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Host-derived amino acids support the proliferation of symbiotic bacteria

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ABSTRACT **Animals are typically colonized by diverse bacterial symbionts, many of which are commensal and, in numerous cases, even essential for their host's proper development and growth. In exchange, the host must supply a sufficient array and quantity of nutrients to support the proliferation and persistence of its microbial community. In this investigation, we have examined such a nutritional environment by determining the symbiotic competence of auxotrophic mutants of the bioluminescent bacterium *Vibrio fischeri*, and have demonstrated that the host squid *Euprymna scolopes* provides at least 9 aa to the growing culture of symbiotic *V. fischeri* present in its light-emitting organ. We also collected and analyzed the extracellular fluid from this organ, in which the symbionts reside, and confirmed that it contained significant amounts of amino acids. The combined results suggested that host-derived free amino acids, as well as peptides or proteins, are a source of the amino acids that support the growth of the symbionts. This work describes a technique to sample the symbionts and their surrounding environment without contamination by host tissue components and, in combination with molecular genetic studies, allows the characterization of the nutritional conditions that support a cooperative animal–bacterial symbiosis.**

Animals and plants typically exist in association with specific microbial biota (1–3). Occasionally the association is with a pathogen and leads to a diseased condition, but far more frequently the host and its microbes coexist over long periods of time in a state of cooperation that may be obligate to one or both of the partners (2–4). Not surprisingly, a fundamental property of these relationships is the nature and direction of organic nutrient exchange between the two. In many enteric associations (like those in the rumen of cows, or in the termite gut) the animal partner provides a source of refractory organic compounds that its symbionts refine and make useful to both themselves and their host (5, 6), whereas in pathogenic associations the flow of nutrients is believed to be unilateral from the host to the microbial parasite.

Analyses of the nutritional environment of the ubiquitous associations of animals with commensal and cooperative bacterial symbionts have proven very difficult because of the small scale over which the interaction takes place and the complexity of the host tissue's chemical composition (1); similarly, the identities of only a few host-derived compounds that support the growth of animal pathogens have been reported (e.g., refs. 7 and 8). To understand the nature, dynamics, and regulation of nutrient exchange between hosts and their associated microbiota, a simple, experimentally manipulatable model association is needed.

Dozens of species of luminous animals maintain monospecific associations with bioluminescent bacteria that are housed in specialized light-emitting organs (9). The best studied of these symbioses is that between the sepiolid squid *Euprymna scolopes* and the bacterium *Vibrio fischeri* (4, 10, 11). The nascent light organ of a newly hatched *E. scolopes* juvenile is free of bacterial symbionts, and thus each generation of host must be colonized by bacteria from the surrounding seawater. Typically, a few *V. fischeri* cells enter pores on the surface of the light organ and travel down narrow channels leading to epithelium-lined crypts located in the center of the organ (ref. 12, Fig. 1*A*). Within 12 h, the symbionts proliferate rapidly to a population of about 10^6 light-emitting bacteria (13). The animal is believed to use the symbiont-generated luminescence only during its nocturnal feeding behavior (14), and each morning more than 90% of the symbionts are vented from the crypts into the surrounding seawater (15). Experimental manipulation of the ambient photic cycle of the squid has revealed that this expulsion is triggered by the onset of illumination, suggesting that the venting behavior could be artificially induced.

Because each day the unexpelled cells must repopulate the light organ as nightfall approaches (15, 16), the host must provide its symbionts with sufficient nutrients to support both their regrowth and their subsequent bioluminescence activity (16). The identity of the nutrients supplied to light organ symbionts has been the subject of speculation for many years, and two different organic compounds have previously been proposed to support *V. fischeri* in symbiotic associations. Physiological experiments have suggested that glucose could serve as the primary nutrient in some light organs (17). Alternatively, the discovery that *V. fischeri* cells have the unusual ability to utilize cAMP as a sole carbon, nitrogen, and phosphate source has led to the hypothesis that the specificity of their symbiotic associations could be due to cAMP being the only host-derived nutrient released into the crypts of the light organ (18). Both of these theories, which postulate that the bacteria within the crypts have access to only one major nutrient, have remained untested. The recent development of molecular genetic approaches to the study of the symbiotic interaction between *V. fischeri* and *E. scolopes* (10, 19, 20), together with the newly discovered ability (described herein) to sample the contents of the light organ without damaging the surrounding tissue, have allowed an experimental examination of the complexity of the nutritional microenvironment of this association and provide a useful model for the study of other symbioses.

MATERIALS AND METHODS

Isolation of Auxotrophic Mutants of *V. fischeri*. Transposon mutants of the prototrophic *V. fischeri* strain ESR1 (19) were

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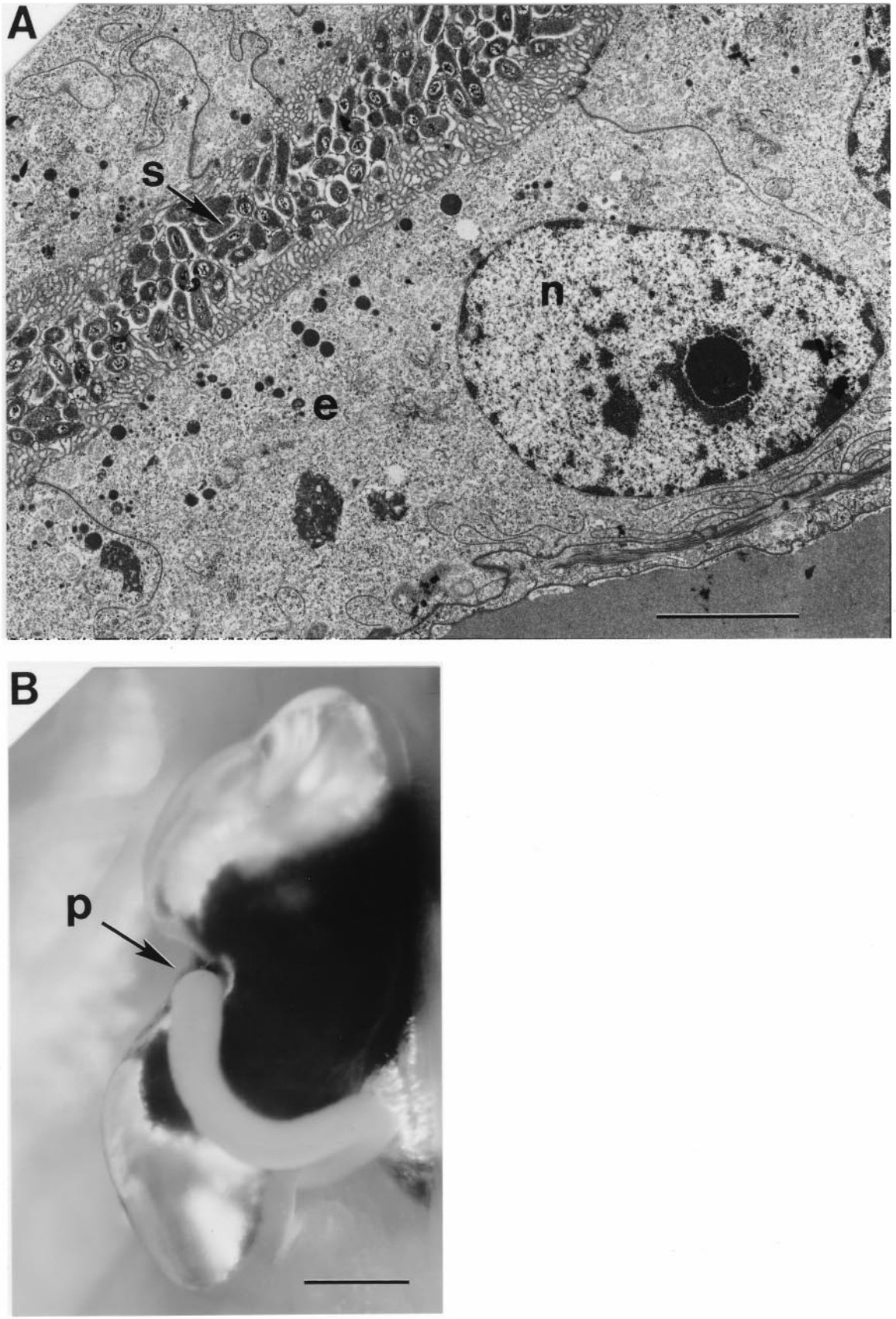

FIG. 1. The light organ environment of symbiotic *V. fischeri* cells. (*A*) Thin-section transmission electron micrograph showing symbionts (s) colonizing a portion of the light organ crypts of an adult *E. scolopes*. The crypts are bounded by microvillus epithelial cells (e), which are believed to supply nutrients to the symbiont population (12). A host cell nucleus (n) can also be seen. (Bar = 5 μm.) In an adult animal, all of these crypts join at and exit through two lateral pores, one on each of the lobes (12). (*B*) One lobe of the light organ of an adult *E. scolopes* squid viewed during the process of venting. The crypt contents can be seen exiting the pore (p) of the light organ, appearing as a whitish cylindrical stream. (Bar = 1 mm.)

obtained by conjugative transposon mutagenesis with *Escherichia coli* strain S17–1 λpir (pUT mini-Tn5 Cm) (21), and selection for transconjugants was performed on a Luria broth and NaCl (LBS) agar medium (22) containing (per liter) 100 mg rifampicin, and 5 mg chloramphenicol. This procedure has been shown to cause single transposon insertions that are stable in *V. fischeri* (23). Amino acid auxotrophs were isolated from this pool of transposon mutants by using a streptozotocin enrichment method (24). Strains that could be complemented by the addition of a single amino acid were identified (25), and nine mutants that were auxotrophic for different amino acids were analyzed further. The growth rate and luminescence of these mutants were indistinguishable from that of strain ESR1 in SWT broth, a complex tryptone-based seawater medium (13); however, each mutant was unable to grow in the minimal-salts medium MSR, a derivative of a Hepes-buffered minimal salts (HM) medium (9), with 50 mM Tris·HCl (pH 7.5) as the buffer and 24 mM ribose replacing glycerol as the sole carbon source. Physiological complementation of the auxotrophic defect was performed on MSR agar medium on which a dense suspension of cells was spread. Crystals of potentially complementing metabolites were then placed on the surface of the agar, where they slowly dissolved. Subsequent diffusion of the metabolites created a concentration gradient across the plate, allowing the auxotrophs to proliferate where the appropriate concentration of an auxotrophy-complementing nutrient was found. Because high concentrations of some metabolites can be toxic, control plates inoculated with strain ESR1 were observed for evidence of zones of growth inhibition.

The likely genotype of each of the auxotrophs (Table 1) was inferred from its ability to grow in the presence of a particular amino acid or its metabolic intermediates, and by assuming that *V. fischeri* uses the same amino acid biosynthetic pathways as those described in *Escherichia coli* or *Salmonella typhimurium* (25, 26). For instance, mutant strain G1 (Table 1) was unable to grow in minimal medium supplemented with either glutamate, ornithine, or citrulline, but did grow with arginosuccinate or arginine; thus, we concluded that the lesion was probably in the arginosuccinate synthetase (ArgG) homolog of *V. fischeri*, and designated the strain to carry the allele *argG1* (27). When physiological complementation could not separate all of the enzymes in a biosynthetic pathway, only the three-letter code of the operon was given. Confirmation of each of these predicted genotypes must await experimental evidence that prototrophy is recovered after genetic complementation with the expected gene or its homolog.

Colonization Competence of *V. fischeri* Auxotrophs. The ability of the amino acid-requiring strains of *V. fischeri* to initiate a colonization, achieve the typical number of symbiont cells, and persist in the light organ of juvenile *E. scolopes* was examined as previously described (13). Briefly, newly hatched juveniles were each placed in 5 ml of seawater containing 10^4 cells of either the parent (ESR1) or one of the nine auxotrophic strains. After 3 h the animals were moved to fresh seawater containing no *V. fischeri* cells and the development of symbiosis was monitored photometrically (13). In the evenings, when the symbiont population reaches its maximum number (15, 16), the extent of colonization was assessed by homogenizing the light organs, spreading dilutions of the homogenates on SWT agar medium, and determining the numbers of colonies arising after overnight growth (13).

Collection of Light Organ Crypt Contents. We used the normal light-induced venting behavior of *E. scolopes* (15, 16) to facilitate collection of the crypt contents as follows. Squids in the size range between 20 and 25 mm in mantle length were anesthetized in seawater containing 2% ethanol, and their light organs were accessed by a ventral dissection under dim to dark conditions. A bright light source was then aimed at their eyes, and within minutes the crypt contents of both sides of the organ were rapidly expelled by muscular contraction (Fig. 1*B*), and 5 to 10 μl was collected with a capillary tube and immediately cooled on ice. The symbiont cells were quickly separated from the surrounding matrix fluid by centrifugation at 14,000 × *g* for 15 min at 4°C. The two fractions, symbionts and matrix fluid, were used for subsequent biochemical analyses.

Table 1. Symbiotic colonization effectiveness of auxotrophic *V. fischeri* strains

Strain	Auxotrophy	Inferred genotype*	Relative colonization effectiveness†, %
ESR1	None	Wild type	100
G1	Arginine	*argG1*	52
G2	Cysteine	*cys1*	5
G3	Glycine	*glyA1*	120
G4	Leucine	*leu1*	22
G5	Lysine	*lysA1*	4
G6	Methionine	*met1*	23
G7	Proline	*pro1*	36
G8	Serine	*ser1*	5
G9	Threonine	*thr1*	5

*The genotype is used to indicate the possible identity of the missing enzyme activity in the biosynthetic pathway for each amino acid. These predictions are based on the identity of the most proximal metabolic intermediate that is able to complement the auxotrophic defect (25). (See *Materials and Methods*.)

†Each value is the mean of determinations made on between four and eight juvenile animals 45 h after inoculation; all values were significant to within ±8% or better. The wild-type level (100%) was 8×10^5 *V. fischeri* cells per light organ.

Biochemical Analysis of the Matrix Fluid and Symbionts. We determined the amino acid composition, both monomeric and multimeric, of the crypt matrix fluid and the symbiont cells obtained from the released crypt contents. Because our analyses did not differentiate between oligopeptides and polypeptides (i.e., proteins), we have used the generic term "peptides" to include both of these multimeric forms. Monomeric or "free" amino acids present in either the fluid or the lysed bacterial cell fractions were extracted with ethanol, derivatized with phenolthiocarbamyl (28), and the concentration of each monomer was determined by HPLC analysis. The total amount of amino acids (free + peptide) present in these same two fractions was obtained by complete acid hydrolysis, followed by ethanol extraction and derivitization before HPLC analysis. All samples were run in triplicate with norleucine as the internal standard and corrected for loss during hydrolysis and derivitization.

RESULTS AND DISCUSSION

If a host organism were to provide its symbionts with only a single nutrient (e.g., glucose or cAMP), then auxotrophic mutants, such as those that cannot synthesize certain amino acids, would not be able to proliferate inside the host. Each of nine mutant *V. fischeri* strains, auxotrophic for a different amino acid, were found to retain the ability to infect and colonize the light organ of juvenile *E. scolopes* (Table 1). Most of the mutant strains reached population levels that were significantly lower than that typical of the parent strain; nevertheless, they all could obtain a sufficient supply of their required amino acid to support the synthesis of more than 30,000 progeny cells (Table 1). This result suggested that the crypt epithelium provides an array of amino acids, and perhaps other nutrients, to the symbionts. Because the glycine auxotroph colonized as well as the wild-type *V. fischeri* strain, it appears that the supply of this amino acid does not limit symbiont growth within the light organ. Glycine is used by some marine invertebrates as a major tissue osmolyte (29) and, thus, may be expected to be present in abundance within the

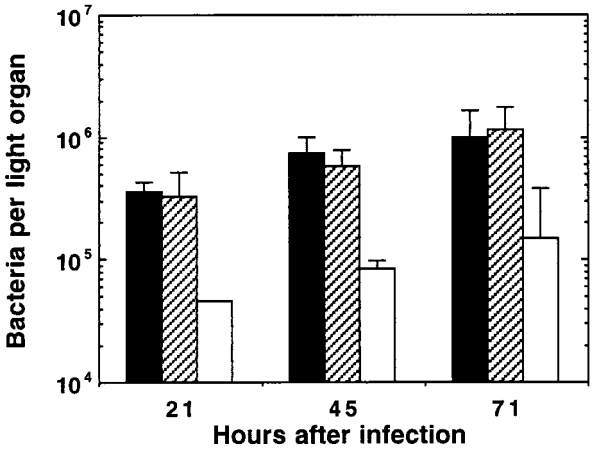

FIG. 2. Persistence of auxotrophic strains of *V. fischeri* in the juvenile *E. scolopes* light organ. The number of bacterial cells present at three times after inoculation was determined in light organs of animals infected with either a glycine auxotroph (hatched bar), a threonine auxotroph (open bar), or the parent strain (solid bar). The error bars (where appropriate) indicate 1 standard deviation.

crypt contents. Interestingly, free glycine has not been detected as a major osmolyte in *E. scolopes* tissue (23) and, thus, may be provided to the symbionts in another form.

To determine the period of time over which amino acids are provided by the host, we examined the persistence of mutant colonizations during the first 3 days of the symbiotic infection. If these nutrients were supplied only during the initiation of the symbiosis, the auxotrophic mutants would be unable to repopulate the light organ after each round of daily venting of the symbiont bacteria, and thus their numbers would decrease over time. In fact, such a decrease does not occur (Fig. 2); instead, the levels of colonization by both the glycine and the threonine auxotrophs mimicked the parent strain, increasing in number by 5 to 10% each day over the 3-day period observed. These results demonstrate that the host supplied these two, and likely other, amino acids as a daily ration to support the normal diel regrowth of its symbiont population.

Our discovery of a procedure for directly sampling the contents of the light organ crypts (Fig. 1*B*) and separating the symbiont cells from the matrix fluid surrounding them allowed us to look for evidence of whether the host provided these amino acids as monomers, as peptides, or as both. Analyses of the bacteria-free crypt matrix fluid revealed that many of the free amino acids were present in the crypt matrix at a concentration below 10 μM. In contrast, these amino acids were present in the crypt matrix fluid in a multimeric form at a 10- to 100-times greater abundance. This difference may indicate either that symbiotic *V. fischeri* cells utilize the monomeric forms as rapidly as they appear, or instead that the major form in which amino acids are presented is as peptides or proteins. In any case, the host must be a net source of at least 9 aa, and their abundance in the multimeric form suggests that they could supply sufficient material to permit the biosynthesis of a large portion of the total symbiont cell protein (Table 2). Thus, that several of the auxotrophs achieve less than 10% of the typical level of colonization may reflect their inability to either rapidly or completely utilize some of these peptides. Studies designed to identify *V. fischeri* extracellular proteases and their possible induction in the symbiosis are underway to clarify this issue. It is also possible that the more abundant amino acids [e.g., alanine, glycine, glutamate/glutamine, or isoleucine (Table 2)] provide a host-derived chemotactic signal that the symbionts use to initially locate and colonize the light organ crypts (19, 23).

Although there are numerous examples of the transfer of essential amino acids and vitamins from microbial symbionts to their animal hosts (1, 30, 31), we show here the inverse relationship, that a host squid provides an array of amino acids, perhaps in the form of peptides or proteins, to its bacterial symbionts, creating a nutritional environment that is considerably more complex than was previously anticipated (17, 18). Many pathogenic bacteria that remain extracellular during

Table 2. Comparison of the concentrations of amino acids present in the released crypt matrix fluid and symbiont cells from the *E. scolopes* light organ*

Amino acid	Crypt matrix fluid		Symbiont cells (free + peptide), mM
	Free form†, mM	Peptide form‡, mM	
Alanine	0.14	0.91	1.79
Arginine	ND	0.67	0.90
Aspartate + asparagine	0.06	1.49	2.08
Cysteine	ND	0.21	0.21
Glycine	0.16	1.21	0.80
Glutamate + glutamine	0.10	1.63	3.40
Histidine	ND	0.36	0.40
Isoleucine	0.12	0.58	0.94
Leucine	0.07	1.10	1.58
Lysine	0.04	1.11	1.44
Methionine	ND	0.67	0.90
Phenylalanine	0.03	0.59	0.86
Proline	ND	0.72	0.79
Serine	0.04	1.01	1.31
Threonine	ND	1.09	1.44
Tyrosine	0.03	0.48	0.69
Valine	0.09	0.77	1.26
Total	0.88	15.25	22.74

ND, Not detected (<0.01 mM).

*Representative values for the concentrations of amino acids present in released crypt contents. Values are calculated based on a 10-μl volume of collected crypt contents. These values may be underestimations because of a slight swelling of the matrix fluid upon its release. Tryptophan concentration was not determined in the analyses.

†Concentration of each amino acid present as free monomers in the sample.

‡Concentration of each amino acid present as peptides (i.e., oligopeptides and proteins) was calculated as the total amino acids minus the free monomers.

their colonization of host tissue (32) are also believed to have available to them a complex nutritional environment, resulting either from the lysis of host cells or the digestion of extracellular tissue matrix material. Unfortunately, we know remarkably little about the identity of these nutrients or their role in colonization (33), although it is becoming clear that the nature of a pathogen's major carbon source can effect the synthesis and expression of virulence factors (34, 35). The regulated pattern of nutrient provision (Fig. 2), as well as the ability to easily quantify the success of metabolically defined mutants (Table 1), will allow these processes to be described in the *Vibrio*–squid association.

In conclusion, we have addressed the fundamental question of the nature and dynamics of nutrient exchange between cooperative bacterial symbionts and their animal hosts by examining the bioluminescent association between *V. fischeri* and the squid *E. scolopes*. The auxotrophic mutants described in this study (with the exception of the glycine auxotroph) constitute examples of what has been termed "symbiosis accommodation" mutants (10), i.e., mutants that are capable of initiating (and persisting in) a host colonization, but only to an extent limited by their inability to fully accommodate to specific conditions of the host's tissues. The use of auxotrophic mutants as environmental probes, combined with the application of a newly developed technique for accessing the fluid environment surrounding the symbionts, has led to the discovery that the host provides a complex mixture of amino acids, perhaps in the form of polypeptides, to its symbiotic *V. fischeri* cells. These nutrients are delivered as a daily ration that supports a periodic repopulation of the light organ after each morning's host-controlled expulsion of symbionts. Characterization of symbiosis-deficient mutants and sampling the symbiotic environment itself for microchemical analyses represent two powerful approaches that will continue to reveal the processes by which an animal host communicates with, and controls the proliferation of, its symbiont population (10, 11). Future investigations focusing on the identity, function, and source of other polymeric compounds present within the crypt matrix fluids may help to elucidate how the animal host regulates the size of the symbiont population.

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