan crystals. Further, crystals were not formed with treatment with 10 mM dichlorophenol indophenol, which selectively inhibits NOS activity (Spessert *et al.*, 1994). Replacement of NADPH with NADP+, NADPH or NAD+ did not result in specific staining. Finally, dicoumarol, which inhibits the NADPH-diaphorase activity of DT-diaphorases and NADPH dehydrogenases, but not that of NOS (Levine *et al.*, 1960), did not inhibit the reaction. Taken together these data provide biochemical evidence that the NADPH-diaphorase activity is attributed to the presence of NOS in the host tissues.

Analyses with the uNOS antibody. By Western blot analyses the universal NOS (uNOS) antibody recognized a major band at approximately 138 kDa in both aposymbiotic (animals exposed to seawater containing non-symbiotic bacteria, but devoid of detectable *V. fischeri*) and symbiotic juvenile light organs (Fig. 3A). The estimated molecular mass was consistent with the subunit molecular masses reported for some invertebrate NOSs

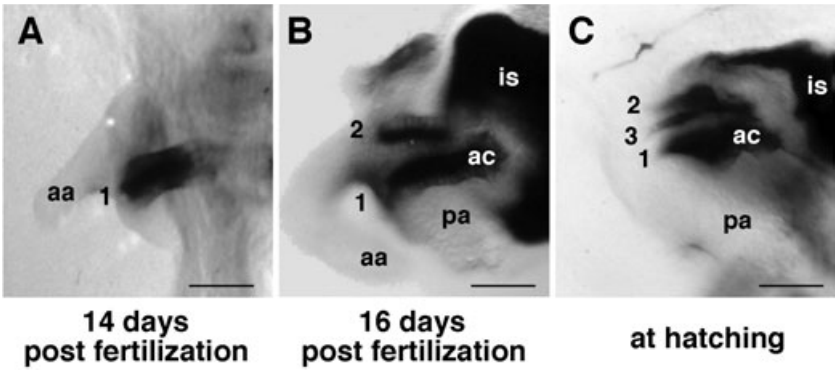

Fig. 2. NADPH-diaphorase staining in the developing light organ of *E. scolopes*. Beginning at approximately two-thirds of the way through embryogenesis the three ducts begin to form in sequence (A–C). The crypt numbers are positioned to the left of the pores that open to the ducts.
A. Embryonic stage A26/27, 14 days or ≈ 2/3 through embryogenesis.
B. Embryonic stage A28, 16 days or ≈ 3/4 through embryogenesis.
C. At hatching. Embryos were incubated at 23°C; stages are according to Arnold *et al.* (1972).
aa, anterior appendage; ac, crypt antechamber; is, ink sac; pa, posterior appendage. Bar, 50 μm.

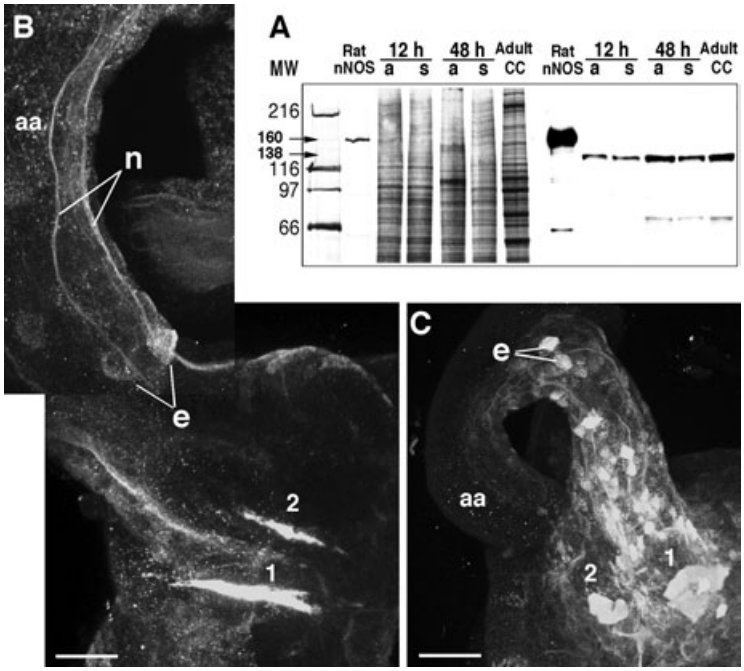

Fig. 3. The pattern of cross-reaction of a universal NOS (uNOS) antibody with the tissues of the light organ of juvenile *E. scolopes*.
A. A silver-stained SDS–PAGE gel (left portion) and Western blot (right portion) of light organ proteins (40 μg lane^{-1}). A major immune cross-reactive subunit was detected in host tissues at 138 kDa. Rat nNOS was detected at 160 kDa (1.2 μg).
B. Confocal microscopy of the light organ of juvenile revealed immune cross-reactive sites in epithelial cells and putative neurones of the anterior appendage of the ciliated epithelial fields. In this confocal section, much of the lengths of two of the three ducts (1,2) were labelled strongly. Bar, 20 μm.
C. A deeper confocal projection revealed strong labelling of the crypt antechambers of two crypts (1,2). Bar, 30 μm.
a, aposymbiotic; aa, anterior appendage; CC, central core; e, epithelial cell; n, neurone; s, symbiotic.

(Regulski and Tully, 1995; Luckhart and Rosenberg, 1999; Lee *et al*., 2000). A smaller, minor band also appeared in most samples, including the rat brain neuronal NOS (nNOS) control, which suggested that these cross-reactive species are breakdown products of the NOS subunits. By this method, no reproducible difference could be detected between aposymbiotic and symbiotic light organs at 12 and 48 h post hatching, known critical time points in bacteria-induced light organ development (Montgomery and McFall-Ngai, 1994; Lemus and McFall-Ngai, 2000). However, because tissues that are not associated with the symbiosis, such as the hindgut and ink gland, would be included in these samples, localized differences in tissues directly associated with the symbiosis might be obscured. The protein extract from the bacteria-containing central core of the adult light organ, which houses the bacteria, also had an immune cross-reactive protein at the same molecular mass as that of the juvenile light organs. This finding demonstrated that the subunit occurs in tissues of the organ complex directly interacting with bacteria and suggested that the presence of NOS in these tissues continues throughout the life of the host. Control blots probed with secondary antibody alone had no cross-reactive bands.

Whole-mount immunocytochemistry with the uNOS antibody strongly labelled sites not only in the ducts and antechambers of the crypts, but also in the superficial ciliated epithelial fields of juvenile animals (Fig. 3B and C). The duct and crypt antechamber epithelia were highly cross-reactive throughout. In contrast, similar to the NADPH-diaphorase staining, the cross-reactivity in the ciliated epithelium was limited, being most pronounced in individual cells scattered throughout the appendages and in two internal tracts (Fig. 3B and C). The latter labelling was similar in appearance to NOS-like NADPH-diaphorase staining in the nerve tracts of molluscs that exhibit NOS activity (Moroz and Gillette, 1996; Kimura *et al*., 1997). As found with the diaphorase staining, the deep crypts did not label strongly with the antibody.

Detection of NO in light organ tissues. Labelling of unfixed embryonic and hatchling light organs with analogues of diaminofluorescein (DAF) provided evidence for the developmental production of high levels of NO in the ciliated epithelial fields, ducts, and antechambers (Fig. 4A and B). The initiation of NO production was concomitant with the beginning of embryonic formation of these tissues. Because NO production continued after a mild fixation, we also labelled tissues with DAF following fixation to provide further evidence that the DAF fluorochrome was labelling NO (Fig. 4C and C′). The pattern of labelling was more localized and punctate in the ciliated fields and along the ducts than that observed in unfixed specimens, but the overall pattern was similar.

Detection of NOS- and NO-containing vesicles in host-shed mucus. As described above, a subpopulation of cells of the superficial ciliated epithelium labelled positively with uNOS antibody. Some of these labelled cells were observed to export uNOS-reactive cell contents into a region where symbionts will aggregate (Fig. 5A and B). Further, DAF-staining of light organs coincubated with rhodamine-labelled wheat-germ agglutinin (WGA), which labels the mucus (Nyholm *et al*., 2000), revealed small (1–5 μm) DAF-positive membrane-bound vesicles associated

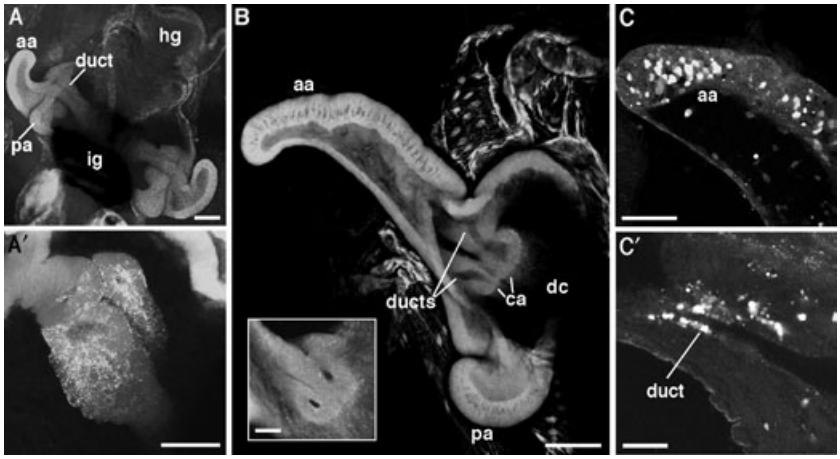

Fig. 4. Visualization of NO in the embryonic and hatchling organ by DAF-labelling and confocal microscopy.
A. A whole light organ of an embryo about half-way through embryogenesis showed DAF fluorescence (bright white) in the developing superficial ciliated epithelia, ducts, and crypt antechambers. Bar, 50 μm. A′. An image projection showing the duct/antechamber of a juvenile before hatching. Bar, 30 μm.
B. A confocal section of one-half of a hatchling light organ at the level of the superficial ciliated appendages, ducts and crypt antechambers showed intense positive signal similar to that of the embryos. In contrast, the deep crypts, which are medial to the antechambers, had no detectable DAF fluorescence. Bar, 40 μm. Inset, section at the level of the pores showing DAF signal in the epithelium surrounding the pores. Bar, 30 μm.
C, C′. Strong DAF fluorescence occurred in individual cells of specimens that had undergone mild fixation. C. The appendages of a ciliated epithelial field. Bar, 30 μm. C′. The duct epithelium. Bar, 20 μm.
aa, anterior appendage; ca, crypt antechambers; dc, deep crypt; hg, hindgut; ig, ink gland; pa, posterior appendage.

both with the epithelium of the superficial ciliated appendages and with the shed mucus (Fig. 5C and D). In hatchlings that had not been induced to shed mucus, these vesicles were rarely observed and, when detected, were associated with the small amounts of mucus that are present before shedding.

The effects of NO scavengers on early infection events. Because all of the NOS inhibitors tested (see *Experimental procedures*) were not tolerated by the animals, attempts to modify NO levels were restricted to the use of NO scavengers, rutin hydrate and N-methyl-D-glucamine dithiocarbamate (MGD). The DAF fluorescent signal in the

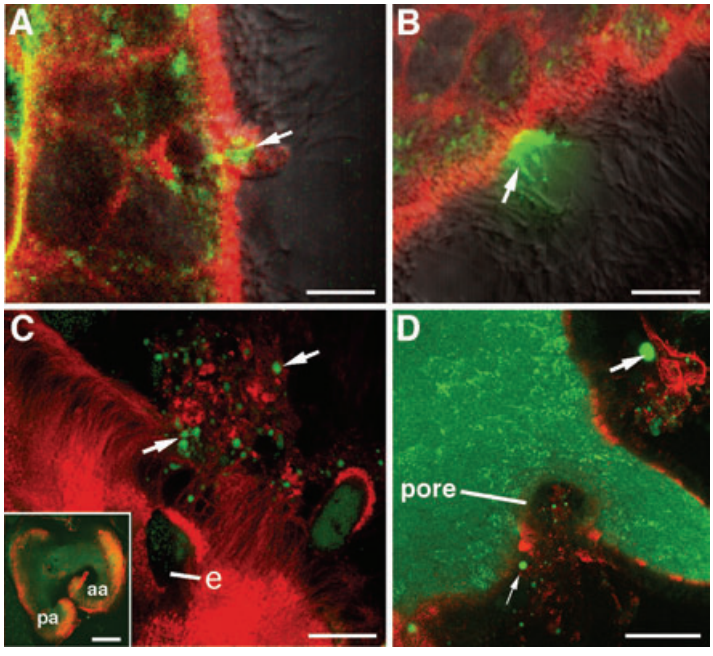

Fig. 5. Vesicle-associated NOS and NO in mucus secreted from the superficial ciliated epithelial fields.
A,B. Superimposed DIC and confocal images of NOS localization (uNOS antibody labelling, green; a counterstain of rhodamine phalloidin, labelling actin in host cytoplasm, red). A. The contents of anti-uNOS-positive cells exporting cell contents (arrow) into the extracellular space, i.e. into the lumen of the body (mantle) cavity where symbionts will aggregate. Bar, 20 μm. B. The anti-uNOS-positive cell contents at the cell surface (arrow). Bar, 10 μm.
C,D. Confocal images of NO and mucus localization (DAF-labelled NO, green; rhodamine-WGA-labelled mucus, red). C. A confocal section at the surface edge of a ciliated field reveals mucus and NO-containing vesicles (arrows) being released from localized areas along the epithelium. Bar, 10 μm. Inset, a view of the whole ciliated field on one-half of a juvenile light organ that has been induced to secrete mucus shows the thin layer of mucus (red) over the appendages and ridge of the field. Bar, 50 μm. aa, anterior appendage; e, epithelial cell; pa, posterior appendage. D. A confocal section through the level of the pore with NO-containing vesicles (arrows) associated with mucus shed from the region of the pores. Bar, 20 μm.

light organ ducts was markedly attenuated in animals exposed to 0.1 mM rutin hydrate (≈ 50% decrease in relative fluorescence); MGD was not confirmed to reduce the NO signal at non-toxic concentrations.

For these experiments, we used the light organ isolate *V. fischeri* ES114 and/or *V. parahemolyticus* KNH1, an isolate from the seawater of the host's habitat. Squid treated with either rutin hydrate or MGD during incubations with either *V. fischeri* or *V. parahaemolyticus* often had aggregates that were significantly larger (hyperaggregates, 50–200 μm in diameter) than those that formed with no scavengers present (<20 μm in diameter) (Fig. 6). Although individual GFP-labelled cells could be seen in other areas of the host's body, they did not occur in aggregations unless associated with host mucus, demonstrating that neither rutin hydrate nor MGD alone causes cells to aggregate with one another. Approximately 50% of the aggregates that formed in light organ mucus in the presence of rutin hydrate ($n = 30$) and MGD ($n = 17$) were hyperaggerates, and occasionally aggregates of *V. parahaemolyticus* covered half of the light organ surface. Hyperaggregates were never observed in any of the animals exposed to these strains in the absence of the scavengers ($n = 50$). Because of the large size of the hyperaggregates, it was not possible to enumerate the bacterial cells in each aggregate; however, the cell packing density was similar to that of normal aggregates, demonstrating that the increase in aggregate size was attributed to an increase in cell number rather than a change in cell spacing. Treatment with NO scavengers did not markedly and reproducibly increase the ability of *V. parahaemolyticus* to enter the light organ.

In previous analyses of light organ colonization, we determined that *V. fischeri* is a competitive dominant in the mucus aggregates (Nyholm and McFall-Ngai, 2003). Thus, we conducted experiments to determine whether scavenging NO might alter the ratio in the mucus of the *V. fischeri* cells to the cells of non-symbiotic environmental bacteria. GFP-labelled *V. parahemolyticus* was used in these studies. (GFP labelling of either symbiotic or non-symbiotic bacteria does not affect their ability to aggregate or their competitiveness within an aggregation; Nyholm and McFall-Ngai, 2003.) The two competing strains were added at 1:1, i.e. 1000 cells ml^{-1} each, into the seawater surrounding the host animal. After a 4 h incubation, the proportion of GFP-labelled *V. parahemolyticus* to unlabelled *V. fischeri* cells in an aggregate was determined by enumerating the GFP-labelled cells by confocal microscopy and unlabelled cells by differential interference contrast microscopy, as previously described (Nyholm and McFall-Ngai, 2003). The presence of rutin hydrate or MGD did not result in a higher proportion of the *V. parahaemolyticus* in the aggregates, suggesting that NO resistance in *V. fischeri* cannot be the sole mechanism by which it exerts dominance in the mucus aggregates.

Symbiont-induced developmental changes in NOS and NO production

The NOS signal in the superficial ciliated epithelium (Fig. 7), as well as the ducts and crypt antechambers (data not shown), of juvenile animals was markedly attenuated by 18 h following hatching, irrespective of symbiotic state. This turndown coincides with the time during which the host sheds mucus from this tissue (Nyholm *et al*., 2002). All areas showed symbiosis-induced attenuation of both the NOS (Fig. 7) and NO (Fig. 8) signals by 18 h.

To determine the timing after initiation of colonization of the NO attenuation in the superficial fields, ducts and antechambers, hatchlings were examined by DAF NO detection between 4 and 12 h after exposure to *V. fischeri*. In addition, to determine if this attenuation was reversible and, if so, when, hatchlings were cured of *V. fischeri* at time points between 6 and 12 h post inoculation. The attenuation of the NO signal in the ducts and antechambers was detected as early as 6 h, and became irreversible after the symbiosis was fully established, i.e. curing the light organ with antibiotics did not return the signal to the levels of aposymbiotic animals. However, irreversibility required that the animals be exposed to *V. fischeri* for at least 8 h before curing. Specifically, squid cured at 6 h following exposure to the symbiont retained high NO lev-

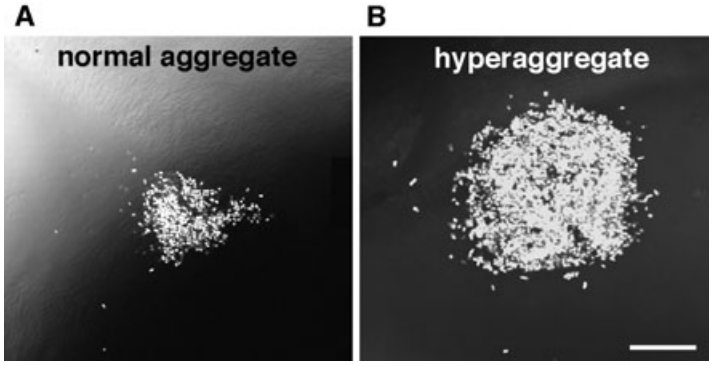

Fig. 6. Influence of NO scavengers on the occurrence of hyperaggregation of *V. parahaemolyticus* cells.
A. A normal *V. parahaemolyticus* aggregate.
B. An example of a hyperaggregate. Bar, 20 μm.

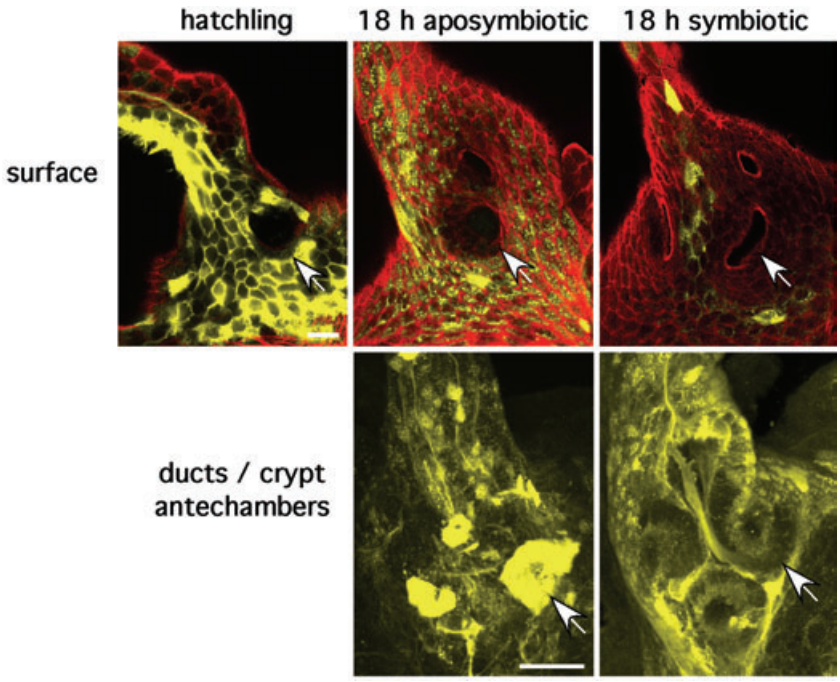

Fig. 7. Changes in NOS protein in different regions of the juvenile light organ in response to non-specific interactions with environmental bacteria (aposymbiotic) and specific interactions with *V. fischeri* colonizing the deep crypts of the light organ (symbiotic). (anti-uNOS antibody labelling, yellow; rhodamine phalloidin labelling, red). Arrows indicate pores, and the corresponding duct or crypt antechambers. Top panels show changes in surface from a hatchling to 18 h aposymbiotic and symbiotic light organ. Bottom panels are projections through the duct and antechamber. Surface bar, 20 μm; duct/antechamber bar, 30 μm.

els through at least 96 h. About 50% of the squid cured at 8 h, 70% of the squid cured at 10–11 h, and 100% of the squid cured at 12 h had attenuated NO signals through 96 h.

Studies have shown that DAF analogues are sensitive to the calcium levels within the tissues being examined and show enhanced fluorescence in the presence of calcium (Broillet *et al.*, 2001). Thus, we conducted control experiments to determine whether the changes in the NO staining that we observed were because of changes in calcium levels in host cells (data not shown). The light organ ducts and crypts had barely detectable labelling with the calcium-sensitive fluorochrome calcium green, and symbiosis did not alter the labelling pattern in these regions. In contrast, symbiosis increased the calcium green staining of the superficial ciliated fields, but the pattern of calcium

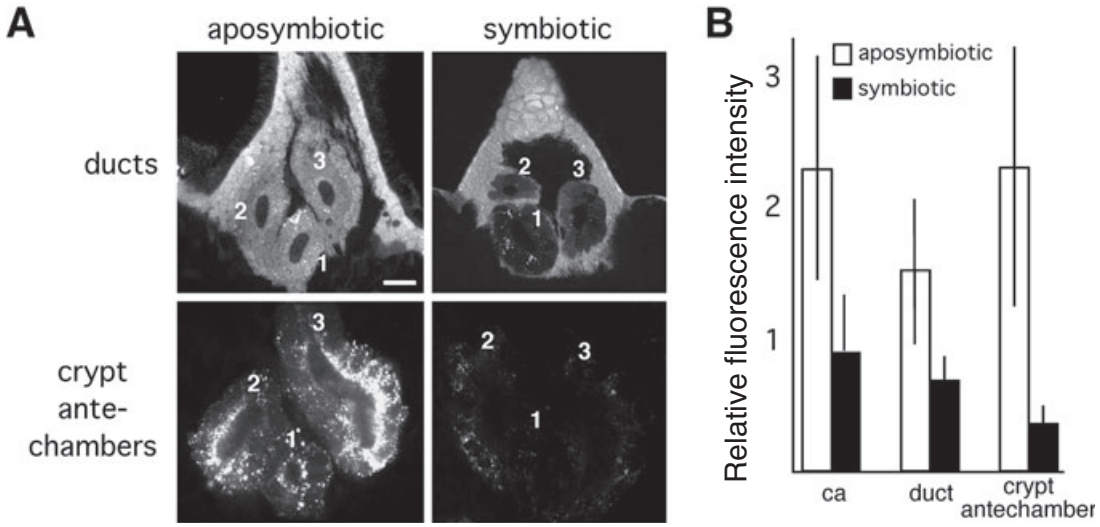

Fig. 8. Changes in NO production in the ducts and crypt antechambers of the juvenile light organ (18 h post hatching) during establishment of the symbiosis with *V. fischeri*.
A. Ducts 1, 2 and 3 of one side of the organ, lead to the antechambers 1, 2, 3. NO is indicated by fluorescence (white).
B. The graph depicts symbiosis-induced changes in DAF fluorescence ($n = 10$ for the ciliated appendages and ducts; $n = 9$ for the antechambers, i.e. all three antechambers for three animals; average ± SEM). Bar, 10 μm.

labelling in this tissue was different from the pattern of NOS and NO distribution, as suggested by antibody and DAF labelling, respectively. Thus, while changes in light organ calcium levels appear to be involved in the symbiosis, they are not correlated with the observed patterns of change in NO and NOS production. In addition, these findings provide evidence that the changes observed in the NO signal in the light organ detected by DAF labelling were not because of changes in calcium levels.

Discussion

The results of this study provide evidence that NO and the enzyme that catalyses its production, NOS: (i) first appear in the *E. scolopes* light organ during embryogenesis of this tissue; (ii) occur in light organ tissues associated with the process of symbiont inoculation; (iii) are incorporated into the mucus aggregates where the specific symbiont is harvested; and (iv) have levels that are irreversibly attenuated during the period of mucus shedding and further attenuated by colonization of the light organ crypts by *V. fischeri*. Taken together these data suggest that NO production is an integral part of a suite of mechanisms involved in orchestrating the initiation and establishment of the squid-vibrio symbiosis.

NO and NOS were detected both in the superficial ciliated fields involved in symbiont harvesting and in the ducts and crypt antechambers through which colonizing symbionts must pass during infection (Figs 2–5). This pattern of occurrence, as well as the onset of NOS synthesis during embryogenesis, suggests that NO production is involved in mediating the earliest events of the symbiosis. The highest levels of NO were detected in the light organ tissues of newly hatched animals, which have not yet begun to shed the mucus in which symbionts gather. Upon sensing the bacteria-specific molecule peptidoglycan (Nyholm *et al*., 2002), the host animal sheds copious amounts of mucus from these fields, which have vesicles containing NO and NOS (Fig. 5); the symbiosis-independent attenuation of NOS/NO is concomitant with this event. Although NO production has been implicated in modulating mucus secretion in a number of systems (Nagaki *et al*., 1995; Rose *et al*., 1997; Morschl *et al*., 2000), the authors are not aware of any reports in which NO and NOS are located in the mucus itself. However, rarely is it possible to view mucus secretion in an intact organ system with the same ease as can be done with the squid light organ.

While there is no precedence for NOS and NO in secreted host mucus, a mechanism has been reported in invertebrates for the persistent activity of NO in cellular secretions. For example, in certain blood-sucking insects, NO is associated with carrier proteins, the nitrophorins, that sequester and transport NO extracellularly into the host blood, where the insect NO is thought to promote vasodilation and inhibit platelet aggregation (Ribeiro *et al*., 1993; Valenzuela and Ribeiro, 1998). In the case of the squid system, the NOS present in the transported vesicles may remain active and/or the NO may be associated with carrier proteins similar to the nitrophorins.

Experiments involving exposure of juvenile *E. scolopes* to NO scavengers provided evidence for a defensive role for the NOS/NO-containing vesicles in the mucus. Specifically, treatments with NO scavengers resulted in the formation of significantly larger aggregates of either symbiotic or non-symbiotic bacteria, suggesting that NO in the aggregates limits the number of bacteria that accumulate. In the normal colonization process, *V. fischeri* cells aggregate in the mucus for about 2 h and then, in response to some as yet unknown cue, migrate into the ducts and eventually into the crypt spaces. This behaviour is serially repeated over the first day following hatching. In contrast when *V. fischeri* cells are absent from the environment, other bacterial species, such as the environmental isolate of *V. parahemolyticus* used in this study, associate with the mucus. These non-symbiotic bacteria might be predicted to continuously aggregate and accumulate in the mucus, because their aggregates do not migrate to the ducts. However, the aggregates remain limited in size. Thus, the presence of NO may serve to prevent the accumulation of large concentrations of non-symbiotic bacteria near sites of colonization.

Although further attenuated by the onset of symbiosis, NOS activity continued to occur in light organ tissues. In the superficial epithelium, this facility may allow the animal to respond to cycles of symbiont gain and loss that can occur over the first few days following hatching (Lee and Ruby, 1994). Specifically, whereas the regression of this epithelium is irreversibly triggered upon first full colonization with *V. fischeri*, the cessation of its mucus shedding and the associated aggregation of bacteria *are* reversible with antibiotic treatment (Nyholm *et al*., 2002). Thus, while symbiont aggregation in host-shed mucus will eventually cease because of the full regression of the superficial fields, retaining portions of the functional, NOS/NO-containing ciliated epithelium during this early period would allow the animal to continue to sample the environment for symbionts.

NOS and NO levels in the ducts and crypt antechambers were also irreversibly attenuated following symbiont colonization of the light organ crypts. The decrease in NOS/NO levels in the ducts and crypts would likely create a less oxidatively stressful environment for both host and symbiont cells. While the cells of the superficial epithelium are transient, studies of cell proliferation in the light organ tissues have shown that the crypts grow by cell divisions at their blind ends, and the ducts and antechamber cells do not rapidly proliferate (Montgomery and McFall-Ngai,

1998). Thus, the duct antechamber cells are likely to have active mechanisms for repairing possible damage caused by high levels of NO. It should be noted that, while the NOS and NO are lowered in these areas, their production is still detectable. Further, the finding of substantial quantities of NOS in the adult symbiotic tissues by Western blot analysis suggests that NOS and NO play a continued role in the symbiosis. In addition, these regions of the light organ possess a highly active halide peroxidase that contributes to an oxidatively stressful chemistry (Weis *et al.*, 1996; Small and McFall-Ngai, 1999). Because the light organ remains open to the environment through its lateral pores throughout the life of the host, mechanisms for continued vigilance against non-specific colonization are likely to be present. The continued imposition of some level of oxidative stress by host tissues, and persistent resistance by the bacterial symbionts, may provide one such mechanism by which partner fidelity is sustained.

Because widely used NOS inhibitors and NO scavengers were not well tolerated by the animal, the use of these agents was of limited value in deciphering the role of NOS activity and NO production in host tissues. For example, the period of colonization by *V. fischeri* that is required for the irreversible attenuation of NO in light organ tissues (9–12 h) was coincident with the irreversible signal for regression of the ciliated fields (Doino and McFall-Ngai, 1995). This finding suggests that a link may exist between these two developmental events. High NO levels have been implicated in the maintenance of the larval state prior to metamorphosis in certain mollusc species (Leise *et al.*, 2001). However, the resolution of whether NO is involved in host light organ development awaits the development of NOS inhibitors that can be tolerated by the host and/or the development of methods (such as RNAi) to interrupt gene expression in the host.

Future application of bacterial genetic approaches should provide a power tool for determining the extent to which the symbiont requires NO resistance to colonize successfully. Such approaches with the squid-vibrio symbiosis have already revealed that the symbiont participates actively in the colonization process. For example, *V. fischeri* with a mutation in a gene encoding a two-component sensor kinase, a bacterial protein involved in responding to the environment, are defective in initiation of the symbiosis (Visick and Skoufos, 2001). This finding suggests that responses of *V. fischeri* to the specific microenvironment presented by the host are required for early colonization competence. Genes encoding putative aerobic and anaerobic NO-inactivating systems are present in the *V. fischeri* genome (see http://www.ergo.integratedgenomics.com/Genomes/VFI/index.html) and will serve as candidates in genetic studies to determine whether they are involved in responses or resistance to NO.

The data presented here implicate nitric oxide in the initiation and maintenance of a beneficial animal–bacterial symbiosis. This finding provides another example in which both beneficial and pathogenic symbioses are controlled by the same biochemical 'language' between host and symbiont (Hentschel *et al.*, 2000). Thus, the eventual outcome of the interaction is because of, at least in part, the differential modulation of the same molecular language.

Experimental procedures

General methods

Adult *E. scolopes* were captured and adult animals and egg clutches maintained as previously described (Montgomery and McFall-Ngai, 1993; Lamarcq and McFall-Ngai, 1998). Juvenile squid used in experiments were collected within 30 min of hatching and placed in scintillation vials containing natural seawater that does not have sufficient quantities of *V. fischeri* to be infective. *V. fischeri* (ES114) cells at 10^3 ml^{-1} (unless otherwise indicated) were added to the seawater to generate colonized animals (symbiotic); control uninfected animals (aposymbiotic) were maintained in vials that contained natural seawater alone. At 12 h, the animals were placed into fresh seawater and the presence or absence of luminescence of the squid was assessed using a luminometer (Turner Designs TD-20/20, Sunnyvale, CA) to confirm the presence or absence of colonization by *V. fischeri* in symbiotic and aposymbiotic animals, respectively. All chemicals, except fluorochromes, were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) unless otherwise noted; fluorochromes were purchased from Molecular Probes, Inc. (Eugene, OR) unless otherwise noted.

The detection of NOS activity by the NADPH-diaphorase reaction

For NBT NADPH-diaphorase assays, both embryonic and juvenile animals were first anesthetized in a 1:1 solution of 0.37 M $MgCl_2$ and seawater, and the light organs were then removed and placed for 30 min in a fixative of 4% paraformaldehyde in marine PBS (mPBS – 50 mM sodium phosphate buffer with 0.45 M NaCl, pH 7.4). Initially a series of fixation and times (15, 30, 45 and 60 min) were tested to define the optimal conditions (Moroz and Gillette, 1996) that did not abolish all staining, but eliminated the background staining in other portions of the squid. After fixation, samples were rinsed 3×5 min in mPBS containing 1% Triton X-100 and then incubated in a solution containing 1 mM NBT and 1 mM βNADPH for 60 min. The samples were then washed 2×5 min in mPBS, incubated in a post-fixation solution of 4% paraformaldehyde in methanol for 1 h, and dehydrated in 100% ethanol 2×5 min. The light organs were then cleared in 100% methyl salicylate for 10 min and mounted on glass slides. In control experiments, the organs were treated as described, but in the presence of either 1 mM dicoumarol or 0.1, 10 or 100 mM 2,6-dichlorophenol-indophenol. To determine substrate and cofactor specificity, reactions were conducted with either the β-NADPH omitted or substituted with the related cofactors 1 mM βNADP+, 1 mM βNAD+, or 1 mM βNADH. Light organs were examined with a Leica MZFL III stereoscope to localize the conversion of the soluble NBT to purple formazan

crystals, which is indicative of NADPH-diaphorase activity under the above-prescribed conditions.

Western blot analysis

We used an antibody generated to a peptide of a conserved region in all known animal NOSs, anti-'universal' NOS (anti-uNOS; Affinity Bioreagents, Golden, CO) for both Western blot analyses and immunocytochemistry (see below). In Western-blot analyses, we determined the number of immune cross-reactive subunits in host light organ tissue extracts, and compared the abundance of the subunits in extracts of symbiotic and aposymbiotic juvenile organs at different times after hatching. In addition, because the juvenile symbiotic tissues of the light organ cannot be separated from associated non-symbiotic tissues, such as the ink gland and hindgut, we also assayed the symbiont-containing epithelia (central cores) of adult light organs to determine the presence or absence of proteins cross-reactive with the uNOS antibody in symbiosis-specific tissues.

To obtain extracts of light organs for electrophoresis, the organs of 50 symbiotic and aposymbiotic juvenile animals per sample were dissected from the host's mantle cavity and homogenized in glass microhomogenizers in 100 μl of 20 mM Tris-HCl buffer with 0.15 M NaCl, pH 7.9, in the presence of a mixture of protease inhibitors. The central cores of adult light organs were removed and homogenized in a similar manner. The samples were then centrifuged at 20 800× *g* in a tabletop centrifuge at 4°C for 20 min. For the blot, the supernatant was concentrated with a Microcon YM-3 (Millipore Corp., Bedford, MA), and the protein concentration was determined spectrophotometrically (Whittaker and Granum, 1980). Samples (40 μg lane^{-1} for light organs; 1.2 μg rat nNOS) were loaded onto a 7.5% polyacrylamide gel and run at 25 mA constant current. A companion gel was run simultaneously, and subsequently silver stained, to confirm equal loading of proteins and to assess levels of sample degradation.

To prepare the blot, the gel then was transferred onto nitrocellulose membrane at 15°C for 16 h at 23 mA in Tris-glycine transfer buffer. To inhibit non-specific binding, the membrane was incubated for 1 h in a blocking solution of 20 mM Tris-HCl with 0.5 M NaCl (TTBS buffer) that contained 0.05% Tween 20 and 4% milk. The membrane was then incubated for 2 h in the blocking buffer with uNOS antibody at a 1:250 dilution, washed (3 × 15 min) in TTBS buffer, and then incubated for 45 min with biotinylated goat anti-rabbit secondary antibody at a 1:3000 dilution and a 1 : 3300 dilution of horse radish peroxidase conjugated to avidin. The membrane was then washed 3 × 15 min in TTBS and immune cross-reactive sites were visualized by chemiluminescence (Renaissance Chemiluminescence kit, NEN Life Sciences Inc., Boston, MA). A control blot was processed in the same manner, with omission of the primary antibody to determine whether proteins would non-specifically bind the secondary antibody.

Whole-mount immunocytochemistry

To determine the location and abundance of sites in the light organ that are immune cross-reactive with the uNOS antibody, and to compare these findings with localization of NOS activity by the histochemical staining for NADPH diaphorase activity, we conducted whole-mount immunocytochemistry (ICC). In addition, we used this method to determine the influence of symbiosis on the abundance of NOS in light organ tissues.

For immunocytochemistry, animals were first anesthetized in 2% ethanol and fixed in 4% parformaldehyde in mPBS for 12 h at 4°C. Samples were rinsed in 2 × 5 min in mPBS, then light organs were removed and permeabolized with mPBS containing 1% Triton-X-100 for 48 h at 4°C. Non-specific binding was blocked by incubation of light organs for 12 h in mPBS containing 1% goat serum, 0.5% bovine serum albumin and 1% Triton-X-100 (blocking solution). Specimens were incubated at 4°C with anti-uNOS (Affinity Bioreagents, Inc.) at a dilution of 1:50 for 4 days. Samples were rinsed 4 × 30 min with 1% Triton-X-100 in mPBS, then placed overnight in blocking solution, followed by incubation at 4°C in the dark with a 1:50 dilution of TRITC-conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) in 1% Triton-X-100 in mPBS for 12 h. Samples were then rinsed 3 × 30 min in 1% Triton-X-100 in mPBS, followed by two rinses in mPBS. Light organs were mounted on glass slides with 90% glycerol and viewed by confocal microscopy.

The detection of NO in light organ tissues

Either 4,5-diaminofluorescein diacetate (DAF-2DA) (Kojima *et al*., 1998; 1999) or 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM DA) (Itoh *et al*., 2000) was used to detect NO production in the light organ directly. These fluorochromes fluoresce in the presence of NO, although DAF-FM DA, which became available during this study, is more sensitive detector of NO; selected early experiments with DAF-2DA were repeated with DAF-FM to confirm that the squid tissues behaviour similarly in response to labelling with these two fluorochromes. No qualitative differences were detected, although DAF-FM DA was about 2× more sensitive with squid tissues. Thus, for simplicity, we will refer to all experiments with these fluorochromes as 'DAF'. Squid embryos (following removal from the egg) and juveniles (aposymbiotic and symbiotic) were incubated in seawater containing 2.5 or 5.0 μM DAF for 15 min to 1 h, depending on the experiment. After incubations, squid were placed in fresh seawater where the residual fluorochrome was rinsed from the tissues by the ventilatory movements of the animals. The animals were then anesthetized in seawater containing 2% ethanol, dissected to expose the ventral surface of the light organ, and viewed with a Zeiss 510 laser scanning confocal microscope (LSM). In experiments for the detection of NO in fixed tissues (Sugimoto *et al*., 2000), hatchlings were fixed as described (see above, *The detection of NOS activity by the NADPH-diaphorase reaction*), rinsed with mPBS, stained with DAF, and viewed by LSM. Physiological analyses of *V. fischeri* have shown that it neither produces NO nor uses it as a terminal electron acceptor (E.G. Ruby, pers. comm.).

To determine whether NO was present in the mucus secreted by the ciliated fields, squid were incubated with DAF (2.5 μM for 20 min). At 5 min into this incubation period, 3.5 μg ml^{-1} rhodamine-labelled WGA was added. WGA is a fluorochrome that binds to the sialated residues in the mucus of the ciliated fields (Nyholm *et al*., 2000). The squid were then anesthetized, dissected and viewed by LSM. To compare the NO signal in mucus to the signal detected in the absence of mucus, newly hatched squid were immediately placed into filter-sterilized seawater to prevent induction of mucus shedding, which occurs in the presence of bacterial cells in seawater. A subset of these

specimens was then induced to produce mucus by adding bacteria to the seawater. The NO signal detected by DAF was compared between squid that had been induced and squid that had not been induced to shed mucus.

For control experiments to determine how patterns of DAF fluorescence were influenced by possible symbiosis-induced changes in calcium, aposymbiotic and symbiotic squid were infected for 24 h and then stained with 8 μM calcium green for 1 h and examined by LSM and the calcium staining patterns were compared with those obtained with DAF.

Determining reversibility of symbiont-induced changes in NO production

To determine whether observed symbiosis-induced developmental changes in NO production are reversible, newly hatched animals were first exposed to 10^3 *V. fischeri* cells ml^{-1} in seawater. Cohorts of this group of animals ($n = 10$ for each treatment) were then placed into 0.2 μM filtered seawater containing 10–15 μg ml^{-1} chloramphenicol at either 6, 8, 12, or 48 h post inoculation to clear the symbiont population from the light organ (Doino and McFall-Ngai, 1995). The aposymbiotic control animals ($n = 10$) were maintained in seawater with chloramphenicol throughout the experiment. The luminescence was monitored and the light organs of a subset of animals were plated to confirm clearance of the symbionts. At 60 h or 96 h post hatching all squid samples were incubated for 1 h with 5 μM DAF and examined by LSM.

Studies with NOS inhibitors and NO scavengers

We performed experiments to determine whether NOS inhibitors or NO scavengers would affect specificity of the colonization. First, preliminary experiments were performed to evaluate the use of various NOS inhibitors and NO scavengers in the squid-vibrio system. In these preliminary experiments, the NO scavengers (Calbiochem, San Diego, CA), rutin hydrate and N-methyl-D-glucamine dithiocarbamate (MGD) were tolerated by the animal, i.e. the animals remained healthy at concentration ranges (0.1–1 mM) within which these pharmacological agents are effective in other systems. Unfortunately, several widely used NOS inhibitors (Calbiochem), including N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NNA) were not tolerated by the animal. Rutin hydrate and MGD were then evaluated in preliminary experiments for their ability to affect the DAF signal produced by NO in the light organ ducts. Newly hatched, aposymbiotic animals were exposed for 1 h to the scavengers and then labelled with 2.5 μM DAF-FM for 15 min and examined by LSM. To determine effects rutin hydrate and MGD on normal colonization by *V. fischeri*, we inoculated the animals as described above in *General methods*, but included the inhibitor/scavengers at concentrations of 0.1–1 mM in the surrounding seawater, and monitored the timing and levels of colonization.

We characterized the effects of rutin hydrate and MGD on the ability of *V. parahaemolyticus* (KNH1, carrying the GFP-expressing plasmid pKV111), a non-symbiotic relative of *V. fischeri* that also occurs in the host habitat of the host, to form aggregates and enter host tissues, alone or in competition with *V. fischeri*. The carriage of the plasmid pKV111 has been determined not to influence either aggregation of Gram-negative bacteria or colonization by *V. fischeri* (Nyholm *et al*., 2003). To evaluate aggregation and entrance of non-specific bacteria into the light organ, newly hatched animals were incubated with or without 0.1 mM rutin hydrate or MGD in the presence of *V. parahaemolyticus* alone at a concentration of 2000 cells ml^{-1} and viewed at 12 h. To evaluate competition of the two species, *V. parahaemolyticus* (GFP-labelled) and *V. fischeri* (unlabelled) were added together to a final concentration of 2000 cells ml^{-1} in the presence of 1 mM MGD and viewed at 4 h. In both experiments, to view the squid, they were anesthetized, dissected and the light organs were visualized by confocal microscopy. The size of the aggregated bacteria was evaluated by epifluorescence (LSM) to visualize the GFP-labelled *V. parahemolyticus* and differential interference contrast microscopy to visualize the unlabelled *V. fischeri*.

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