1 **Are you my symbiont? Microbial polymorphic toxins and antimicrobial compounds as**
2 **honest signals of beneficial symbiotic defensive traits**

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11 Short title: Polymorphic toxins as honest signals in symbiosis

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13 **Abstract:**

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15 In defensive symbioses where microbes benefit their host by killing competitors, predators or
16 parasites, natural selection should favor the transmission of microbes with the most beneficial
17 defensive traits. During the initiation of symbiosis, the host's ability to accurately pre-assess a
18 symbiont's beneficial traits would be a selective advantage. We propose that one mechanism by
19 which a host could recognize and select a beneficial partner would be if the latter displayed an
20 honest signal of its defensive or other symbiotic capabilities. As one example, we suggest that
21 polymorphic toxins and their surface receptors, which are involved in inter-microbial competition
22 and predator killing activities, can be honest signals that facilitate partner choice in defensive
23 symbioses.

Introduction

Microbes are fundamental components of all life on earth. Their metabolic and chemical diversity affords abundant potential for goods and services that may be exchanged with, or used against, other organisms they encounter [1]. In mutualistic microbial symbioses, microbes associate intimately with hosts (plants, animals, or other microbes) and provide activities that promote host health and reproductive fitness. Experimental evidence in diverse microbial symbioses has revealed that in numerous associations, a particular species or strain of microbe is faithfully transmitted to the next generation, while others are excluded. Here we review findings from recent and past literature, encompassing binary and consortial symbioses and those of invertebrates and vertebrates, to suggest the concept of honest signaling as a unifying model for molecular mechanisms by which partner selection and specificity is achieved.

Main text

Symbiont selectivity and partner choice

In many symbioses, the association between the host and microbe is specific, such that only one strain or species of microbe associates with or is transmitted by a particular species of host [2-6]. Evolutionary theory suggests that in environmentally transmitted symbioses, in which the symbiont is recruited anew each host generation, the host may select best performing symbionts from a population of possible partners, in a process known as partner choice [7]. While myriad data support the concept of partner choice, the mechanisms underlying this process remain obscure for the majority of symbioses [8].

Partner choice in some systems will depend on the host's ability to assess symbiont performance before engaging in the symbiosis. How are beneficial partners distinguished from the many other possible associates present in an environment? How are the distinctive characteristics of a beneficial microbe transduced to promote their exclusive physical

association with an as-yet un-colonized host? The answers to these questions will help connect the concept of partner choice to the molecular events occurring during symbiont transmission.

The key characteristic that must be assessed in partner choice is the beneficial function(s) provided by the symbiont, two prominent categories of which are nutritional and defensive [9,10]. In the former, microbial symbionts provide essential nutrients and can enable expansion of plants and animals into diverse nutrient-poor or toxic niches that would otherwise be uninhabitable (Table 1). For instance, hydrothermal vent mussels from the genus *Bathymodiolus* are fed by a horizontally acquired gill-tissue symbiont that uses inorganic oxidation to fix carbon [11,12], and land plants acquire essential nutrients, such as nitrogen, through symbiosis with arbuscular mycorrhizal fungi or bacteria [13]. Symbiosis can also contribute to degradation of inaccessible nutrient stores, such as recalcitrant plant material that is made accessible to some types of ants through cultivation of herbivorous fungi [14]. Similarly, nematodes in the genera *Steinernema* and *Heterorhabditis* can kill living insects for use as a food source by virtue of symbiosis with *Xenorhabdus* and *Photorhabdus* bacteria, respectively, which contribute to killing and tissue bioconversion of the insect [15].

In defensive or protective mutualisms, the microbial symbionts help defend the host organism or its progeny from predators and pathogens [9], including by producing chemically diverse small molecules or protein toxins that can kill aggressor or competitor organisms [16] (Table 1). As just one example of many, *Alteromonas* bacteria on the surface of shrimp embryos produce istatin, which protects the embryo from the fungal pathogen *Lagenidium callinectes* [17]. Also, *Hamiltonella defensa* provides protection to its host pea aphid *Acyrtosiphon pisum* by producing a phage-encoded toxin effective against parasitoid wasps [18]. Inter-microbial competition can also be a defensive symbiotic trait, since it can protect niche resources from opportunistic invaders. For instance, arthropods associate with antimicrobial-producing

Actinobacteria that help protect food or progeny from pathogens [19-22] and the nematode symbiont *Xenorhabdus nematophila* provides an advantage to its entomopathogenic nematode host by producing an R-type phage tail bacteriocin that kills the obligate symbiont of a competitor nematode [23].

In systems where antimicrobial or anti-predator activities are an essential component of the mutualism, natural selection will favor the inter-generational transmission of microbes with the strongest toxicity toward the target. Recent work in beewolf predatory wasps indicates partner choice is occurring in the transmission of the symbiont from the female antennae to the brood cell in which progeny cocoons develop. In experimental crosses, the antennae of female beewolves could be colonized by the symbiont of another beewolf. However, those females did not deposit antennal gland secretions into the brood cells, effectively blocking transmission of the non-native symbiont to progeny [6]. Similarly, fungus-cultivating *Acromyrmex* ants exhibit self-grooming behavior, which may function to maintain the symbiont on the cuticle surface, more frequently when colonized by their native strain of antibiotic-producing defensive symbiont, relative to strains isolated from other ant populations [22]. Such crossing experiments also demonstrated *Steinernema* spp. nematode adaptations and specificity for native species or strains of their *Xenorhabdus* bacterial symbionts [24,25]. Similarly, strains of the dominant gut symbionts of honeybees and bumblebees show specificity for their respective hosts and can outcompete non-native strains even when numerically disadvantaged [5].

Honest signaling

To provide a framework to investigate mechanisms of partner choice, we propose a model in which assessment occurs through honest signaling, wherein the symbiont, at some cost to itself, displays or sends a signal to the host of its ability to cooperate (e.g. provide nutrients or defensive functions). Here, we define a signal as a product of the symbiont that has evolved to

be recognized by the host, and to which the host has evolved to respond. In our model, the honest signal presented to the host directly and accurately (i.e. non-deceptively) represents the beneficial activity the symbiont provides [26]. For example, honest signaling for nutritional and defensive symbioses might occur through the evolution of processes that lead to specialized accumulation and secretion of a metabolite characteristic of a nutritional pathway and an antimicrobial compound/toxin, respectively. The model requires that the host has evolved mechanisms to recognize the honest signal and to respond by promoting association with, or transmission of, the appropriate signal-producing symbiont. Below we expand on this model by describing how symbiont-produced toxins and antibiotics could serve as honest signals, functioning both as defensive factors that benefit their host and as distinctive traits enabling recognition and physical association during partner choice (Fig. 1).

Defensive polymorphic toxins of microbial symbionts

Microbes produce a dizzying array of bioactive secondary metabolites and proteins, many of which have functions in defensive symbiosis. Indeed, symbionts from aquatic and terrestrial environments are being mined for pharmaceutically useful natural products [16,27,28]. Diverse classes of protein toxins can have anti-microbial and anti-eukaryotic activities, with target specificity typically residing in a strain-variable domain of the protein [29-35]. Recently, several classes of protein toxins were grouped into a family known as polymorphic toxin systems (PTS) based on several unifying characteristics including their mosaic multi-domain structures and wide allelic diversity both within and between genomes [32,35]. Furthermore, recent research has highlighted the ubiquity of genes predicted to encode polymorphic toxins in the genomes of beneficial bacterial and archaeal symbionts [5,12,25,29,36]. Of particular note, the genomes of vertically transmitted endosymbionts of hydrothermal vent mussels are enriched for toxin-encoding genes, including members of the classes noted above [12]. Three toxin classes were

predominant: repeats in toxins (RTX), multifunctional autoprocessing RTX toxins (MARTX) and Rhs/YD repeat toxins (Table 1).

Based on their ubiquity among symbionts and prominent roles in inter-microbial competition and toxicity against eukaryotes, some PTS are likely mediators of defensive activity in many symbioses. This possibility may explain the presence of toxins in the mussel endosymbionts; the authors of that study suggest PTS defend the host against intra-nuclear pathogens or eukaryotic parasites, and offer as evidence the fact that the majority of the identified toxin genes are highly variable across isolates, as might be expected of genes encoding defensive activities [12]. Similarly, honeybee and bumblebee dominant gut symbionts encode numerous PTS and natural product loci. Recent work has suggested that variation in these loci may account for the ability of the bee hosts to differentiate among strains of these gut symbionts [5]. Defensive roles of PTS have been established in several symbioses, such as *Drosophila neotestacea* protection from a nematode parasite through the action of a Shiga-like toxin produced by a spiroplasma symbiont [37]. In the pea aphid symbiont *A. pisum*, phage regions can encode one of three different classes of toxin effective against parasitoid wasps: Shiga-like toxin, cytolethal distending toxin (*cdtB*), or YD-repeat toxins, each with the potential for allelic diversity among strains [30]. Similarly, most *Xenorhabdus* and *Photorhabdus* strains encode a class of PTS known as Tc toxins that are thought to contribute to the insect-killing activity that supports the nutrition and reproduction of their mutualistic nematode hosts [29]. Also, *X. nematophila* utilizes another type of PTS, a bacteriocin encoded by a P2-like remnant phage, to compete with closely related bacteria and confer a competitive advantage to its nematode host [23,38].

As currently defined, PTS systems typically comprise three components: a modular toxin, an activity that confers self-immunity to the expressed toxin variant, and associated pseudogenes encoding variations of toxin and immunity modules (orphan modules) [29,32,35]. The modules

within each toxin protein can include a secretion domain, a target receptor binding domain, and a toxin effector domain that can be nucleases or nucleic acid or protein modification enzymes. Genomic analyses suggest that recombination between the primary toxin and the pseudogenes, as well as horizontal gene transfer gives rise to different variations of toxins expressed by closely related species or strains of bacteria [29,34,35], which likely promotes evolution of new targets and recognition of self from non-self. For example, bacteriocin-encoding P2-like remnant phage loci are conserved among *Xenorhabdus* species and include a variable region that encodes truncated tail fiber ORFs that appears to have led to diversification of bacteriocins through recombination [38]. Overall, PTS inter-strain variability may enable their function in some systems as honest signals of diverse target specificity.

Linking honest signals to the initiation of symbiont-host interactions

Our model requires that the honest signal of the symbiont will directly link the defensive behavior (e.g. expression of a specific toxin or anti-microbial small molecule) with the characteristics necessary for successfully initiating colonization (e.g. adherence to a host surface) or transmission to progeny (Fig. 1). The beewolf-*Streptomyces* symbiosis may offer a concrete example of this idea. The *Streptomyces* symbionts, which are physiologically diverse, live in reservoirs in the female antennae and are transmitted to the progeny cocoon [6,39]. It has been suggested that the host may alter the nutritional content of the antennal gland environment to promote competition among symbiont strains and to increase antibiotic production by the winners of this competition. The symbiont with the greatest competitive fitness and greatest antibiotic production would thereby be selected and transmitted to the cocoon [40,41]. While compelling with respect to host-mediated promotion of the most competitive symbiont, this scenario still lacks an explanation for the mechanism by which female beewolf behavior is modulated such that they only spread gland secretions in cocoons when a beneficial symbiont is present, not when a non-native strain is present [6].

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181 How might the host specifically recruit the honest-signaling symbiont while excluding less
182 desirable candidates? One route by which this may occur is through direct physical interaction
183 between a microbial surface-localized toxin and the symbiont host cell surface (Fig. 1A). In this
184 simplest scenario, the host may have evolved a receptor with specificity for a specific toxin type
185 or class, and binding leads to adherence of the symbiont to the host cell surface. Specific
186 receptor-mediated recognition of a secreted antibiotic or its transporter could similarly lead to a
187 change in symbiont host gene expression or protein activity that triggers altered behavior of the
188 host (Fig. 1B). Indeed, substantial literature has established that sub-inhibitory levels of
189 antibiotics can serve as inter-cellular signals that modify target cell gene expression [42].
190 Similarly interactions of toxins with host cell proteins could have differing effects on predators,
191 where they lead to death, and symbiont hosts, where they elicit developmental or cell
192 morphology changes that promote interaction with the symbiont (Fig. 1C). Recent work offers
193 intriguing circumstantial evidence to support this last idea. The symbiont "*Candidatus*
194 *Endobugula sertula*" produces secondary metabolites called bryostatins that are thought to help
195 protect from predation the larvae of its marine bryozoan host [43]. Host parents inoculate larvae
196 with the symbiont as they develop within an ovicell, and although not necessary for defense at
197 this stage, bryostatin production is higher in adults with ovicells than without. At this stage, the
198 symbiont induces host actin-related gene expression and the authors suggest that this may be
199 caused by the defensive bryostatin, which modulates gene expression in target predator hosts
200 [43]. If true, it is possible that the production of bryostatin may be an honest signal of defensive
201 capabilities that directly modifies symbiont host cell morphology to mediate transmission to
202 progeny (Fig. 1C).

203

204 For toxins that mediate inter-microbial competition another potential for honest signaling exists
205 in the toxin or antibiotic surface receptors and transporters (Fig. 1A and B). This may be

occurring in the association between rodent forestomach epithelia and *Lactobacillus reuteri*, which can compete with other bacteria through the production of bacteriocins [44]. Rodent-derived strains of *L. reuteri* specifically form biofilms on mouse forestomach epithelia while strains from other animals do not, and a rodent-specific bacteriocin ABC-type transporter is necessary for this phenotype [4]. In the context of an honest signaling model, this finding suggests that the ability to transport a bacteriocin, as indicated by the presence of a specific, surface-localized transporter, could serve as an honest indicator of competitiveness. The transduction of that signal to host association is mediated by the requirement of this peptide for biofilm formation on the specialized host cell surface.

In general, polymorphic toxins have co-opted conserved surface proteins with important functions in the target cell as receptors. *Escherichia coli* contact-dependent inhibition (CDI) toxin binds to BamA, an outer membrane protein (OMP) component of the β -barrel assembly machinery conserved among gram-negative bacteria [45]. Despite the conservation of BamA among potential target cells, the range of CdiA is limited by BamA surface loop variation; the *E. coli* EC93 CdiA toxin can only bind to some BamA alleles and not others [46]. Interpreted within our model of honest signaling, the CdiA-BamA case supports the idea that OMPs that are resistant to competitor toxins could be signals of competitive fitness that can also directly mediate binding to host cell surfaces (Fig. 1A). OmpA, which facilitates import of *Escherichia coli* colicin K toxin [47] is necessary for *Sodalis glossinidius* and *Vibrio* spp. to colonize their tsetse fly and zebrafish hosts respectively [48,49]. Furthermore, variability in OmpA surface loops determines if the *S. glossinidius*-tsetse fly colonization phenotype [50]. Similarly, the *X. nematophila* OMP NilB is a specificity determinant for colonization of *S. carpocapsae* nematodes [3,24]. While the majority of *Xenorhabdus* species lack a *nilB* homolog, at least one other, *X. innexi* encodes a *nilB* homolog with sequence differences from *X. nematophila nilB* in surface loops necessary for colonization ([51]; H. Goodrich-Blair, unpublished data). If NilB is an

honest signal we predict that it represents some aspect of the beneficial activities of *X. nematophila*. The genomic contexts of *nilB* genes may provide clues to the identities of these traits. In *X. nematophila*, *nilB* is flanked by genes encoding synthesis machinery for lysine-rich peptides with antimicrobial activity [52,53], while *X. innexi nilB* is adjacent to PTS-encoding loci. Intriguingly, phylogenomics indicate that *nilB* is co-inherited with several classes of PTS (H. Goodrich-Blair and G. Suen, unpublished data). These genetic links indicate that NilB may be an honest signal with dual roles as a receptor or transporter for toxins or antibiotics and as an adhesin mediating host colonization. In any of the cases described above, the consideration of OMP toxin-resistant variants as honest signals requires that their expression without concomitant expression of the beneficial defensive toxin must come at some cost to the symbiont. A hypothetical example of such a cost is that in the absence of the symbiont-produced toxin, a competitor may survive and produce its own toxin that recognizes the honest signaling OMP variant, leading to symbiont death.

Conclusions

Two repeated themes are emerging from the study of invertebrate and vertebrate models of microbial defensive symbiosis such as those reviewed here. First, co-evolved host-microbe partners that are specialized for each other can discern native partners from even very closely related strains. Second, the genomes of many mutualistic microbial symbionts encode a diverse array of polymorphic toxins and small molecules that are known or proposed to function in either defense or mutualistic host association. The concept of honest signaling suggests that symbiotic partner choice has selected for a direct link between cooperative functions and the initiation of symbiotic host colonization. Indeed, there is mounting evidence to support the idea that toxins and metabolites that confer a competitive advantage within a symbiotic association also play a dual function in direct interactions with a host to initiate those associations (Fig. 1). The potentially overlapping functions of molecules in both defense and symbiont host interactions

258 could facilitate the future discovery of host-range specificity determinants and novel bioactive
259 natural products.

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Using experimental crosses this study demonstrated that strain-level bacterial diversity impacts the fitness of nematode-bacterial pairs and provides experimental evidence for partner fidelity feedback in the evolution of these associations. A comparative genomics analysis highlighted PTS as important factors in strain diversification and symbiotic maintenance.

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Table 1. Symbiotic systems: Host, microbial partners, and their associated interactions

Host Organism	Microbial Symbiont	Nutritional or Defense Symbiont	PTS/Antibiotic Effectors	Reference
<i>Bathymodiolus azoricus</i>	Sulfur-oxidizing (SOX) symbiont	Both	YD-repeats RTX	[12]
<i>Bugula neritina</i>	<i>Candidatus</i> <i>Endobugula sertula</i>	Defensive	Bryostatin	[43,54]
<i>Acromyrmex sp.</i>	<i>Pseudonocardia sp.</i>	Defensive	Antimicrobials	[22,55]
<i>Philanthus sp.</i> (beewolf)	<i>Candidatus</i> <i>Streptomyces philanthi</i> ' (CaSP)	Defensive	Antimicrobials	[6,56]
<i>Acyrtosiphon pisum</i> (pea aphid)	<i>Hamiltonella defensa</i>	Defensive	Shiga-like toxin, YD-repeats	[30,57]
<i>Drosophila neotestacea</i>	<i>Spiroplasma</i>	Defensive	Shiga-like Toxin	[37]
<i>Steinernema carpocapsae</i> (nematode)	<i>Xenorhabdus nematophila</i>	Both	YD-repeats, bacteriocin, lysine rich peptide	[2,23,24]
<i>Heterorhabditis</i> sp.	<i>Photorhabdus</i>	Both	Tc Toxins	[29,58]
<i>Steinernema feltiae</i>	<i>Xenorhabdus bovienii</i>	Both	Predicted Shiga- toxin	[59]
<i>Danio rerio</i> (Zebrafish)	<i>Vibrio</i> (ZWU0020)	Likely Defensive	YD-repeats RTX	[49]
Germ-free Swiss Webster Mice	<i>Lactobacillus reuteri</i>	Likely Defensive	bacteriocin	[4,44]

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Figure Legends

Figure 1. The dual potential of polymorphic toxins and antibiotics in defense and honest signaling.

Polymorphic toxins and antibiotics (orange symbols) can be either exposed on the surface of symbiotic cells (gray rounded rectangles) or secreted into the environment or host cells (green). From these locations, the molecules can interact in a specific way with either target cells for defensive activity (left) or as honest signals interacting with symbiotic hosts during initiation of symbiosis (right). (A) The interaction of surface localized toxins with the receptor (blue surface triangles) on competitor cells leads to cell death (dashed line and skull/crossbones symbol). The symbiont cell is resistant to toxicity due to a specific receptor with a variable surface loop (red triangle) or due to an immunity protein (not shown). Association with the symbiotic host is mediated by adherence of the toxin or OMP to specific receptors (blue and red respectively) on the host cell surface. (B) Antibiotics (orange circles) secreted by the symbiont through secretion systems (blue rectangles with arrows) can mediate interference competition. An immunity protein (red half circle) mediates survival of the symbiont strain. The binding of antibiotics or their surface-exposed transporters to symbiotic host surface receptors (red symbols on host cell surface) could trigger signal transduction cascades that alter host behavior. (C) Toxins (orange symbol) have eukaryotic host cell modifying activities through interactions with host cell proteins (blue or red triangles). The same activities could have lethal effects in predators (cell death indicated by dashed lines and skull) but in symbiotic hosts could induce necessary morphological changes (e.g. endocytosis) that promote association with the symbiont. In all cases, the molecular diversity of these molecules enables specialization for both defensive targets and symbiotic hosts.

Figure