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Managing Amphibian Disease with Skin Microbiota

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The contribution of emerging amphibian diseases to the sixth mass extinction is driving innovative wildlife management strategies, including the use of probiotics. Bioaugmentation of the skin mucosome, a dynamic environment including host and microbial components, may not provide a generalized solution. Multi-omics technologies and ecological context underlie effective implementation.

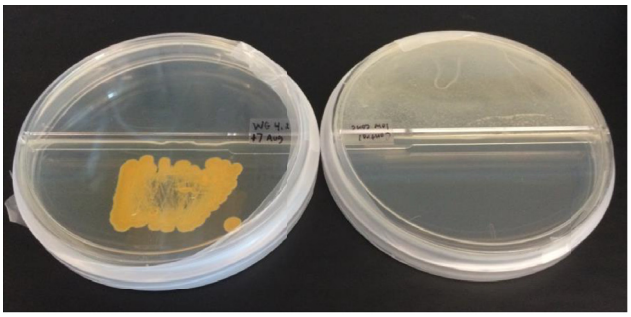
Global population declines and extinctions of over 200 amphibian species have been linked to the fungus *Batrachochytrium dendrobatidis* (*Bd*). The hydrophilic chytrid fungus infects amphibian skin, causing the disease chytridiomycosis. Recent work puts forth the hypothesis that a hypervirulent Global Pandemic Lineage of *Bd* (*Bd*-GPL) may have emerged, in part, as a result of hybridization among disparate *Bd* genotypes brought into contact by human movement of amphibians [1], and that this lineage is associated with global amphibian declines. A newly described salamander chytrid, *Batrachochytrium salamandrivorans* (*Bsal*), is causing recent declines in Europe and threatens both other European salamander populations and the highly diverse North American salamanders. Evidence again points toward human-facilitated transcontinental movement of animals in spreading *Bsal* to a new geographic region of susceptible amphibians [2]. In addition to proposed trade restrictions and containment of *Bsal*, development of new tools for disease management is urgently needed.

There are a variety of disease management strategies, including removal of disease reservoirs (e.g., *Bd*-tolerant, invasive amphibians), habitat alteration by reducing canopy cover to increase basking opportunities, management of zoospore micro-predators, and fungicide applications. However, many of these strategies are either not feasible or are not effectual in amphibian wetland habitats, particularly in remote and pristine regions. A relatively new strategy harnesses the natural enemies of fungal pathogens, which reside right on the amphibians' skin as symbiotic microbiota, that are capable of inhibiting pathogen colonization and establishment. Thus, we consider this approach to be a novel application of probiotics or beneficial bacteria that provide pathogen defense for the amphibian host [3]. We have recently produced a database of such beneficial bacteria with a corresponding culture collection maintained at UMass Boston and the Culture Collection of Switzerland [4]. Most of these bacteria were tested in co-culture assays against *Bd*, and some have been used successfully to promote disease resistance when applied to frog and salamander skin [3].

The mechanisms by which bacteria (and other symbionts, including viruses, fungi, and protozoans) are able to promote disease resistance are not well known. Some bacteria are able to promote host immune defenses, and others compete with invading pathogens directly. Direct competition among microbes often leads to secreted toxins, such as small antibiotic molecules. In several cases, *Bd* inhibition was associated with the production of bacterial secondary metabolites. For example, *Pseudomonas fluorescens* produces the compound 2,4-diacetylphloroglucinol (DAPG) and *Janthinobacterium lividum* produces indole-3-carboxaldehyde (I3C) and violacein, all of which can inhibit *Bd* growth [3].

The production of bacteriocins and volatile organic compounds (VOCs), while less explored, may also be responsible for bacterial inhibition of *Bd*. Bacteriocins are a group of antimicrobial peptides produced by bacteria and archaea. In human systems, bacteriocins produced by resident bacteria are known to have a bacterial pathogen-inhibiting function [5], and some bacteriocins are antifungal. The same could be true for amphibians' bacterial symbionts; however, this remains unexplored. In addition to the use of the bacteriocin-producing bacteria as probiotics for amphibians, purified bacteriocins (e.g., pheromonicins [6]) could also be used to treat *Bd* infection, especially in captive situations, and may be safer than traditional antifungal compounds for amphibians. Currently, itraconazole is the best antifungal treatment option for amphibians when used correctly. Antifungal treatments include potential side effects and negative impacts on non-target microbiota.

In addition to antibiotic production and antifungal activity when in co-culture [4], some bacteria appear to have long-distance effects facilitated by VOCs. Here we show that bacteria confined to one side of a split-Petri plate are able to inhibit *Bd* growth on the other side of the plate, without physical contact (Figure 1). Thus, we suspect that aerosolized VOCs inhibit fungal growth. Indeed, the botany literature has many examples of soil bacteria that are able to promote plant health and limit fungal disease by VOC production. For example, some *Pseudomonas* bacteria produce compounds, including phloroglucinols and hydrogen cyanide, that suppress plant disease [7]. Multiple soil bacteria with VOC-mediated antifungal activity [8] have been found on amphibians' skin. Similarly, researchers studying *Pseudogymnoascus destructans*, the etiological agent of white-nose syndrome in bats, have recently proposed using



Experimental isolate	Representative image of *Bd* (10x, 400 μm scale bar)	Mean % *Bd* area (SD)	T (df = 4)	P	Cyantesmo test strip (blue indicates HCN)
Janthinobacterium lividum		9.5 (3.4)	8.439	0.001	
Pseudomonas sp.		9.0 (2.5)	8.87	0.001	
Rhodococcus fascians		0.0 (0.0)	11.23	<0.0001	
Control		52.0 (8.0)			

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Figure 1. Amphibian Skin Bacteria Producing Volatile Antifungal Compounds. Preliminary data show that amphibian skin bacteria produce aerosol volatile compounds capable of inhibiting growth of the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*). Top: Split-Petri dishes (VWR) show a bacterium on the bottom half of the left plate isolated from a southern leopard frog, *Lithobates sphenocephala*, (*Rhodococcus fascians* – matching GenBank accession number HG796188) and no growth of *Bd* (top half of plate), and control (right plate) with abundant *Bd* growth. Note that secondary metabolites from *R. fascians* collected in liquid media did not inhibit *Bd*. The antifungal volatile organic compounds (VOCs) released from the bacteria are under investigation and may lead to disease-management strategies to improve amphibian conservation. Hydrogen cyanide (HCN) production was not indicated based on Cyantesmo strip tests (Macherey-Nagel GmbH & Co.), but was indicated in a similarly tested *Pseudomonas* isolate from Colombia (VF35). Bottom: We quantified the area of *Bd* growth in split-Petri dish experiments with VOC-producing antifungal isolates from amphibian skin compared to control growth (experimental design adapted from [8], artificially colored red area calculated with ImageJ, statistics with IBM SPSS Statistics v.23). *R. fascians* completely inhibited *Bd*. Inhibition of *Bd* by isolates producing VOCs is included in updates to the Antifungal Isolates Database (4).

VOC-producing soil bacteria (*Rhodococcus*) in hibernation caves to kill spores and reduce the fungal pathogen [9]. *In vivo* studies are needed to examine live amphibians for the production of antifungal compounds identified in cultures, and to test treatment of infected amphibians. Perhaps VOC-producing bacteria can be deployed at amphibian-rich habitats (i.e., breeding sites) to reduce the zoospore pool, thereby facilitating population persistence. Instead of the stench of disease, recollecting miasma theory, perhaps host odors can signal the presence of antifungal microbial defenses and contribute to behavioral ecology and sexual selection of resistant hosts. In other words, frogs may be able to smell VOC-producing microbiota, and if odor is linked with disease resistance, protected partners may have a selective advantage in mate choice.

The goal of microbial therapy is to increase survival of amphibians through augmenting skin microbes that inhibit pathogens directly or indirectly by promoting host immunity. Probiotic bioaugmentation may lead to herd immunity in amphibian populations or communities [3]. The ecological context and biotic resistance to probiotics – climate, host immune response, and microbial community composition or diversity – will shape their effects [10]. A primary criterion for an effective probiotic is the ability to colonize and persist on the amphibian's skin at an abundance high enough to achieve host protection [3]. For some species this has been a major obstacle, such as the extinct (in the wild) Panamanian golden frog, *Atelopus zeteki* [11] or the Panamanian rocket frog, *Colostethus panamansis* [12]. In a number of other systems, microbial communities become established early in development, even in the human womb [13]. Thus, probiotics applied early in development may colonize open niches and establish in concert with host immunity. We found that some amphibian skin-associated bacteria can persist through life-history transitions from aquatic larvae through terrestrial adult stages (Figure 2, data from [14]). Identification of core antifungal community members, and augmentation of these members early in development, may prove effective. An antifungal *Chryseobacterium* sp. was one core member found across life stages. While not a core member, *J. lividum* has

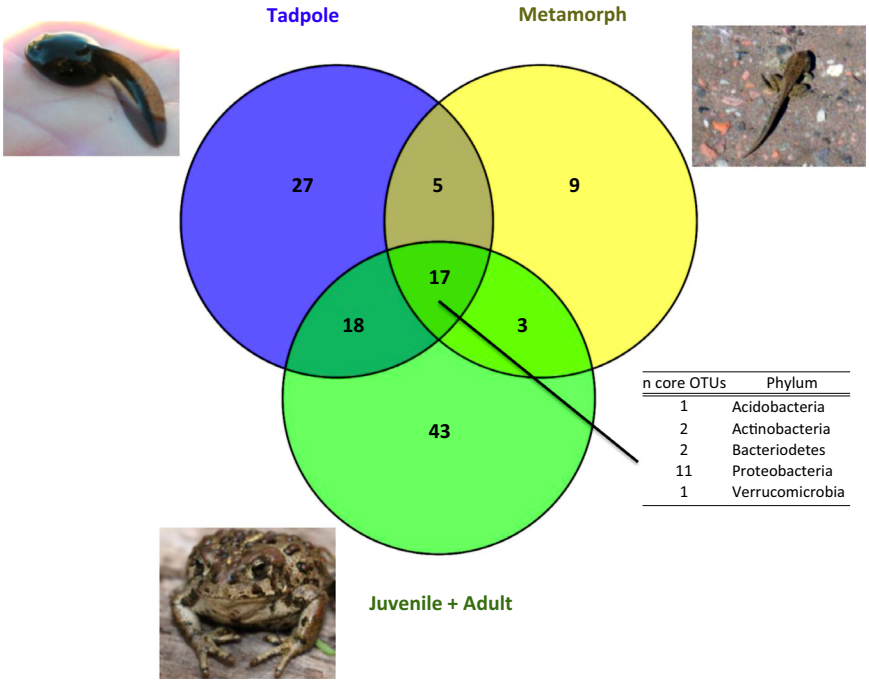

Figure 2. Prevalent Skin Bacteria Persisting through Life-history. The bacteria found on the skin of boreal toad, *Anaxyrus boreas*, aquatic tadpoles, recent metamorphs, and terrestrial juveniles and adults demonstrate a shifting community with a significant overlap in membership. The Venn diagram includes numbers of operational taxonomic units (OTUs) found in ≥90% of individuals in each life-history stage (n = 8 tadpoles, 8 metamophs, and 16 juveniles/adults) field-sampled concurrently at Trout creek, Colorado. For example, in the tadpole lifestage, there are 27 core bacterial OTUs that are only found on that lifestage, 5 core OTUs are found on both tadpoles and metamorphs, and 18 core OTUs are found on both tadpoles and adults. Seventeen core bacterial OTUs are found across all boreal toad life stages including a *Chryseobacterium* sp. identified in culture with anti-*Bd* metabolite production. Details of the study are found in [14], and indicate persistant potential probiotics closely associated with the host. Photos from D. Brewbooks and J. N. Stuart.

violacein-producing antifungal strains that can reduce mortality from chytridiomycosis, and is occasionally present on tadpoles. Thus, augmentation of *J. lividum* at the tadpole stage by probiotics or prebiotics (nutrients favoring beneficial bacteria) may prove effective. Furthermore, in one study, *Escherichia coli* was engineered to produce violacein [15], and common amphibian skin bacteria could be similarly manipulated, leading to more consistent probiotic establishment on hosts. Biotic resistance theory may not apply perfectly to microbiota in understanding colonization resistance because therapies avoid introducing novel organisms into naïve populations and there are both host (environmental) and microbial (competition) factors. That said, microbial diversity and composition may have an understudied influence on probiotic effectiveness.

The current focus on individual bacteria as probiotics to mitigate chytridiomycosis is likely over-simplistic. The use of multispecies probiotics or whole-community transplants [12] are avenues for future exploration. Additionally, multi-omics approaches that integrate functional aspects of interacting microbial communities [14], metagenomic descriptions of the microbial communities, and metabolomic descriptions of the active mucosal compounds have great potential to advance this field. For example, network analyses used by Kueneman *et al.* [14] show that some bacterial groups, including the Burkholderiales and Pseudomonadales, negatively co-occur with fungi. These correlational analyses, along with culture and *in vivo* studies incorporating the ecological context of the mucosome, help focus probiotic development. One frontier is the identification of bacterial genes associated with antifungal function. Antifungal genes are unlikely to be expressed consistently under common culture conditions, and isolate identification by 16S rRNA gene sequencing is not necessarily a reliable indication of the antifungal capacity. Whole genome sequencing may thus lead to better diagnostics of antifungal function of microbial communities.

In addition to developing conservation strategies, future studies may also be directed toward testing ecological theory. For example, to what extent are hosts and microbiota co-evolved? Is the microbiota heritable? To what extent are recovering amphibian populations adapting to *Bd* by changes in microbiota or microbial-immune interactions? Are there ecological trade-offs between a microbiota-based defense and a host immune system defense? Can microbial VOCs signal the immune condition of hosts, or affect behavior of conspecific hosts?

In addition to these research questions, a common bioethical concern of conservation biologists is: What are the ecological consequences of probiotic applications, particularly in sensitive habitats with other endangered wildlife species? This concern is balanced by the consequence of no action and continuing population losses. Current standards for ecological use of bioaugmentation strategies include: (i) the use of native microbes only, perhaps cultured from the skin of an amphibian co-existing with *Bd*. The host may either tolerate low-level infection, or resist infection [3]. (ii) Tailored management within systems should also examine non-target effects on aquatic and soil microbes, and the microbiota of non-target hosts. Furthermore, there are many potential

applications of probiotic approaches to amphibians in aquaculture systems and the pet-trade that could have a significant impact toward reducing the spread of fungal pathogens via these trade routes. Novel tools that target the pathogen but limit toxicity to the host, such as probiotics, are sorely needed in these sectors. While there is much to learn in the field of wildlife microbial ecology, probiotics hold great potential for directed disease management and conservation intervention.

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Spotlight

HIV-1 Envelope Under Attack

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The human immunodeficiency virus type 1 (HIV-1) envelope (Env) plays a critical role in viral replication and represents a potential target for host antiviral factors. Recent work by Tada and colleagues identifies membrane-associated-RING-CH8 (MARCH8) as a potent anti-HIV factor blocking virion incorporation of Env. Thus, MARCH8 joins a growing list of host factors attacking HIV-1 Env.

The HIV-1 Env glycoprotein plays critical roles in viral replication, tropism, and pathogenesis. Env is first synthesized as a polyprotein, gp160, and is subsequently cleaved by cellular proteases in the Golgi apparatus into gp120 and gp41. The proper synthesis, modification, folding, cleavage, cell surface targeting, and virion incorporation of Env are all essential steps in the generation of infectious HIV-1 particles. Consequently, these steps are all vulnerable targets for attack by host defense factors (Figure 1).

A recent study by Tada and colleagues, published in *Nature Medicine*, has illustrated the importance of this concept of host defense against HIV-1 Env [1]. These authors identify MARCH8 as a novel antiviral factor suppressing HIV-1 Env surface expression and virion incorporation. Like many scientific discoveries, their finding came from an unexpected observation: that the transduction efficiency of lentiviruses produced from MARCH8-expressing cells was 65-fold lower than that of control lentiviruses, suggesting an antiviral role for this protein. Tada and colleagues demonstrated, in a logical series of experiments, that MARCH8 is a novel antiviral factor against diverse viruses in virus-producing cells. MARCH8 expression in target cells showed no effect on viral infectivity, indicating that its antiviral activity is the result of its expression in the producer cells. Also, MARCH8 expression in producer cells did not influence the production of HIV-1 virions, as monitored by viral Gag proteins. The blockage in the infection of the viruses produced from MARCH8-expressing cells occurred prior to reverse transcription and integration, and in fact was due to a marked defect in viral entry. Finally, detailed analysis of released viral particles revealed that the level of Env proteins on HIV-1 virions produced from MARCH8-expressing cells was dramatically decreased.

MARCH8 is a member of the MARCH family of RING-finger E3 ubiquitin ligases and is mainly found in endosomes, lysosomes, and vacuoles, but also on the plasma membrane. MARCH8 induces the ubiquitylation of substrate proteins and regulates their vesicular transport between membrane compartments. Cell-surface expression of several cellular glycoproteins is specifically downregulated by MARCH8 [2]. The anti-HIV-1 activity of MARCH8 is dependent on its E3 ubiquitin ligase activity. As a potential viral restriction factor, MARCH8 is highly expressed in monocyte-derived macrophages (MDM) and dendritic cells (MDDC). The expression of endogenous