THE ROLE OF CUTANEOUS BACTERIA

IN RESISTANCE OF AUSTRALIAN TROPICAL RAINFOREST FROGS

TO THE AMPHIBIAN CHYTRID FUNGUS

BATRACHOCHYTRIUM DENDROBATIDIS

Thesis submitted by

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STATEMENT OF THE CONTRIBUTION OF OTHERS

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This project was co-supervised by Professor Ross Alford, Professor Lin Schwarzkopf, Dr Annette Thomas and Dr Stephen Garland. Ross Alford had significant input into experimental design and statistical analyses and provided editorial assistance. Lin Schwarzkopf input ideas and useful editorial comments. Technical and editorial support on Chapters Three, Four and Six was provided by Annette Thomas and Stephen Garland. A number of people acted as research assistants in both the field and laboratory and they are named, and their contributions listed, in the Acknowledgements. Sarah Sapsford collected the swab samples that formed the basis of Chapter Seven while conducting her mark-recapture M.Sc. project, and will be included as a co-author when this chapter is submitted for publication. Additional editorial comments on individual chapters were provided by Julia Hazel, Ian Bell, Andrea Phillott, Jamie Voyles, Robert Puschendorf and Tim Harvey.

Chapter Four has been accepted for publication in the journal "Diseases of Aquatic Organisms". It is my own work with intellectual and technical input from co-authors Ross A. Alford, Stephen Garland, Gabriel Padilla and Annette D. Thomas. Three anonymous reviewers also provided valuable comments, and these have been included.

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DECLARATION ON ETHICS AND PERMITS

This research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th Edition, 2004, the Queensland Animal Care and Protection Act, 2001 and the Queensland Nature Conservation Act, 1992. The proposed research study received animal ethics approval from the JCU Animal Ethics Committee under approval numbers A1316 and A1420, and approval from the Queensland government under scientific permit number WITK05922209.

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ABSTRACT

Fungal diseases pose a serious threat to animal and plant health. The emergence of the disease chytridiomycosis, caused by the fungus *Batrachochytrium dendrobatidis* (*Bd*), has resulted in the declines or extinctions of many amphibian species worldwide, although the severity of its effects varies widely among host species. Antibiotic-producing bacterial symbionts present on amphibian skin can protect their hosts from disease-related mortality. Bioaugmentation of naturally occurring bacteria that are antagonistic to *Bd* offers great potential for disease prevention in field settings. However, there is a paucity of knowledge on the ecological interactions of cutaneous bacteria with their hosts and *Bd*; this needs to be addressed prior to development and application of probiotics. This thesis addresses technical issues associated with culture and testing of bacterial isolates in the laboratory, investigates variation among rainforest-dwelling frogs in the activity of their bacterial symbionts against *Bd*, and assesses the temporal stability of frog bacterial assemblages.

One factor that might limit the development of effective probiotics is the potentially low proportion of bacteria that can be cultured from environmental samples; commonly believed to be approximately 1%. If the great majority of bacteria on amphibian skin are not culturable, many species with desirable properties are likely to be unavailable for bioaugmentation research. I used both culture and culture-independent techniques to estimate the proportion of isolates that are not culturable and determine the proportion of cultured bacteria that are missed during the process of colony selection for isolation. On average, 75% of the bacteria on individual green-eyed treefrogs, *Litoria serrata*, were culturable. My results thus indicate that the proportion of culturable bacteria present on frog skin is much higher than the traditional 1%. However, of those culturable bacteria, only 53% were selected for isolation. This discrepancy is likely due to morphologically identical colonies present on agar plates but not selected for isolation. Therefore, apparently morphologically identical colonies should be over-selected during initial isolation to reduce the proportion that are missed, and ensure that most bacteria with potential for bioaugmentation research are likely to be cultured. Another factor that might affect the detection of bacterial symbionts with activity against *Bd* is the method used to screen bacterial isolates for inhibition. Previous studies have used agar-based in vitro challenge assays to identify candidates for bacterial supplementation trials. However, agar-based assays can be difficult to set up and to replicate reliably. To overcome these difficulties, I developed a semi-quantitative spectrophotometric challenge assay technique. Cell-free

supernatants were prepared from filtered bacterial cultures and added to 96-well plates in replicated wells containing *Bd* zoospores suspended in TGhL broth medium. Plates were then read daily on a spectrophotometer until positive controls reached maximum growth in order to determine growth curves for *Bd*. I tested the technique by screening skin bacteria from *L. serrata*. Thirty-one percent of bacteria tested showed strong *Bd* inhibition, while some may have promoted *Bd* growth, a previously unknown effect. My technique avoids a number of issues associated with agar-plate assays and thus provides a useful contribution to the expanding field of bioaugmentation research.

One aspect of community ecology theory suggests that complex communities, with higher numbers of taxa and hence more potential interactions, are more resistant to invasion than simple communities with fewer taxa. In the Australian Wet Tropics, the severity of *Bd* effects varied among sites and frog species, and some species have since reappeared or recolonised, despite *Bd* now being enzootic. The available range of variation in history among species and sites provided an opportunity to investigate the role of anti-fungal cutaneous bacteria in protection of frogs against *Bd* infection. I conducted in vitro challenge assays to determine the capacity of bacteria, isolated from five species of rainforest frogs at five sites in northern Queensland, Australia, to inhibit *Bd*. I then used DNA sequencing to identify *Bd*-inhibitory bacteria and determine whether cutaneous bacterial taxa were associated with particular frog species, sites, infection status of frogs or intensity of *Bd* infection. Ninety-four percent of bacterial isolates came from just three families; Pseudomonadaceae, Enterobacteriaceae and Xanthomonadaceae, which were present across all frog species and sites. *Bd* infection intensity was negatively correlated with number of inhibitory genera present on frogs, suggesting that increased diversity of *Bd*-inhibitory taxa may play a role in reducing the intensity of *Bd* infections, hence facilitating frog coexistence with enzootic *Bd*. There was evidence suggesting that *Bd*-inhibitory bacteria may have facilitated the reappearance of frogs at one of two upland Wet Tropics sites I surveyed. Frogs at this site had greater cultured bacterial isolate richness, a greater number of inhibitory bacterial genera, and a higher proportion of inhibitory isolates than their lowland conspecifics. They also had a significantly higher proportion of individuals with one or more *Bd*-inhibitory bacterial species. My results also suggest that alternative mechanisms for decreasing susceptibility to *Bd* infection are likely to have evolved at the other Wet Tropics upland site I monitored where frogs have reappeared.

While the possibility of bioaugmentation offers hope for frogs threatened with extirpation, the stability of the symbiotic microbial assemblage is likely to affect its susceptibility to

manipulation, and thus the success of bacterial supplementation. A microbial assemblage that changes little with time may have highly stable species interactions and therefore be difficult to supplement, while a dynamic microbial assemblage may be more amenable to manipulation. I collected swab samples from 14 individual rainforest frogs captured multiple times over the course of a year, and compared the bacterial assemblage profile generated for each swab sample among frogs grouped by date sampled and by patterns of *Bd* infection. Twenty-five of 114 unique bacterial strains found eight times or more constituted the dominant assemblage members, with five core strains each occurring in greater than 65% of samples. The proportion of core bacterial strains was significantly higher in frogs that were not infected with *Bd* prior to sampling than in frogs that were infected or had recently lost infection. The taxonomic composition of the dominant microbial assemblage also differed significantly between these two groups of frogs. Bacterial species richness decreased over the year, with a corresponding statistically significant change in assemblage composition. The temporal changes observed suggest that the microbiota would be amenable to manipulation through bioaugmentation of *Bd*-inhibitory bacteria. However, turnover of core strains was low, suggesting that bioaugmentation success might be enhanced by application of dominant assemblage members.

I have presented evidence for the importance of amphibian cutaneous microbiota in protection of wild amphibians against *Bd*; greater taxonomic richness of cutaneous bacteria is associated with lower *Bd* infection intensity. With this knowledge, an improved technique to screen bacteria for inhibitory effects against *Bd*, and an understanding of the natural changes that occur in bacterial assemblages over time, there is now further support for the use of bioaugmentation as a tool to protect amphibians against the devastating effects of chytridiomycosis.

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CHAPTER ONE : INTRODUCTION

Host-microbe symbioses

Microbes have historically been viewed by humans chiefly as causes of disease, but only a very small fraction cause ill health (Dethlefsen et al. 2007). In nature, the majority of microbes perform vital ecosystem functions, such as nutrient cycling and decomposition. Gaining insight into interactions among microbes and between metazoans and microbes is essential to understand ecosystem dynamics (Konopka 2009). Microbial community ecology aims to elucidate the richness, abundance, structure and function of microbes within communities. Recent findings demonstrate that taxonomic diversity in such communities can impact disease dynamics (Keesing et al. 2006), and functional redundancy can provide community stability during periods of environmental stress (Wittebolle et al. 2009).

Microbes are an extremely successful group of organisms that have colonised the majority of surfaces on the planet. One of the reasons for their success is their ability to form symbioses; permanent or long-lasting associations with other living organisms. They do this in three principal ways; commensalism, mutualism, and parasitism. In a commensal relationship, one organism receives benefits from the relationship while the other does not, but is not harmed by it. In a mutual relationship, both partners benefit but not necessarily equally, and in parasitic relationships, one partner benefits at the expense of the other. Symbioses can be obligate, where the symbiont is unable to live without its partner, or facultative where it is also free-living. Here, we focus on mutualistic relationships where metazoan hosts and their bacterial symbionts provide benefits to one another.

Vertebrate hosts have well-developed innate and acquired immune systems that prevent colonization by potentially pathogenic micro-organisms. Mutualist microbes have developed adaptations to suppress or otherwise evade host immune defences (Hooper 2009). For example, the mammalian immune system senses both pathogenic and non-pathogenic bacteria using pattern-recognition receptors (PRRs) that trigger the innate and the acquired immune systems (Medzhitov 2007). Toll-like receptors are the best known PRRs, and these activate inflammatory or antimicrobial activity in response to an invading bacterium. Bacteria may adapt to their host by expressing adaptation factors, encoded on genomic islands (Steinert et al. 2000; Hacker and Carniel 2001). The expressed genes may encode cell-surface receptors, such as polysaccharides (see D'Haeze and Holsters 2004) that facilitate host

colonisation. In the mammalian gut, *Bacteroides* spp. that decorate their polysaccharide receptors with the free sugar L-fucose can colonise hosts, while mutant bacteria that cannot do this are excluded (Coyne et al. 2005). The host also expresses L-fucose on the surface of gut epithelial cells, so using this sugar as camouflage may ensure that bacterial symbionts are not recognised as foreign (Coyne et al. 2005).

Some symbiotic interactions are based on the transfer of nutrients from symbiont to host. For example, bacterial symbionts on corals may assimilate nutrients from low nutrient niches, such as water surrounding coral reefs, and pass the nutrients to the host coral via the mucus layer where the symbionts reside (Rohwer et al. 2002). Similarly the human gut benefits from nutrients provided by its microflora (Backhed et al. 2005). For example, the majority of vitamin K is derived from bacterial biosynthesis in the gut (Bentley and Meganathan 1982). Nutrient flow can also occur in the opposite direction. The squid *Euprymna scolopes* provides at least nine amino acids to the bacterial symbiont, *Vibrio fischeri*, which lives within its light organ (Graf and Ruby 1998). Symbiotic microbes can also play a role in angiogenesis and the post-natal development of the human gut, in metabolism of toxins, and in reinforcement of the gut immune system by providing a barrier against infection and disease (See Box1 in Hentschel et al. 2003). They may also provide protection from predators via chemical defence systems (Moran 2006).

Many bacterial symbioses with plants, animals and humans are well described. For example, mammalian herbivores house microbes in their guts to digest cellulose; fish and squid use luminescent bacteria held in light organs to communicate; and deep sea tube worms use microbes to fix CO_2 (see Paracer and Ahmadjian 2000). Clearly microbes have a number of co-evolved roles. Hosts can select for certain microbes while defending themselves against others. *Hydra* spp. shape their microbial communities (Fraune and Bosch 2007), as do squid, *E. scolopes* (Kimbell and McFall-Ngai 2003). Fungus farming ants house symbiotic bacteria in specialised cuticular crypts, showing a highly evolved relationship between the ant, bacteria, farmed fungus, and a pathogen (Currie et al. 2006). These microbial assemblages are specific to the host and can reflect millions of years of coevolution (Moran and Telang 1998). In specific associations like these, coevolution of microbe and host often involves the vertical transfer of symbionts from generation to generation (Moran 2006). In other symbioses, the resident microbial community can vary substantially among individual hosts within the same population due to restricted migration, strong interaction with the host and host genetic differences

(Dethlefsen et al. 2007). In these cases the hosts are primarily inoculated with their symbionts through horizontal or environmental exposure (Dethlefsen et al. 2007).

There are many factors that shape symbiotic microbial communities. The initial order of microbial colonisation, the taxa involved, and the subsequent history of a given host habitat can all affect bacterial community composition (Dethlefsen et al. 2006; Turnbaugh et al. 2007). Once bacteria have colonised a given niche, they communicate with conspecifics by regulating gene expression in response to bacterial cell density; a process known as quorum sensing (Hastings and Greenberg 1999; Miller and Bassler 2001). Quorum sensing regulates processes such as expression of virulence genes, antibiotic production, motility and biofilm formation (Miller and Bassler 2001; Waters and Bassler 2005).

Temperature and pH also act as natural environmental factors that shape microbial communities (Thakur et al. 2004; Ritchie 2006; Sharon and Rosenberg 2008). For example, water temperature increases can shift the balance towards overgrowth of pathogenic bacteria in coral ecosystems (Ritchie 2006; Sharon and Rosenberg 2008). However, the coral probiotic hypothesis proposes that the relationship between corals and their symbionts is dynamic, and that corals can adapt to changing environmental conditions by altering their mutualist bacteria (Reshef et al. 2006). It is, therefore, possible that, even with changes in the symbiotic microbial community, community function will remain the same. For example, the bacterial assemblage on the sponge *Ircinia fusca* changed over time, most likely as a result of water temperature change (Thakur et al. 2004). However, one bacterial species persisted throughout the water temperature changes; this species had antifouling properties (Thakur et al. 2004), suggesting that resistance to colonization of fouling organisms may be a principal role of the bacterial community on this sponge.

In another example of functional stability, Rawls et al. (2006) collected gut microbiota from zebrafish and mice, and conducted intertaxonomic transplants to axenic recipients. The recipient community resembled the donor community in terms of phyla present, but the relative proportions of the taxa changed to reflect those found in the normal gut microbiota of the recipient, suggesting that selective pressures in the host gut habitat are responsible for structuring the microbial community (Rawls et al. 2006). McFall-Ngai (2006) proposed that these findings show that guts of particular vertebrate species support functionally similar species that are shared between gut communities of different vertebrates, despite the different habitats and conditions to which the hosts are exposed. Maintaining the function of the microbial community is, therefore, likely to be important to the health of the host.

A number of recent reviews have reinforced the importance of ecological interactions between microbes and hosts (Belden and Harris 2007; Dethlefsen et al. 2007; Zilber-Rosenberg and Rosenberg 2008). There are several common threads from these reviews: 1) All animals and plants have evolved with diverse symbiotic microbial communities whose individual members constantly interact to maintain a balance essential for host health; 2) Symbionts play a role in the adaptation and evolution of the host; 3) Ecological or genetic changes which disrupt the balance of the microbial community may lead to decreased fitness of the microbe-host unit and cause disease; and 4) Exploring microbial-host interactions may reveal new ways to prevent disease or maximize host health. Therefore, host defences, which include symbiotic microbes, evolve to overcome pathogens, and pathogens evolve to overcome host defences in an ongoing process driven by evolutionary and environmental forces (Brunham et al. 1993; Poulsen et al. 2010).

Disease resistance by bacterial symbionts

There has been much interest in mutualisms that involve bacterial protection against disease. If disease protection mechanisms can be elucidated, it may be possible to manipulate these systems to benefit host health. There are two principal ways that symbiotic microbes can protect their hosts against pathogens; by host immunomodulation, and through competitive exclusion (Fuller 1989; Verschuere et al. 2000).

Immunomodulation by bacterial symbionts resident in the mammalian gut has received substantial attention (Wold 2001; Jarchum and Pamer 2011). Recent research has found that commensal filamentous bacteria can activate helper T-cells, thereby enhancing mucosal immunity (Gaboriau-Routhiau et al. 2009; Ivanov et al. 2009). Immunomodulation by skin symbionts has received comparatively little attention. However, resident cutaneous bacteria can modulate the activation of inflammation following skin damage (Lai et al. 2009) and provide protection against pathogens by modulating T-cell function (Naik et al. 2012). These findings may eventually lead to natural treatments for the control of inflammatory skin diseases, such as psoriasis.

Competitive exclusion of pathogens by bacterial symbionts includes preferential competition for nutrients, blocking of pathogen adhesion sites, or production of inhibitory compounds, and these are not necessarily mutually exclusive. *Pseudomonas* spp. can sequester iron by producing iron-chelating structures, known as siderophores (Kloepper et al. 1980; Neilands 1981). These bacteria can outcompete others in iron-poor environments; this phenomenon has been demonstrated extensively in aquaculture studies (Smith and Davey 1993; Gram et al.

1999). Other bacteria can displace or exclude pathogens by preferentially occupying epithelial or mucosal adhesion sites (Blomberg et al. 1993; Lee and Puong 2002; Chabrillon et al. 2006). They may do this by using specific attachment mechanisms. For example, the presence of bacterial pili, associated with host-attachment, were correlated with a protective effect against a pathogenic bacterium in the gut of zebrafish larvae, where they promote the colonization of beneficial symbionts (Rendueles et al. 2012). However, of all the competitive exclusion mechanisms, production of inhibitory compounds is, perhaps, the best studied. There are many types of inhibitory compounds produced by bacteria, such as antibiotics, bacteriocins, lysozymes, proteases, organic acids, hydrogen peroxide or ammonia (Neilands 1981; Williams and Vickers 1986; Bruno and Montville 1993; Boskey et al. 1999). Of these, antibiotic compounds have received the majority of attention, because of the constant demand for novel pharmaceutical drugs.

Antibiotic-producing bacterial symbionts that provide disease resistance to their hosts are widespread in nature. In plants, bacterially-produced antibiotics occur in the spermosphere and rhizosphere and enhance plant root growth by suppressing pathogens (Raaijmakers et al. 2002). Perhaps the success of *Pseudomonas* as the principle bacterial genus involved in antibiotic production has been their ability to competitively exclude other microorganisms, possibly as a result of siderophore production (Haas and Défago 2005; Weller 2007; Santoyo et al. 2012). A number of insects also use antibiotic production by symbiotic bacteria to protect against fungal parasites. For example, fungus farming ants use antibiotic-producing bacteria to control a fungal pathogen that parasitises their fungal “gardens” (Currie et al. 1999; Currie et al. 2006). Another very similar symbiosis occurs in beewolf wasps, in which *Streptomyces* spp., housed in specialised antennal glands, produce a potent antibiotic cocktail that protects the wasp larvae from parasitisation (Kaltenpoth et al. 2005; Kroiss et al. 2010). This recurring theme of *Streptomyces* spp. in fungal biocontrol has also been demonstrated in pine beetles that also use a specialised structure, known as a mycangium, to house the bacterial mutualists that protect a fungal symbiont (Scott et al. 2008). In insects, these specialised structures, used to house beneficial symbionts, indicate a co-evolved relationship between host, symbiont and pathogen.

In the aquatic environment, shrimp embryos gained protection against a fungal pathogen through mutualist *Alteromonas* sp. that produced the antibiotic, isatin (Gil-Turnes et al. 1989), and a bacterial symbiont of American lobster embryos effectively controlled the same fungal pathogen by producing the antibiotic tyrosol (Gil-Turnes and Fenical 1992). Coral mucus

symbionts also confer antibiosis (Ritchie 2006), with *Pseudoalteromonas* spp. offering strong protection against a number of potential pathogens (Nissimov et al. 2009; Shnit-Orland and Kushmaro 2009). In marine sponges, antibiotic-producing bacteria are associated with antifouling properties. For example, the sponge *Ircinia fusca* preferred a *Bacillus* sp. symbiont (Thakur et al. 2004), while *Dendrilla nigra*, housed *Nocardiopsis* sp. (Selvin et al. 2009); both bacteria produce antibiotics.

In vertebrates, bacterial antibiosis for protection against pathogens is poorly studied. However, in two bird species, the European Hoopoe and the Green Woodhoopoe, nestling birds harbour antibiotic-producing bacteria in their uropygial glands that control feather-degrading pathogens (Soler et al. 2008; Martín-Vivaldi et al. 2010). There is also growing evidence for the role of antibiotic-producing bacterial strains in the control of the amphibian pathogen, *Batrachochytrium dendrobatidis* (Harris et al. 2009a; Becker and Harris 2010).

Manipulation of symbioses for disease control

The discovery of widespread use by host taxa of mutualist bacteria that produce antibiotics has resulted in much effort to mimic natural systems by manipulating the microbiota to reduce infection and control disease. There is great interest in the development of biocontrol solutions to replace commercial antifungal drugs in agriculture and aquaculture, thereby eliminating the associated issue of antibiotic resistance, or at least replacing it with a system that, itself, evolves. There have been many successful agricultural trials involving beneficial microbiota. For example, an antibiotic-producing *Streptomyces* sp. bacterium coating pea seeds provided protection against the fungal pathogen *Pythium ultimum* (Yuan and Crawford 1995), and antibiotic-producing *Pseudomonas* sp. suppressed canola stem rot, caused by the fungal pathogen *Sclerotinia sclerotiorum* (Fernando et al. 2007). Similarly, in aquaculture systems, which are highly prone to disease because of overcrowding, *Pseudomonas* spp. strains that produced antibiotic compounds in iron-depleted conditions improved survival against the pathogen *Vibrio anguillarum* in rainbow trout (Gram et al. 1999; Spanggaard et al. 2001).

Occasionally, an *in vitro* effect against a pathogen does not translate to *in vivo* activity, demonstrating that detailed knowledge of the host-pathogen system is essential to develop effective biocontrol agents (Gram et al. 2001). Abiotic factors such as temperature (Shanahan et al. 1992), pH (Ownley et al. 1992), nitrogen, carbon and other nutrients provided by both host and environment, can influence antibiotic production (Raaijmakers et al. 2002). For example, antibiotic production by a potential *Pseudomonas fluorescens* biocontrol strain for

crops was repressed by phosphate in laboratory trials (Duffy and Defago 1999); this effectively ruled out this strain as a biocontrol option, because phosphate is widely used as a fertiliser in agriculture.

Many studies have found that biocontrol agents can be inconsistent in their performance, possibly due to difficulty colonising the host (Gram et al. 2001; Tagg and Dierksen 2003; Corthesy et al. 2007). This is known as "colonisation resistance". The term was originally used to describe resistance by the resident microbiota to an invading pathogen (van der Waaij et al. 1971). However, it is now also used to describe the resistance of the native microbiota to colonisation of an "outsider" microbe, irrespective of whether it is pathogenic or beneficial. Therefore, development of probiotic bacteria for biocontrol needs to take into account the host, pathogen and environment where it will be used. Very often, repeated applications of the biocontrol agent are needed to maintain pathogen control, as the observed beneficial effect is frequently short-term (Gram et al. 1999; Robertson et al. 2000; Tagg and Dierksen 2003). However, there is considerable interest in the natural control of pathogens given the increase in incidence of emerging infectious diseases.

Emerging infectious disease and amphibian declines

Worldwide, infectious disease outbreaks are being reported with increasing frequency (Daszak et al. 2000; Jones et al. 2008) and are often driven by human activities including globalisation, and environmental and social change (Aguirre and Tabor 2008; Cunningham et al. 2012; Lindgren et al. 2012). Diseases that threaten human health, such as SARS, HIV, Ebola, West Nile fever and Hendra virus, typically receive much attention; however, many wildlife species are also affected by emerging infectious diseases (EIDs) (Daszak et al. 2000; Smith et al. 2009). While documented cases of species extinctions caused by disease are infrequent (Smith et al. 2006), susceptible species that are restricted in range, have small population sizes, and that occur with other species that act as reservoirs for the pathogen, can be driven to extinction (de Castro and Bolker 2005; Smith et al. 2009). Amphibians are one group of animals that have recently suffered extensive population declines and extinctions (Collins 2010).

Concern about the speed and severity of worldwide amphibian declines has been voiced by herpetologists since the late 1980's. Many causes have been suggested for these declines, including habitat loss, over-harvest, introduced species, ultraviolet radiation, pesticide use, pollution, climate change and disease (Alford and Richards 1999; Collins and Storfer 2003; Alford 2010; Collins 2010). A Global Amphibian Assessment undertaken to document the status of all described species of amphibians found that neotropical montane, stream-dwelling

species were the most severely threatened; of those species most rapidly declining, "enigmatic" causes, such as disease and climate change, were the most prevalent (Stuart et al. 2004). Recent declines of amphibians, along with similar though lesser declines of other taxa, have been so severe that some scientists have postulated that we are currently experiencing the planet's sixth mass extinction event (Wake and Vredenburg 2008).

In northern Queensland, Australia, Wet Tropics frog population declines were first observed in the early 1990s at elevations above 400m (Richards et al. 1993; Trenerry et al. 1994; McDonald and Alford 1999). The patterns of frog declines frequently differ for each species and site surveyed. The precise dates of population declines at many sites are unknown, although some sites were surveyed during the declines (Richards et al. 1993; McDonald and Alford 1999). *Litoria serrata* did not appear to suffer permanent upland population declines, although reduced numbers of frogs were observed on some surveys during the peak decline years from 1989-1993 (Richards et al. 1993; Richards and Alford 2005). However, *L. nannotis*, *L. rheocola*, *Nyctimystes dayi, L. nyakalensis, Taudactylus rheophilus* and *T. acutirostris* all disappeared from upland sites between March 1990 and January 1994 (Richards et al. 1993; Laurance et al. 1996). Some species, such as *L. nannotis*, and *L. rheocola,* have subsequently begun to recover (Retallick 2002; pers. obs.; K. McDonald, per.comm.), possibly through recolonisation from stable lowland populations (Woodhams and Alford 2005), while *N. dayi* has mostly remained restricted to lower elevation sites (pers. obs.). Other species, such as *L. nyakalensis, T. rheophilus* and *T. acutirostris,* which were restricted to high-elevation sites, have not been seen since (McDonald and Alford 1999). However, *L. lorica*, another upland rainforest endemic, believed to have disappeared at the same time, was recently rediscovered (Puschendorf et al. 2011), providing hope for the discovery of further remnant populations.

Batrachochytrium dendrobatidis

While a number of microparasites can cause disease and death in amphibian hosts, few are associated with population declines or species extinctions (Collins 2010). However, iridoviruses and fungi have been linked to localised population declines, although the former are not known to cause species extinctions (Pessier 2002; Collins 2010). A recently emerged pathogenic chytriodiomycete fungus, *Batrachochytrium dendrobatidis* (*Bd*) (Longcore et al. 1999), is the cause of many recent enigmatic amphibian declines and species extinctions (Berger et al. 1998; Berger et al. 1999; Daszak et al. 2003; Skerratt et al. 2007). *Bd* has been found in wild amphibians surveyed in North, South and Central America, Africa, Australia and New Zealand, Europe and Asia (Berger et al. 1998; Morell 1999; Pessier et al. 1999; Ron and

Merino 2000; Bosch et al. 2001; Parker et al. 2002; Bell et al. 2004; Kusrini et al. 2008). Frog population declines in northern Queensland, Australia are correlated with the arrival of *Bd* (Berger et al. 1998). In Australia, chytridiomycosis, the EID caused by *Bd,* is listed as a key threatening process under the Government's Environmental Protection and Biodiversity Conservation Act (1999).

The structure and life cycle of *Bd* were initially described by Longcore et al (1999). The infective motile stage consists of a flagellated zoospore that settles on the host epidermis, where it encysts, produces rhizoids and grows into a zoosporangium. The zoosporangium enlarges with developing zoopores until a discharge papillae forms and zoospores are released into the environment. Released zoospores may reinfect the same animal, thus repeating the life cycle and increasing infection levels within an individual host (Piotrowski et al. 2004).

Bd is the only chytrid fungus known to parasitise a vertebrate (Longcore et al. 1999). It uses keratin in amphibian skin or tadpole mouthparts as a nutrient source (Berger et al. 1998). Little is known about how it survives in the environment, but reservoirs may exist that facilitate its spread. While transfer of zoospores does not appear likely to occur through shared substrate use (Rowley et al. 2007), *Bd* has been found in water (Kirshtein et al. 2007; Walker et al. 2007), on a number of reptile species (Kilburn et al. 2011), on wild geese (Garmyn et al. 2012) and in crayfish guts (McMahon et al. 2013). Tadpoles and non-susceptible adults (Woodhams et al. 2008b; Schloegel et al. 2010; Reeder et al. 2012) can also act as reservoirs for infection.

Bd may kill frogs through the production of toxic compounds, or by epidermal disruption leading to osmotic imbalance (Berger et al. 1999). While no toxins have been found, *Bd* supernatants contain proteases and lipases that disrupt intercellular junctions (Brutyn et al. 2012). This disruption causes osmotic imbalance (Voyles et al. 2007) which leads to asystolic cardiac arrest and death (Voyles et al. 2009). Symptoms of chytridiomycosis in adult hosts usually only develop a few days before death and can include reddening of the ventral surface, lack of movement or interest in food, an increase in skin sloughing, and loss of righting reflex (Berger et al. 1999). In tadpoles, mouthpart loss may prevent feeding and lead to weight loss, but mortality only occurs in some species (Blaustein et al. 2005).

Variation in host susceptibility to *Batrachochytrium dendrobatidis*

Despite the growing body of knowledge on the pathogenicity and epidemiology of *Bd*, much remains unknown about the origins and spread of this fungal pathogen, and why some amphibian species are more susceptible to infection and disease than others. Over the last

decade, studies have revealed that pathogen virulence, host health, host and environmental temperatures, habitat selection and host innate immune defences may all contribute to variation in host susceptibility to some extent (Berger et al. 2005; Woodhams and Alford 2005; Rowley and Alford 2007b; Fisher et al. 2009; Richards-Zawacki 2010; Woodhams et al. 2010; Puschendorf et al. 2011).

Pathogen virulence can vary with *Bd* strain. Berger et al. (2005) exposed *L. caerulea* to three different Australian *Bd* strains and found that the time to death varied with the strain and possibly the time that the strain had been in culture. Similarly, *Bufo bufo* toads exposed to three different European strains of *Bd* (Fisher et al. 2009), and *Pseudacris triseriata* frogs exposed to two North American strains (Retallick and Miera 2007), showed a significant difference in mortality among treatment groups. There is also some evidence that host body condition can also affect susceptibility to infection and disease. In one trial, lighter *B. bufo* toads died earlier than heavier toads when infected with *Bd* (Fisher et al. 2009), and *Lithobates sphenocephalus* tadpoles showed a lower prevalence of *Bd* infection when fed on a high protein diet (Venesky et al. 2012).

Temperature and behaviour

Environmental temperatures can impact both *Bd* growth and host ability to respond to pathogens. *Bd* is tolerant of a range of temperatures between 4 and 30^{o}C, but grows optimally in culture from 17-23^{o}C (Johnson et al. 2003; Piotrowski et al. 2004). It is unable to tolerate complete desiccation (Johnson et al. 2003). At temperatures from 7 - 10^{o}C, *Bd* can increase fecundity with zoospores remaining active, and therefore infective, for longer (Woodhams et al. 2008a). This might partially explain why Australian tropical frogs at higher elevation sites in winter are more strongly impacted by *Bd* infection than frogs at lowland sites (Woodhams and Alford 2005; Woodhams et al. 2008a). Experimental exposure of infected *L. chloris* frogs held in a naturally fluctuating temperature regime with two, eight hour temperature increases to 37^{o}C, can eliminate *Bd* infection (Woodhams et al. 2003). While air temperatures this high do not occur in many rainforest environments, substrate temperatures well above the thermal limits for *Bd* growth can be experienced by frogs in open forest habitats (Puschendorf 2009; Daskin et al. 2011), and frogs basking in patches of sunlight may attain temperatures well above 30^{o}C (Rowley 2006). The effect of a one-hour daily temperature spike of 33^{o}C, on *Bd* cultures grown at a constant 15^{o}C, significantly reduced *Bd* growth compared to controls (Daskin et al. 2011). Therefore, any ability of frogs to elevate body temperatures above the thermal optimum for *Bd*, even for short periods, may affect disease outcomes (Daskin et al. 2011). In this way, dry forest habitats may act as refugia against disease, providing a source

population from which to recolonise upstream rainforest sites following declines (Puschendorf 2009). Temperature may also affect the host immune response, and, therefore, the ability to reduce *Bd* infection. Lower temperatures are associated with reduced host ability to respond to potential pathogens (Raffel et al. 2006; Ribas et al. 2009).

The temperature constraints that limit *Bd* growth are linked to the behaviour of individual hosts. For example, frogs that bask may raise their body temperature beyond the thermal optimum for *Bd* (Rowley 2006). Different species may prefer different basking temperatures (Gantz and Sheafor 2012) which could account for variation in susceptibility to, or fate from, *Bd* infection. Different microhabitat use was correlated to *Bd* susceptibility in three species of Australian frogs that suffered declines to different extents (Rowley and Alford 2007a). Behavioural fever, in which individual ectothermic animals behaviourally adjust body temperature to combat infection by a pathogen, may be used by the Panamanian golden frog, *Atelopus zeteki* (Richards-Zawacki 2010), and the boreal toad, *B. boreas* (Murphy et al. 2011), to control *Bd* infection.

Acquired and innate immune responses

Amphibians have well developed immune systems, with both innate and adaptive components (Rollins-Smith and Woodhams 2011). The role of the adaptive immune system in defence against *Bd* has received much attention, with some studies indicating a possible adaptive response and others demonstrating no such evidence (Richmond et al. 2009). Major histocompatibility complex (MHC) allele frequencies varied significantly between infected and uninfected natterjack toads, *B. calamita*, suggesting a possible adaptive immune response (May et al. 2011). Adaptive immunity may also be important in the frog, *X. laevis*, which has a demonstrated an antibody response to *Bd* infection (Rollins-Smith et al. 2009; Ramsey et al. 2010). However, the same methodology failed to produce an antibody response in *B. boreas* (Rollins-Smith et al. 2009), suggesting species-specific variation in this ability. Little evidence of an adaptive response to *Bd* was found in *X.* (*Silurana*) *tropicalis* (Ribas et al. 2009; Rosenblum et al. 2009); and immunisation of juvenile *Rana muscosa* was not effective at generating adaptive immunity (Stice and Briggs 2010). However, a similar immunisation experiment with *B. boreas* found that toads with prior exposure to *Bd,* housed in dry environments, survived three times as long following a second exposure compared with toads that had not previously been exposed (Murphy et al. 2011).

Anti-microbial peptides (AMPs), secreted by the skin glands of a number of amphibian species, are part of the host innate immune defence against pathogens, and can be effective *in vitro*

against *Bd* (Rollins-Smith and Conlon 2005; Rollins-Smith 2009; Conlon 2011). However, despite a large number of *in vitro* studies demonstrating their potential (Rollins-Smith et al. 2002a; Rollins-Smith et al. 2002b; Woodhams et al. 2006a; Woodhams et al. 2006b; Woodhams et al. 2007a; Kenyon 2008; Woodhams et al. 2010), the role that they play *in vivo* is not fully understood (Conlon 2011). One recent study has demonstrated that AMPs on the skin of resting northern leopard frogs, *R. pipens*, are of sufficient concentration to inhibit the growth of *Bd*, suggesting that AMPs are indeed likely to play a role in the innate immune response against *Bd* in this species (Pask et al. 2012). Further studies of this type, linking the constitutive amounts of AMPs present on amphibian skin to that necessary for *in vitro Bd* inhibition, will be useful to elucidate their role in vulnerable host species.

Bacterial symbionts

Frogs may also gain protection against *Bd* through mutualist bacteria present on their skin. If *Bd* has only recently emerged, a topic under much debate (Morehouse et al. 2003; Fisher et al. 2009; James et al. 2009; Farrer et al. 2011; Schloegel et al. 2012), there may have been little time for evolution of specific host defences against it. However, frogs carry a suite of resident cutaneous bacteria that do not harm them, some of which may be antagonistic to invading pathogens. In line with many of the ideas presented in the Hologenome Theory of Evolution (Zilber-Rosenberg and Rosenberg 2008; Rosenberg et al. 2010; Rosenberg and Zilber-Rosenberg 2011); if a frog with all its microbial symbionts is viewed as a holobiont (one unit of evolutionary selection; Reshef et al. 2006), then the more rapid evolution of the bacterial symbionts, compared with the host's innate and acquired immune defences, can lead to improved host fitness. This improved fitness can facilitate coexistence of host and pathogen (Belden and Harris 2007). It therefore seems possible that frogs that have recovered or recolonised at sites where they once existed prior to *Bd*-related population declines, may have done so due to improved fitness caused by selection acting on the beneficial symbiotic microbiota they carry.

It is entirely possible that multiple mechanisms may act synergistically allowing coexistence of amphibian populations with enzootic *Bd* infection. However, these mechanisms will not necessarily be the same among species, or even for the same species at different sites, due to the different selection pressures that shape amphibian populations. For example, in one population, behavioural thermoregulation may be a primary method of *Bd*-antagonism with other mechanisms less important; while in another population symbiotic bacteria and AMPs may work synergistically to protect host species against *Bd* (see Myers et al. 2012).

Strategies to mitigate Batrachochytrium dendrobatidis

While the maintenance of threatened amphibian populations in assurance colonies can prevent the immediate extinction of species (Gagliardo et al. 2008; Zippel et al. 2011), reintroduction of these animals into areas where *Bd* is enzootic presents challenges. However, a number of potential strategies have been proposed for the control and treatment of *Bd* infection *in situ* (Australian Department of Environment and Heritage 2006; Woodhams et al. 2011). Strategies that have shown some potential in early trials, through reduction of infection intensity in susceptible hosts, include pond drying to treat potential disease reservoir habitat (Lubick 2010), and exposure of frogs to weak sodium chloride environments (Stockwell et al. 2012). Another strategy that has met with partial success is host immunisation against *Bd*; trials have stimulated a protective effect in some species (Rollins-Smith et al. 2009; Ramsey et al. 2010; Murphy et al. 2011), while they have been completely unsuccessful in others (Rollins-Smith et al. 2009; Stice and Briggs 2010). Further investigations in this research area may yet prove successful.

Other strategies are still at the concept stage or do not translate well to large-scale field applications. For example, a novel idea for reducing *Bd* loads in pond ecosystems is the introduction of predatory zooplankton, which are naturally present in many water bodies inhabited by amphibians (Woodhams et al. 2011). Laboratory trials have demonstrated that *Daphnia* sp. can consume *Bd* zoospores, but it is not yet known whether they will do so in the environment (Buck et al. 2011; Woodhams et al. 2011). Treatment of amphibians with antifungal drugs or heat has been extremely successful in eliminating *Bd* infection in laboratory trials (Woodhams et al. 2003; Berger et al. 2010; Chatfield and Richards-Zawacki 2011; Geiger et al. 2011; Martel et al. 2011). However, the toxicity of antifungal drugs, and their potential effects on non-target species, does not make them ideal for use in the natural environment. It has also been suggested that thermal habitat modification, through provision of heating stations, may increase the opportunity for behavioural reduction of *Bd* infection (Woodhams et al. 2011). This idea shows potential for small-scale pond ecosystems, but is largely impractical for large tracts of inaccessible rainforest.

Bioaugmentation for amphibians

There are no known mitigation strategies that offer as much hope for long-term amphibian defence as bioaugmentation with protective bacteria (Harris et al. 2009b). Amphibian skin is colonised by microbial communities that may confer disease resistance to their hosts (Bettin and Greven 1986; Austin 2000; Harris et al. 2006; Lauer et al. 2007; Woodhams et al. 2007c; Banning et al. 2008; Lauer et al. 2008). Bettin and Greven (1986) were the first to suggest that bacteria found on salamander skin might contribute to host innate immune defence by producing compounds that inhibit pathogen colonisation, but it was many years before Austin (2000) demonstrated that bacteria isolated from salamander skin had *in vitro* antimicrobial activity against possible environmental pathogens. In a more recent study, Lauer et al. (2007) found that 89% of redback salamanders, *Plethodon cinereus*, had culturable skin bacteria that had *in vitro* activity against *Mariannaea elegans* var. *punicea*, a fungus pathogenic to salamander eggs.

It is now known that many bacteria present on amphibian skin can produce compounds with *in vitro* fungicidal activity against *Bd* (Chapters Four and Five; Harris et al. 2006; Woodhams et al. 2007b; Lam et al. 2010; Walke et al. 2011). These bacteria could be harvested from amphibians in populations that are more resistant to *Bd* and applied to individuals from susceptible populations, thus conferring disease resistance (Harris et al. 2006). This addition of bacteria is known as "bioaugmentation", and has tremendous potential to provide a natural, persistent and self-disseminating tool to assist vulnerable amphibian populations while they adapt to *Bd* presence. Disease mitigation through bioaugmentation should target two main groups of amphibians; those to be released from survival assurance colonies into *Bd*-enzootic areas, and natural populations threatened with extirpation due to imminent arrival of *Bd*.

There is strong evidence for the role of cutaneous bacteria in host protection against *Bd*. In one laboratory experiment, salamanders that had their natural microbiota depleted prior to *Bd* exposure, lost a significantly greater amount of weight (one symptom of infection) than those that had a complete microbiota, showing that changes to the microbial community can impact disease resistance (Becker and Harris 2010). A subsequent laboratory study has shown that *P. cinereus* can be successfully inoculated with a bacterium that can produce *Bd*-inhibitory compounds *in vitro*; this mitigated the weight loss often observed following *Bd* infection (Harris et al. 2009b).

Several experimental studies have focussed on the inhibitory characteristics of the bacterium *Janthinobacterium lividum*, which has been found on amphibian skin in a number of field

surveys (Lauer et al. 2007; Woodhams et al. 2007c; Lauer et al. 2008; Lam et al. 2010). Two antibiotic metabolites produced by *J. lividum*, identified as violacein and indole-3-carboxaldehyde, are both capable of inhibiting *Bd in vitro* (Brucker et al. 2008b). Violacein can be present on *P. cinereus* skin in concentrations sufficient to totally inhibit *in vitro Bd* growth (Brucker et al. 2008b). Inoculation of *P. cinereus* with *J. lividum* increased the concentration of cutaneous violacein; salamanders survived experimental infection with *Bd* provided that the violacein concentration was at least 18μM (Becker et al. 2009). This provides evidence for antibiosis as the mechanism of *Bd*-inhibition in this host-symbiont partnership.

The strongest evidence for the potential of bioaugmentation as a preventative technique comes from two recent experiments where *J. lividum* was added to the skin of the frog *R. muscosa* (Harris et al. 2009a; Vredenburg et al. 2011). In the first experiment, the bacterial addition successfully prevented morbidity and mortality from experimental *Bd* infection in captive-raised frogs (Harris et al. 2009a). In the second experiment, a field trial on a population predicted to go extinct with the arrival of *Bd*, a higher proportion of frogs treated with *J. lividum* survived than untreated controls (Vredenburg et al. 2011).

The long-term success of broad-scale applications will largely depend on the ability of probiotic bacteria to persist on the host or in the host's environment. However, only a small proportion of bacteria from the surrounding environment are found on amphibian skin (Austin 2000; Culp et al. 2007; Banning et al. 2008; McKenzie et al. 2012), suggesting that host colonisation is controlled, probably by the innate and acquired immune response, and by other resident cutaneous symbionts. A probiotic bacterium should therefore ideally be present in the environment, and not be recognised as foreign by the host. The success of *J. lividum* in the above trials is possibly attributable to its specificity to the host amphibians tested. Treatment of a captive colony of the Panamanian golden frog, *A. zeteki*, with *J. lividum* failed to provide any protective effect against *Bd*, possibly because *J. lividum* may not naturally be present in this Panamanian species or its habitat (Becker et al. 2012). This highlights the possibility that probiotics need to be developed for each species and habitat. Treating multiple species within a habitat offers particular challenges, especially where species harbour different bacterial communities (see McKenzie et al. 2012). A bacterial cocktail comprising a number of bacteria, each specific to a host species, may be necessary to address this issue.

J. lividum can survive in the digestive tract of *P. cinereus*, suggesting that the gut may act as a reservoir for reinoculation of skin or soil (Wiggins et al. 2011). *J. lividum* can also be passed from the soil to the salamander, subsequently inhibiting *Bd* colonisation (Muletz et al. 2012);

this suggests that environmental bioaugmentation may be a viable application strategy. Bacteria can also persist by horizontal or vertical transmission among individuals. There is evidence for vertical transmission of bacterial symbionts in species that exhibit parental care. For example, the bacterial microbiota of adult glass frogs, *Hyalinobatrachium colymbiphyllum*, and the egg masses that they guarded were similar, suggesting that the bacterial microbiota was transferred from adult to embryos (Walke et al. 2011). Horizontal transmission has not yet been demonstrated but it may occur during mating, or among individuals that share hibernacula or communal retreat sites.

The protective effect that bioaugmentation can confer on susceptible amphibian populations may result from herd immunity. This is where a high proportion of a population has immunity against a pathogen (usually 70-80%), which reduces pathogen transmission thus protecting the population as a whole (Anderson and May 1985). A study by Woodhams et al. (2007c) investigated the presence of *Bd*-inhibitory bacteria on two threatened *R. muscosa* frog populations that had been exposed to *Bd* for different periods of time. They found that more individuals within the population with enzootic *Bd* infection had one or more anti-*Bd* bacteria than a population recently exposed to the pathogen. This newly exposed population was subsequently extirpated (Lam et al. 2010). A further study resurveyed both the surviving population and a second *Bd*-naïve population, predicted to be extinguished if *Bd* emerged, and found that approximately 80% of frogs in both populations had antifungal bacteria (Lam et al. 2010). When *Bd* later emerged in the naïve population, it survived, suggesting that the presence of antifungal bacteria within a high proportion of an amphibian population may provide a protective affect analogous to herd immunity (Lam et al. 2010). A similar effect might be observed in other amphibian populations that have enzootic *Bd* infection.

Despite the enormous progress in this research area to date, a number of important questions remain unanswered. There are some minor problems with the methodology used to select and test bacteria for antibiotic production that could easily be addressed by relatively small alterations to current techniques. In addition, the stability of the cutaneous bacterial assemblage over time has not been determined and this could potentially affect bioaugmentation success. Furthermore, it is not known whether bacterial assemblages differ among hosts with and without *Bd* infection, or whether particular characteristics of bacterial assemblages are associated with intensity of *Bd* infection.

Aims of this thesis

Previous research on variation in amphibian susceptibility to *Bd* infection among populations has focussed on host behaviour, exposure to environmental temperature extremes, and innate immunity (Rowley and Alford 2007b; Puschendorf 2009; Woodhams et al. 2010). The patterns of frog population declines and recovery in the Australian Wet Tropics rainforests provide an ideal study system to test the hypothesis that the resident cutaneous bacterial microbiota has adapted since the arrival of *Bd* to benefit host survival. This thesis broadly aims to expand current knowledge of the cutaneous bacterial ecology of rainforest frogs. It has three specific aims:

1. To determine effectiveness of techniques used to select and test the bacteria resident on frog skin for *Bd*-inhibitory potential.

2. To determine whether the amphibian cutaneous bacterial microbiota may be important in the coexistence of *Bd* with a variety of frog species at various sites in the Australian Wet Tropics.

3. To investigate the stability of the resident cutaneous bacterial assemblage with time and changing infection status of individual animals.

This thesis consists of eight chapters. Chapter One (this chapter) provides historical background and context to the study. It outlines the aims and structure of the thesis. The description of amphibian declines and the ecology of *Batrachochytrium dendrobatidis* are presented here alone and are not duplicated in the introductions to each chapter. Chapter Two presents general methods common to many individual chapters and thus removes much redundancy from the remaining methods sections. Chapters Three to Seven are prepared in manuscript format for submission to journals; details pertaining to journal submission are provided at the start of the chapter where applicable. Chapters Three and Four address issues related to laboratory techniques used to date. Chapter Three addresses whether the current methods used to select bacterial isolates are sufficient to capture the culturable bacterial assemblage present on agar plates. It also assesses the proportion of bacteria that are unculturable using standard aerobic culture techniques. Previous studies have investigated potential inhibition of *Bd* by anti-fungal bacterial metabolites using an agar-based challenge technique, and this technique can present a number of issues leading to inconclusive results. Chapter Four describes and trials a new method for testing bacterial isolates for the production of antifungal metabolites active against *Bd*, which avoids many of the problems

associated with agar-based techniques. Having resolved a number of methodological issues in Chapters Three and Four, revised techniques are used to generate data for Chapters Five and Six. Chapter Five describes the effectiveness of cutaneous bacterial metabolites in inhibiting *Bd* in frog species and sites with different population decline histories, while Chapter Six identifies the bacteria capable of complete inhibition of *Bd* and investigates potential for bacterial control of *Bd* in wild frog populations. Chapter Seven tracks changes in the bacterial assemblage over time in individual members of a wild population of rainforest frogs, and investigates differences relating to changing *Bd* infection status. Chapter Eight provides overall discussion and directions for future research in this area.

CHAPTER TWO : GENERAL METHODS

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Chapter Overview

Many materials and methods used in this thesis are common to a number of the chapters. This chapter describes them in detail and each data chapter thereafter refers here when summarising the methods used, and only describes methods that are unique to the data presented therein.

Materials and Methods

Field sites and study species

Sampling was conducted in the Wet Tropics and Cape York Peninsula bioregions in northern Queensland, Australia. Three study species endemic to the Wet Tropics bioregion were selected for their susceptibility to infection by the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) and their different decline histories; *Litoria serrata,* the green-eyed tree frog, *L. rheocola*, the common mist frog, and *L. nannotis* , the waterfall frog (Figure 2.1). Two species of frogs were selected from the Cape York Peninsula bioregion to represent populations thought to be unexposed to *Bd*; *L. eucnemis* , the fringed tree frog, and *L. longirostris* , the long-snouted tree frog (Figure 2.1). The Wet Tropics species selected are not present on Cape York, however, *L. eucnemis* is a sister species to *L. serrata*, distinguishable principally by call (Richards et al. 2010), and is therefore likely to be comparable in many respects. All species are found predominantly in rainforest (Figure 2.2), but their ranges can extend to bordering wet and dry sclerophyll forest, and all are usually associated with flowing streams.

Four principal field sites were chosen in the Wet Tropics bioregion, with one upland and one lowland site in each of two latitudinally separated national parks. The upland sites were subject to extensive frog population declines in the early 1990s, while frog populations at the lowland sites persisted. One additional upland field site was selected at Upper Peach Creek in Kulla National Park in Cape York Peninsula Bioregion. Wet Tropics sites were Kirrama Bridge 11 and Kirrama Bridge 8 Creeks in Murray Falls, Girramay National Park; and Windin North and Frenchman's Creeks in Wooroonooran National Park. Site locations and habitat types are presented in Table 2.1 and Figure 2.3.

Table 2.1. Field sites and habitat description

Site	National Park	Lat/Long	Elevation (m)	Habitat
Windin North Creek	Wooroonooran National Park	17°22’01.6”S 145°42’58.3”E	750	Complex mesophyll vine forest
Frenchman's Creek	Wooroonooran National Park	17°18’32.8”S 145°55’04.2”E	40	Complex mesophyll vine forest
Kirrama Bridge 11 Creek	Murray Falls, Girramay National Park	18°12’49.9”S 145°47’52.9”E	750	Simple mesophyll vine forest
Kirrama Bridge 8 Creek	Murray Falls, Girramay National Park	18°11’44.8”S 145°52’05.4”E	170	Simple mesophyll vine forest
Upper Peach Creek	Kulla National Park	13°44’13.7”S 143°20’20.1”E	550	Mesophyll vine forest

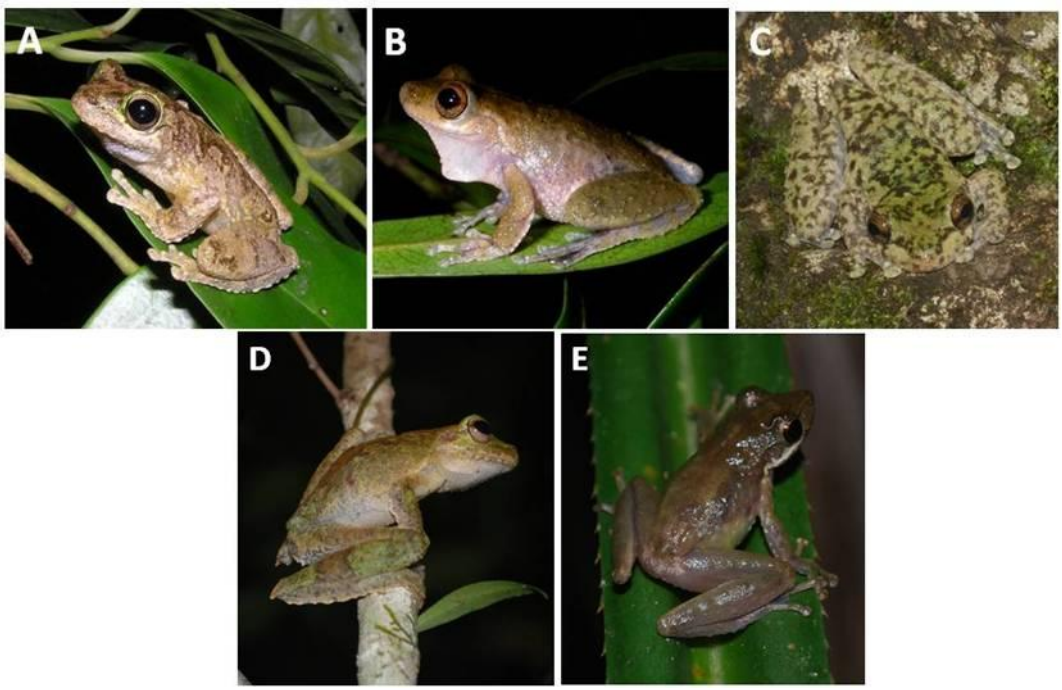

Figure 2.1. Study species. A *Litoria serrata*; B *Litoria rheocola*; C *Litoria nannotis*; D *Litoria eucnemis*; E *Litoria longirostris*.

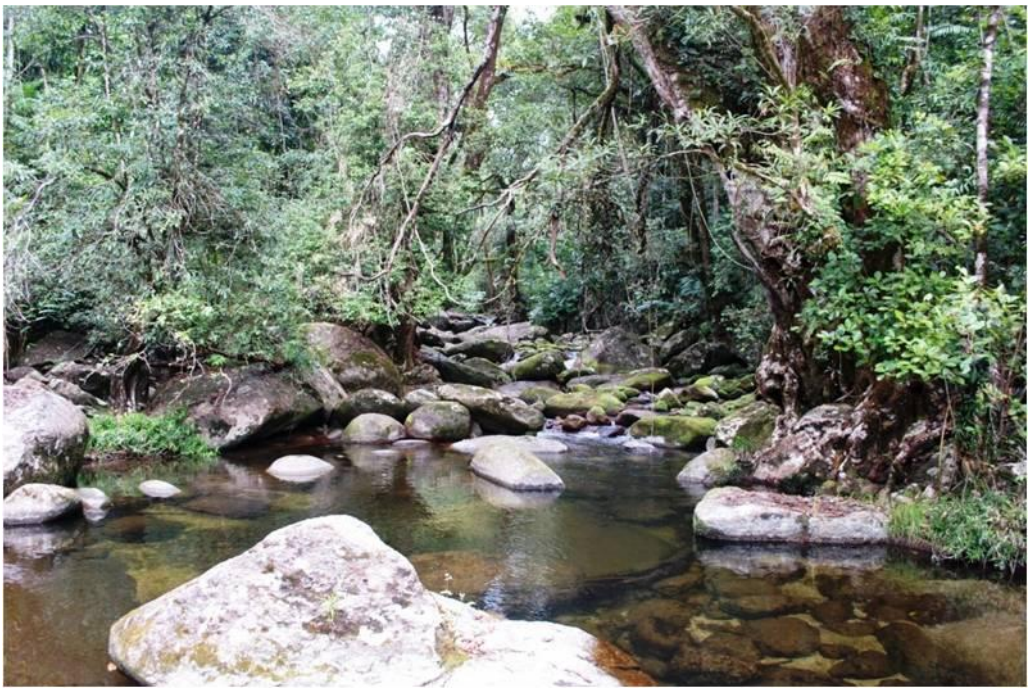

Figure 2.2. Typical rainforest stream habitat at Frenchmans Creek, Wooroonooran National Park, northern Queensland, Australia.

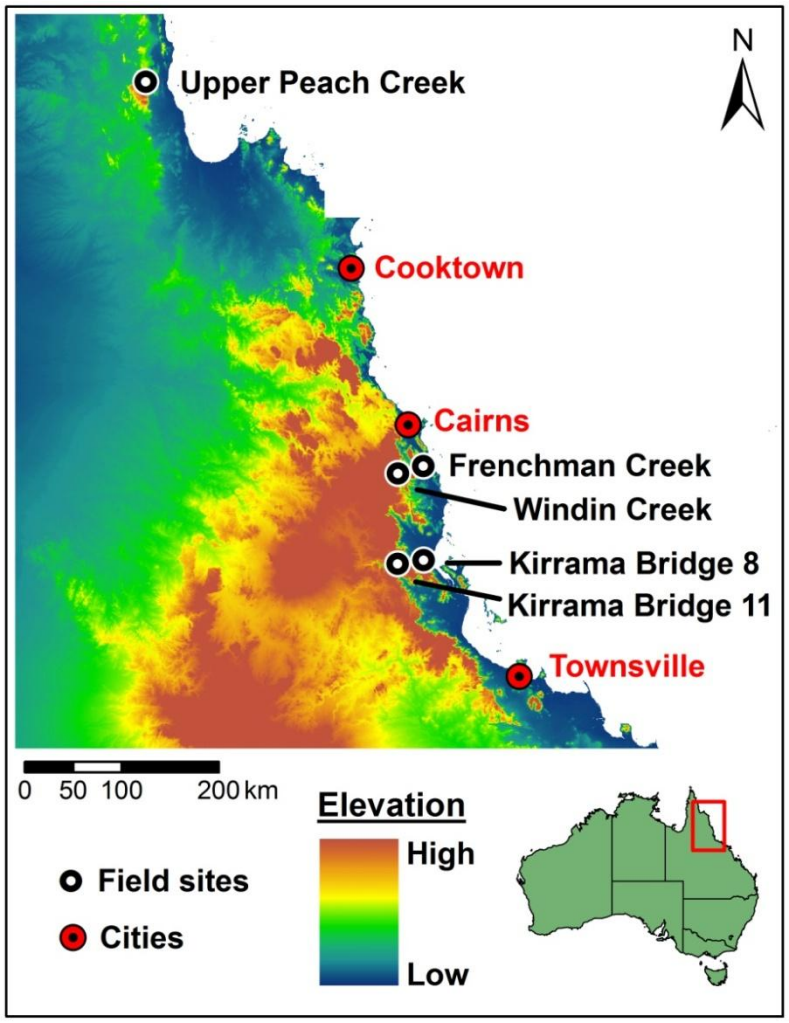

Figure 2.3. Location of field sites in northern Queensland, Australia.

Collection of samples

Sampling was conducted in winter, when *Bd* infection is typically more prevalent, to maximize chances of finding *Bd*-infected frogs, and to make the most of the opportunity to examine potential interactions of *Bd* infection with the cutaneous microbiota. Frogs were hand-captured from vegetation and rocks bordering the creeks, in the winters of 2009 and 2010, using new plastic bags for each individual to prevent cross-contamination of samples. Subsequent handling was carried out with a new pair of vinyl gloves for each animal to prevent contact with human skin bacteria and to preclude the transfer of pathogens or symbionts between animals. Animals were gently restrained in the hand and rinsed twice with a stream of sterile distilled water from a wash bottle to remove transient bacteria, which can differ from the resident microbiota (Lauer et al. 2007). Swab samples were collected with sterile rayon swabs (MW112, MW&E, Bath UK) moistened with sterile distilled water. Often two swab samples were collected at once. The swabs were wiped and rotated over the dorsal and

ventral skin from knee to neck five times and one swab was placed in a sterile microtube and frozen immediately in a flask of dry ice. The second swab's bacteria were immediately transferred to a low-nutrient agar plate (R2A, BD, New Jersey, US) by rotating the swab on the plate surface in a wide zig-zag pattern. Agar plates were sealed with parafilm (Parafilm "M", Pechiney Plastic Packaging Co. Chicago, US), held at ambient temperature (10-25^{o}C) and returned to the laboratory within 72 hours. Finally, an additional swab sample (MW100, MW&E, Bath UK) was collected by rotating the swab over the abdomen, hands, feet and thighs twice for analysis of *Bd* infection status. The individual was weighed and then released at the point of capture. Frozen samples were transported to the laboratory and placed in a -80^{o}C freezer until further DNA analysis could be performed.

Isolation, purification, and storage of microbial cultures

Inverted R2A agar plates were incubated for 48-72 hours at ambient temperature (22-25^{o}C) in the laboratory until microbial culture growth was observed. Plates were examined daily for five days and colonies with different morphological characteristics were identified using a dissecting microscope. One sample of each morphologically unique colony was then isolated to pure (axenic) culture using standard microbiological techniques (Salle 1961). Briefly, each unique colony was selected using a sterile toothpick and streaked multiple times on non-overlapping sections of a new R2A agar plate to thin out the number of bacterial cells. The plate was then left to grow for 24 - 48 hours. This process was repeated until no contaminating organisms were observed. Each putatively axenic isolate was further checked for purity by examining Gram stained cells using oil immersion light microscopy. If contamination was present, the mixed culture was subject to further purification until no contaminating organisms were observed. Axenic isolates were held on R2A agar slants at room temperature until challenge assays were conducted. A copy of each isolate was also frozen at -80^{o}C in 1ml trypticase soy yeast broth with 20% glycerol (2g trypticase soy broth, 1g yeast extract, 200ml glycerol, to 1L deionised water, autoclaved).

Diagnosis of *Batrachochytrium dendrobatidis* infection

Quantitative PCR (Real-time TaqMan® assay) was used to diagnose *Bd* infection status as per Boyle et al. (2004). While it was impossible to determine whether detection of extremely low levels of *Bd* DNA truly represented low-level *Bd* infection, cross-contamination was ruled out as a source of apparent infection as sampled animals were generally not found in close proximity to one another or in contact with water, and new gloves were worn for each animal handled. In addition, stringent laboratory protocols and controls were used reduce potential contamination. Therefore, I considered frogs to be positive for *Bd* infection when more than

zero zoospore equivalents were recorded in at least two of three replicate wells, because it is conceivable that one zoospores detected in just one well could be caused by an aerosol in the laboratory, but would be considerably less likely to be detected in two or more wells.

Bacterial DNA extraction

Axenic isolates

A sterile toothpick was used to inoculate each axenic isolate into a 1.7ml sterile microtube containing 400µl molecular grade water. Samples were vortexed to create a cell suspension and subjected to three freeze-thaw cycles (70^{o}C/-80^{o}C; 10 minutes each), and then centrifuged at 7500 x g for 5 minutes to pellet the cell debris. The supernatant was used directly as template in the DNA amplification reaction. If this was unsuccessful, isolates were extracted using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Doncaster, VIC, Australia) as per the manufacturer's protocol, with pre-treatment for Gram-negative bacteria.

Swab samples

DNA was extracted from bacterial swab samples using a Qiagen DNeasy Blood and Tissue Kit with the following modifications. Swabs were pre-treated as per the recommended Gram-positive bacteria DNA extraction procedure by incubation at 37^{o}C for 30 minutes with 180 µl enzymatic lysis buffer (20 mM Tris·Cl adjusted to pH 8.0, 2 mM sodium EDTA , 1.2% Triton® X-100 , 20 mg ml^{-1} lysozyme). Prior to addition of ethanol, microtubes containing swabs were inverted and a fine hole was pierced in the base with a red hot probe. The tube was then placed inside a new microtube and both tubes were centrifuged for a few seconds in order for the liquid from the swab to pass to the new tube. The tube containing the dry swab was then discarded and the procedure then continued with addition of ethanol to the new tube. DNA was eluted with 100µl Buffer AE and stored frozen at -20^{o}C until required.

Amplification of 16S rRNA gene

The 16S ribosomal DNA gene is used widely in studies of bacterial diversity (Olsen et al. 1986; Sánchez et al. 2007; Youssef et al. 2009). 16S rDNA from pure bacterial isolates was amplified by Polymerase Chain Reaction (PCR) on Bio-Rad C1000/S1000 thermal cyclers (Bio-Rad, Hercules, CA) with the bacteria-specific primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane 1991). The PCR reaction mix contained 0.2 µM of each primer, 0.2 mM dNTPs, 3 mM $MgCl_2$, 0.2 mg ml^{-1} BSA, 1.25U HotStar Taq polymerase (Qiagen, Doncaster, Victoria, Australia) with 1x buffer and <1 ug template DNA. The thermocycling parameters were: 95^{o}C for 15 minutes followed by 35 cycles of 94^{o}C

for 1 minute, 48^{o}C for 1 minute, 72^{o}C for 1.5 minutes and a final elongation for 10 minutes at 72^{o}C.

DNA from swab samples or axenic isolates to be analysed on denaturing gradient (DG) gels was amplified in triplicate by PCR using the bacteria specific primer 1055F (5'-ATGGCTGTCGTCAGCT-3') and the GC clamped primer 1392R (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCACGGGCGGTGTGTAC-3') as described by Ferris et al. (1996) to amplify the variable V7 and V8 regions of the 16S rRNA gene (Baker et al. 2003). Samples were amplified in triplicate PCR reactions to increase the chances of amplification of low copy number DNA fragments. The PCR reaction mix comprised 0.4 µM of each primer, 0.2 mM dNTPs, 4 mM MgCl2, 0.2 mg ml^{-1} BSA, 1.25U Qiagen HotStar Taq polymerase with 1x buffer and <1 ug template DNA. The thermocycling parameters were: 95^{o}C for 15 minutes followed by 10 cycles touchdown PCR comprising 94^{o}C for 1 minute, 58^{o}C for 1 minute incrementing by -0.5^{o}C per cycle, and 72^{o}C for 1 minute, followed by 25 cycles of 94^{o}C for 1 minute, 53^{o}C for 1 minute, 72^{o}C for 1 minute and a final elongation for 20 minutes at 72^{o}C to reduce double banding that can sometimes be observed in DG gels (Janse et al. 2004). Presence of PCR products was examined by electrophoresis in 1.5% agarose gels stained with GelGreen™ (Biotium, Hayward, CA).

Purification and concentration of PCR product

PCR product amplified from frog swab extracts was not typically of a high enough concentration to visualize clearly on a denaturing gradient gel. Therefore, sufficient PCR product was produced and concentrated so that bands could be clearly seen. Triplicate PCR products (each from 50 µl PCR reactions) were combined and purified by passing through a Sephadex™ G-50 column to remove surplus potential contaminants such as unincorporated primers and dNTPs. Purified PCR product was placed in a vacuum concentrator (Savant SpeedVac, Thermo Fisher, Waltham, MA) to reduce total volume to a level that could be loaded on the DG gel without wells overflowing.

Marker Ladder development

A marker ladder was developed from 16S rRNA PCR amplicons of axenic isolates with the aim that this would be run alongside samples on each DG gel to enable comparison of results between gels. Twenty axenic bacterial isolates were tested for their ability to migrate different distances in a DG gel. DNA extraction and PCR amplification was conducted as described above. PCR amplicons were run on a DG gel (see below section on Denaturing Gradient Gel

Electrophoresis for details). Six isolates that migrated different distances were selected and their PCR amplicons combined to form a marker ladder.

Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) of PCR amplicons for bacterial profiling was carried out on a Bio-Rad DCode system (Bio-Rad, Hercules, CA) as per manufacturer's instructions. 6.5% acrylamide gels with 40 – 60% gradients were formed (where 100% denaturant contained 7 M urea and 40% formamide) and placed in 1 x TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) preheated to 60^{o}C. Gels were loaded with marker ladder in three positions; second lane from left, second lane from right and approximately centrally. Outer lanes were not used due to the curving of bands that often occurs with this technique. Gels were run at 70V for 17¾ hours at 60^{o}C; the recirculating buffer pump was switched on after 15 min of electrophoresis to ensure DNA had been adequately drawn into the gel. Gels were stained in 3x GelRed^{TM} (Biotium, Hayward, CA, USA) with 0.1 M NaCl for 60 minutes, and visualized and photographed on a UV transilluminator (Syngene, Cambridge, UK).

CHAPTER THREE : DO STANDARD MICROBIOLOGICAL CULTURING TECHNIQUES ADEQUATELY CAPTURE THE RICHNESS OF BACTERIAL TAXA PRESENT ON RAINFOREST FROG SKIN?

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Chapter Overview

There are a number of technical issues associated with culture and assay of bacterial isolates in the laboratory. This chapter addresses problems associated with laboratory culture and detection of bacterial isolates collected from frog skin.

Abstract

It is commonly believed that 99% of bacteria present in environmental samples cannot be cultured in the laboratory due to their highly specific growth requirements. This could be a serious problem for research in bioaugmentation; if the great majority of bacteria on amphibian skin are not culturable, many species with desirable properties are likely to be unavailable. Few studies have compared the culturable and uncultured bacteria present on amphibian skin, and none have determined the proportion of cultured bacteria that are missed during the process of colony selection for isolation. The objectives of my study were, firstly, to determine the proportion of initially cultured bacteria that are not subsequently selected for isolation because selection is based on gross colony morphology, and secondly, to compare the cultured and uncultured bacteria present on each frog to estimate the proportion of isolates that are not culturable. I collected two skin swab samples from each of ten green-eyed tree frogs, *Litoria serrata,* on Kirrama Bridge 8 Creek in northern Queensland, Australia. One swab was immediately frozen and the other was transferred to an agar plate. After two days and before seven days of growth, I selected all morphologically different bacterial colonies from the agar plate and passaged them to axenic culture. After seven days growth, I swabbed the agar plates to collect samples of all bacteria present. DNA was extracted from all frozen swabs and axenic isolates, amplified by PCR, and run on denaturing gradient gels to produce a banding pattern for each sample, with each band representing a unique species-level ribotype. For each frog, I compared the number of bacterial ribotypes isolated from plates, total ribotypes present on plates, and total ribotypes on frog swabs. On average, 75% of the bacteria on individual *L. serrata* were culturable; this is far higher than the commonly assumed 1%. However, of those culturable bacteria, only 53% were selected for isolation, which suggests that apparently morphologically identical colonies should be overselected during initial isolation to reduce the proportion that is missed. If this is done, my results indicate that

this standard culture technique will capture the majority of bacteria resident on frog skin, so that most bacteria with potential for bioaugmentation are likely to be cultured and screened.

Introduction

Environmental bacteria can be difficult to culture, with up to 99% non-culturable (Sharma et al. 2005). There has been much effort to understand the reasons behind this phenomenon and to find ways to culture increasing numbers of fastidious organisms, in order to understand their physiology. These "unculturable" bacteria may have specific requirements for particular nutrients, temperatures, pH, or oxygen levels, and multiple media types or culture conditions may be required to maximize the chances of their recovery (Vartoukian et al. 2010). In addition, many microbes are likely to be dependent upon others, and are not always possible to culture in isolation (Moran 2006). Conversely, competition within mixed communities may also inhibit the growth of some species (Vartoukian et al. 2010). The ability to culture bacterial isolates is important for the field of bioaugmentation research; if only a low proportion of bacteria are culturable, screening programs are likely to fail to detect potentially important isolates. Therefore, many bacteria important in combating targeted pathogens may be unusable because they cannot be cultured.

A number of culture-independent molecular techniques, using the bacterial 16S ribosomal DNA gene, can be used to create total bacterial assemblage "ribotype" fingerprints. These can provide comparisons with the richness of culturable species. While all techniques have advantages and disadvantages, denaturing gradient gel electrophoresis (DGGE; Muyzer et al. 1993) offers a rapid and economical means of creating and comparing multiple community DNA fingerprints (Green et al. 2009). The 16S ribosomal DNA gene is used widely in studies of bacterial diversity (Sánchez et al. 2007; Youssef et al. 2009).

In several published studies on the bacterial microbiota of amphibian skin, morphologically different bacterial colonies have been selected from mixed microbial communities cultured on a single low-nutrient medium, R2A agar (BD, New Jersey, US) (Lauer et al. 2007; Woodhams et al. 2007c; Banning et al. 2008; Lauer et al. 2008; Lam et al. 2010; Walke et al. 2011). Although this is a logical culture method to use, it is probable that some bacterial species will be missed because a single medium is unlikely to capture all species. However, it is often impractical to use multiple media due to budget and time constraints. The results from previous studies of amphibian microbiota, that used DGGE as a bacterial community fingerprinting technique, suggest that this may not be necessary in any case, as it appears that the proportion of

bacteria present on amphibian skin that are not captured by culture on R2A agar is low (Lauer et al. 2007; Lauer et al. 2008; Walke et al. 2011).

Three previous studies have broadly compared the species richness of cultured and uncultured bacteria found on amphibian skin. Lauer et al. (2007) compared DGGE fingerprints between swabs taken from individual eastern red-backed salamanders and all cultured isolates from the same individuals. They found 4 - 19 cultured morphotypes from each animal and 6 - 15 ribotypes (putative species) by DGGE. Similarly, Lauer et al. (2008) found 1 - 21 cultured morphotypes per animal and 5 - 20 species by DGGE. However, they did not directly compare the uncultured and cultured bacteria on individual animals to determine the proportion of uncultured species present. Both studies suggest that the number of unculturable bacteria found on salamander skin may not be very high. In another study, Walke et al. (2011) compared numbers of cultured and uncultured bacteria from glass frogs in Panama and found that 63% of the total bacterial species richness present was detected through culture. Thus far the evidence suggests that a high proportion of bacterial species present on amphibian skin are culturable, however only two species of salamander and one species of frog have been studied. In addition, there is one possible source of differences between the numbers of isolates cultured from an amphibian and the number detected using molecular techniques that has not been accounted for in previous studies. Standard isolation approaches rely on differences in colony morphology to initially select isolates. It is well known that many bacterial species can produce colonies that are morphologically indistinguishable (Jacques and Morris 1995; Chandler et al. 1997; Lebaron et al. 1998). Therefore, it is possible that the proportion of culturable isolates is underestimated because some bacteria are not detected during the initial phases of isolation and culture.

This chapter has two objectives: First, to determine whether selection of morphologically different bacterial colonies adequately captures the bacterial species richness present on R2A agar plates; and second, to compare the species richness of culturable and unculturable bacteria from the green-eyed tree frog, *Litoria serrata,* to determine how many unculturable bacteria are missed by focusing research efforts on culturable bacteria alone.

To address the first objective, I used DGGE to compare DNA fingerprints of individual isolates cultured and selected from R2A agar plates with swabs subsequently wiped across the same plate surface. Swabs from plates should theoretically contain all culturable isolates. To address the second objective, I compared the DGGE fingerprints obtained above, with the fingerprints obtained from swabs collected directly from frog skin.

Materials and Methods

I hand-captured ten *L. serrata,* in single-use plastic bags, from streamside vegetation and rocks along Kirrama Bridge 8 Creek, and collected two bacterial swab samples from each frog as previously described in Chapter Two. For culture of bacterial isolates, I immediately transferred one swab to an agar plate by rotating the swab on the plate surface in a wide zig-zag pattern, and froze the second swab in dry ice for later molecular analysis. Agar plates, stored at ambient temperature, and frozen swabs were returned to the laboratory within 48 hours of collection. Frozen swabs were transferred to a -20^{o}C freezer.

Agar plates were incubated for 48 - 72 hours at ambient temperature (22 - 25^{o}C) in the laboratory until microbial culture growth was observed. Plates were examined daily for five days and colonies with different morphological characteristics were identified by systematically scanning the plate back and forth across the inoculated area using a dissecting microscope. Morphologically unique isolates were selected and cultured as described in Chapter Two. In addition, seven days after initial inoculation, I swabbed each of the original agar plates with a sterile rayon swab (MW112, MW&E, Bath UK). The swab was rolled over the entire surface of the agar plate to collect all cultured bacterial colonies present and then frozen at -20^{o}C for later molecular analysis.

DNA extraction, PCR, and gel electrophoresis techniques are described in detail in Chapter Two. Briefly, I obtained DNA from three types of samples for each individual frog: samples from each morphologically distinct axenic isolate, which were combined into a single “isolate ladder”, a sample from the swab taken from the original culture plate at day seven, and the original duplicate swab sample taken from the frog and immediately frozen. I extracted DNA from axenic isolates and swab samples, and conducted PCR. Agarose gel electrophoresis revealed that DNA from plate swabs was highly concentrated, so I used a 1 in 10 dilution of template DNA in molecular grade water prior to PCR. I combined 5 µl of each PCR product from axenic isolates from each individual frog to form the frog-specific isolate ladders. As described in Chapter Two, I combined triplicate PCR products from each swab sample and purified and concentrated those from frog swabs prior to loading samples on denaturing gradient gels.

I loaded the three PCR product sample types for each individual frog in sequential lanes on two denaturing gradient gels, with a marker ladder (30 µl PCR product with 15 µl 2x gel loading dye) on the left, right and middle of the gels to enable samples from each gel to be compared.

Data Analysis

I analysed and compared DGGE images using Bio-Rad Quantity One software (Bio-Rad Laboratories, Hercules, CA USA) according to the User Guide. Indistinct bands present at the very top of the gel were not included in the analyses as they are likely to represent heteroduplex PCR products (Liu and Shyu 2006).

As a basis for analysis, I considered each 16S rDNA sequence to be a unique ribotype. It is possible that this either under- or overestimates taxonomic richness, since bacterial species and strains can have more than one 16S rDNA gene (Kang et al. 2010) or, less commonly, can have a 16S rDNA gene in common with other species (Muyzer and Smalla 1998). For each frog, I compared the number of colonies selected, and the resulting number of ribotypes, with the number of ribotypes obtained from swabs of agar plates. In addition, I compared the number of ribotypes found in the original swab samples with those from the plate swabs to calculate the proportion of ribotypes present on each frog that were non-culturable.

Results

Although I collected swabs from frogs in the same way, very different patterns of growth were observed on agar plates. Three agar plates, from frogs 1, 4 and 5, showed very little bacterial growth compared with plates from the remaining frogs, which produced similar patterns of microbial growth but at varying densities (Figure 3.1).

A total of fifty-five unique ribotypes, each representing a bacterial 16S ribosomal gene fragment, were present on the ten frogs swabbed. I found the most common ribotype (number 32) in samples from all frogs (Figure 3.2). An additional six ribotypes (numbers 23, 24, 25, 26, 37 and 40) were present in samples from 50 - 70% of frogs.

In eight out of ten frogs, the number of colonies selected was greater than the number of ribotypes arising from these colonies (Table 3.1), while in two *L. serrata* (LS4 and LS10) the opposite was true. However, for all frogs, there were additional ribotypes present on the initial agar plate that were not selected for isolation. The mean proportion of culturable ribotypes that were selected for isolation was 0.53 (Table 3.1; n = 10, SD = 0.23, range 0.17 - 0.83). Eight of ten frogs sampled had bacterial ribotypes that were not culturable. The mean proportion of non-culturable ribotypes per frog was 0.25 (Table 3.1; n = 10, SD = 0.24, range 0 – 0.73).

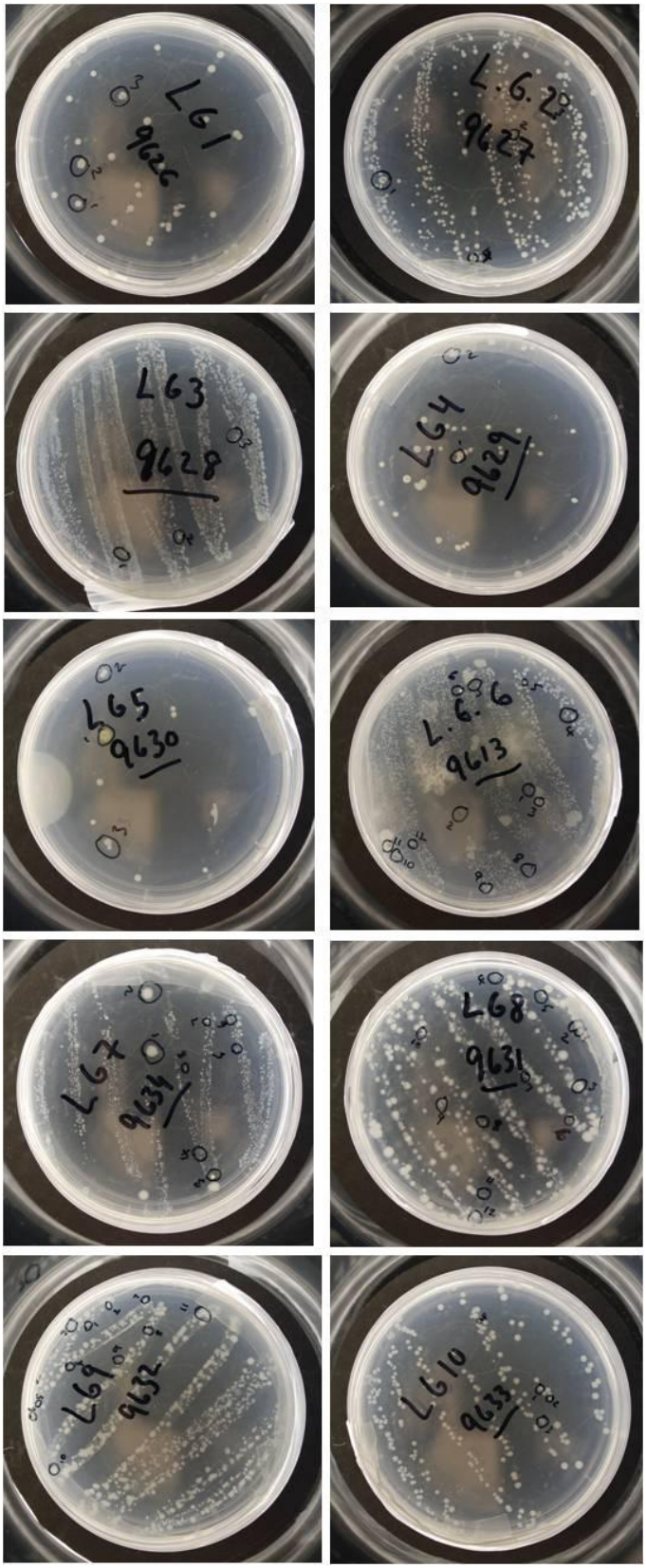

Figure 3.1. Photographs of agar plates after 48 hour microbial growth at 24 - 26^{o}C. Numbered and circled bacterial colonies appeared morphologically different and were selected for isolation.

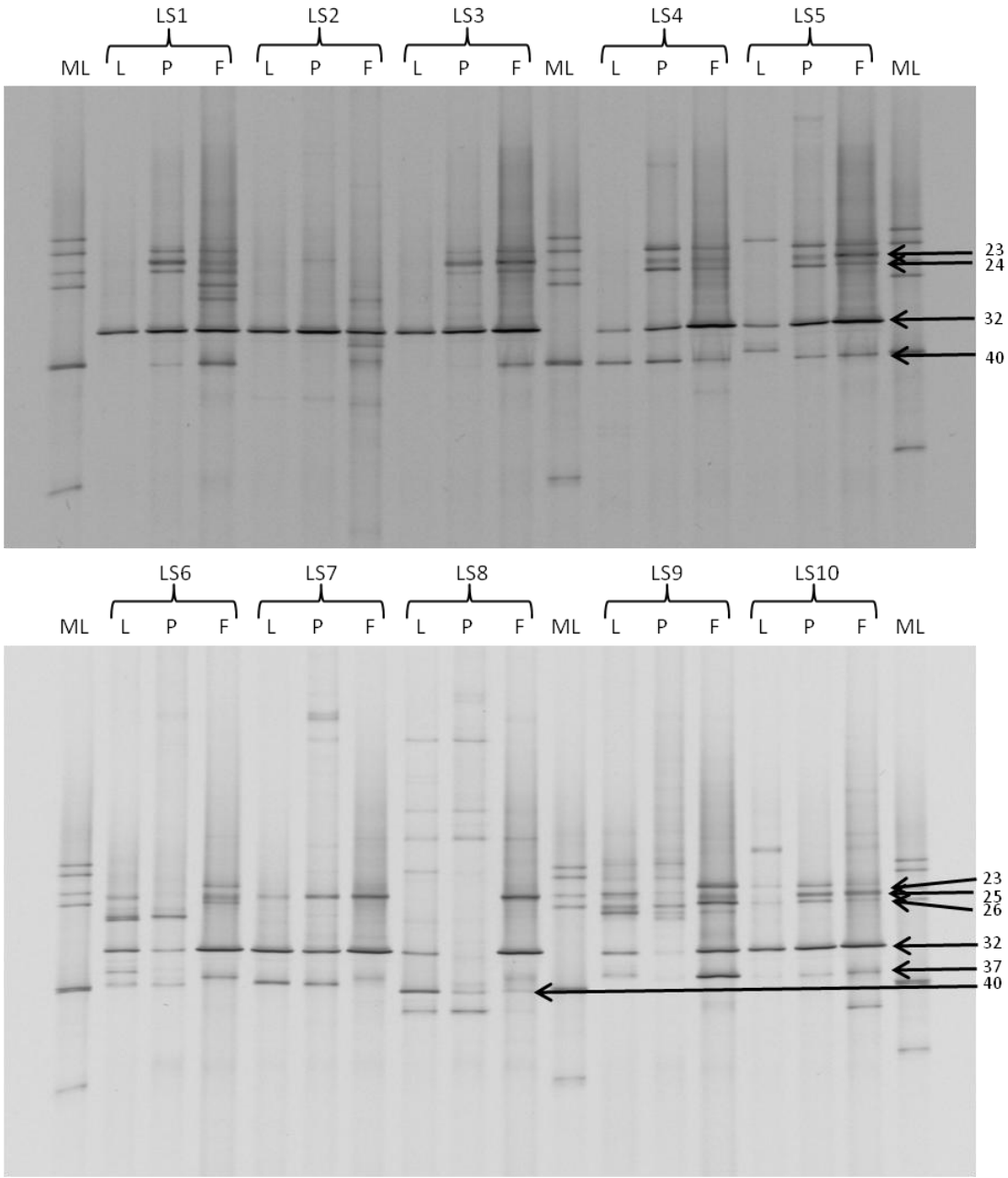

Figure 3.2. Images of denaturing gradient gels. *Litoria serrata* 1 to 5 (LS1 - 5) can be seen on the top gel, and *Litoria serrata* 6 to 10 (LS6 - 10) on the bottom gel. ML = marker ladder, L = isolate ladder, P = plate swab, F = frog swab. Ribotypes occurring in greatest numbers are indicated with arrows.

Table 3.1. The number of ribotypes for individual *Litoria serrata*, representing the 16S ribosomal gene fragments present in denaturing gradient gels. The number of ribotypes from agar plate swabs does not always equal the sum of the number of ribotypes from selected isolates and the number of ribotypes present on agar plate but not selected, because some ribotypes were present in axenic isolates cultured from a plate but absent from the corresponding plate swab.

Frog ID	Number of colonies selected	Number of ribotypes from selected isolates	Number of ribotypes from agar plate swab	Number of ribotypes present on agar plate but not selected	Proportion of culturable ribotypes selected	Number of ribotypes from frog swab	Number of non-culturable ribotypes	Total number of ribotypes	Proportion ribotypes not culturable
LS1	3	1	5	4	0.20	10	5	10	0.50
LS2	4	2	3	1	0.67	10	8	11	0.73
LS3	3	1	6	5	0.17	5	0	6	0.00
LS4	3	4	6	4	0.50	6	1	9	0.11
LS5	3	3	6	5	0.37	5	0	8	0.00
LS6	9	9	8	3	0.75	6	3	15	0.20
LS7	8	5	10	6	0.45	5	3	14	0.21
LS8	15	13	16	8	0.62	8	4	25	0.16
LS9	12	10	10	4	0.71	6	2	16	0.13
LS10	4	5	5	1	0.83	11	5	11	0.45

Discussion

My objectives were to evaluate whether selection of morphologically different bacterial colonies results in adequate representation of culturable bacterial species richness on R2A agar plates, and to determine the proportion of unculturable bacteria present in frog skin swab samples. I found that, on average, 75% of ribotypes present on frogs were cultured on R2A agar plates, and of those, 53% were selected for isolation based on colony morphology.

Bacteria were cultured from all ten frogs but DNA was not always predictably detected by DGGE. In theory, if selection of morphologically different isolates captures all species present on the agar plate, and represents a subset of the bacteria present on the frog, then each resulting ribotype should be present in each of the three samples types examined. In practice, this study demonstrates that this is not the case, with a wide range of possible outcomes, probably due to a combination of human error, culture issues and PCR biases. It is possible that additional bacterial species were present on frogs and remained undetected throughout this study as they were not present in sufficient numbers to culture or amplify through PCR. If a high proportion of isolates had not been detected, this could have affected the overall proportion of culturable or unculturable bacteria present. However, given that the ultimate aim of future bacterial collection, culture and isolation processes is to develop bioaugmentation tools, it seems unlikely that uncommon bacterial isolates, or those that are difficult to culture, would be of sufficient interest to warrant further investigation.

The number of bacterial colonies selected from initial culture plates overestimated the bacterial species richness present in those isolates. This over-selection of isolates was probably caused by perceived differences in colony morphology that were not genuine. Over-selection is not necessarily a problem as long as all morphotypes are selected. However, plate swabs revealed that, for all frogs sampled, there were additional ribotypes present on the initial agar plate that were not selected. An average of just 53% of the total number of ribotypes present on the initial culture plate for each frog were selected for isolation. This results in an overall underestimation of the number of bacterial species present, and might have occurred for several reasons. First, a colony might have been missed if it was morphologically identical to other colonies present, was only present in very low abundance (Vartoukian et al. 2010), was extremely tiny, or was flat and transparent (see Figure 3.1 for the range of bacterial growth patterns obtained on agar plates). Second, a slow-growing colony might not have grown sufficiently well to be visible for selection. Third, close contact with other bacterial colonies on the agar plate might have reduced the probability of detection, and finally, obligate dependencies on other bacteria could have caused some bacterial species to fail to grow alone

(Moran 2006). The presence of heteroduplex gel bands from plate swab samples, which might have caused apparent additional ribotypes, was ruled out as heteroduplex formation does not appear to be a major problem during DGGE (Murray et al. 1996).

This study suggests that the use of bacterial colony morphology alone to capture all bacteria present may not be entirely reliable, and a number of checks are therefore necessary in order to ensure adequate selection of colonies present. Plates should be carefully examined daily, under a dissecting microscope, for the appearance of new colonies before overgrowth by other spreading microorganisms occurs. Deliberate over-selection of visually similar colonies will likely decrease the proportion of culturable bacteria that are not isolated and cultured.

In two *L. serrata* (LS4 and LS10), more ribotypes were present in isolate ladders than the number of colonies selected, indicating that more than one 16S rDNA gene was present in one of the ladder isolates. It is not uncommon for bacteria to have more than one 16S gene; this can result in overestimates of bacterial species richness in mixed species samples (Crosby and Criddle 2003; Kang et al. 2010). Bacterial contamination is highly unlikely to be the cause of this observation as Gram stains of each isolate were examined to confirm purity.

Occasionally, ribotypes from selected isolates were not present in plate swab samples. It is possible that this occurred because of the high abundance of contaminating non-target organisms, such as fungi, that were also present on the agar plate after seven days growth. These contaminating organisms can cause inhibition during PCR (Cruz-Perez et al. 2001). It is also possible that uncommon bacteria from on the agar plate may not have been present in sufficient numbers to be detected following PCR amplification (Crosby and Criddle 2003).

The proportion of culturable isolates in this study was high compared with that of other well-studied environments (Stewart 2012), but was similar to the findings of a previous study on rainforest frogs in Panama (Walke et al. 2011). On average, 75% of ribotypes found on frogs in this study were present on the initial agar plates. Because most bacteria on frog skin appear to be culturable, it is likely that screening programs will not miss many bacterial taxa that are important in immune defence, and hence potential candidates for bioaugmentation. The fact that 25% of ribotypes did not appear to be culturable could be due to inappropriate culture conditions (eg. aerobic versus anaerobic) or media, as some bacteria have specific environmental or nutrient requirements (Vartoukian et al. 2010). It could also be caused by sampling error, either in the initial stages of growth in culture or in the various stages of swabbing when collecting samples. It is possible that detection of additional bacterial ribotypes could be increased through culture on a number of different media types or under

different conditions, but this is probably impractical during a high throughput screening process because of the additional costs and time involved.

The average proportion of unculturable ribotypes may have been higher than the 25% reported here, as the choice of primers used to amplify the 16S rDNA gene fragment can affect the apparent species richness present (Baker et al. 2003). Initial DGGE assessment, using the primers to amplify the V3-V5 regions of the 16S rDNA gene that provides the best representation of species richness when compared to the full length gene (Sánchez et al. 2007; Youssef et al. 2009), did not result in well-defined bands on the DG gels. Therefore, the primers used by Ferris et al. (1996) that amplify the 323 bp V7 and V8 regions of the gene were used for this study. Unfortunately, this region of the gene can underestimate species richness (Sánchez et al. 2007; Youssef et al. 2009), so ribotype richness may be underestimated here. In addition, all PCR-based fingerprinting techniques for mixed species 16S rDNA genes are biased towards amplification of higher copy number DNA (Crosby and Criddle 2003), and any bacterium representing less than 1% of the total assemblage may not be amplified sufficiently to be detected by DGGE (Muyzer et al. 1993). This may also result in underestimates of species richness. Some of these issues may be partially resolved through the use of next generation DNA sequencing technologies (Margulies et al. 2005).

In this study, the most common band (ribotype number 32) was present in all frog swab samples and represented the most intense band present on both DG gels. Band intensity on DG gels is not a reliable indicator of ribotype dominance because of PCR biases that cause differential amplification of mixed species DNA (Farrelly et al. 1995; von Wintzingerode et al. 1997; Suzuki et al. 1998). Nonetheless, it is likely that this represents the most dominant bacterium on all frogs in this study. Six other frequently occurring bands (ribotype numbers 23, 24, 25, 26, 37 and 40) were each present in 50-70% of frog swab samples. Together, these seven putative species are likely to comprise core members of the frog bacterial assemblage. They may form an integrated symbiotic community, with each filling a different role. However, it is also possible that these bacterial species are prevalent within the environment at this site and are simply picked up in greater numbers by the frogs.

Summary

This is the first study on amphibian cutaneous bacteria to document how the richness of bacterial ribotypes differs between the frog, the initial culture plates, and eventual isolates chosen by colony morphology. Although every effort was made to select all morphologically different colonies present on the agar plates, on average, almost half of all ribotypes present

on initial culture plates were not selected for isolation. Therefore, it may be best not to base initial selection of bacterial colonies entirely on morphological characters. In addition to morphological similarity, cultured bacteria can be missed when extremely low numbers of bacterial cells are present, or when overgrowth by more dominant organisms takes place, and these are largely beyond control. I therefore recommend selection of multiple colonies with similar appearance to minimize the proportion of culturable isolates that are inadvertently not selected. With this in mind, this technique was used, with care, to select bacterial colonies present on agar plates for further study in Chapters Four and Five of this thesis.

CHAPTER FOUR : SCREENING BACTERIAL METABOLITES FOR INHIBITORY EFFECTS AGAINST *BATRACHOCHYTRIUM DENDROBATIDIS* USING A SPECTROPHOTOMETRIC ASSAY

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Chapter Overview

In Chapter Three, I addressed technical issues associated with laboratory culture and detection of bacterial isolates collected from frog skin. In this second technical chapter, I present an assay technique to screen bacterial isolates for inhibitory effects against *Batrachochytrium dendrobatidis*. This chapter has been accepted for publication in the journal "Diseases of Aquatic Organisms" and is my own work with intellectual and technical input from co-authors Ross A. Alford, Stephen Garland, Gabriel Padilla and Annette D. Thomas. Three anonymous reviewers also provided valuable comments.

Abstract

Certain bacteria present on frog skin can prevent infection by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd*), conferring disease resistance. Previous studies have used agar-based *in vitro* challenge assays to screen bacteria for *Bd*-inhibitory activity and to identify candidates for bacterial supplementation trials. However, agar-based assays can be difficult to set up and to replicate reliably. To overcome these difficulties, I developed a semi-quantitative spectrophotometric challenge assay technique. Cell-free supernatants were prepared from filtered bacterial cultures and added to 96-well plates in replicated wells containing *Bd* zoospores suspended in TGhL broth medium. Plates were then read daily on a spectrophotometer until positive controls reached maximum growth in order to determine growth curves for *Bd*. I tested the technique by screening skin bacteria from the Australian green-eyed tree frog, *Litoria serrata*. Thirty-one percent of bacteria tested showed some degree of *Bd* inhibition, while some may have promoted *Bd* growth, a previously unknown effect. My cell-free supernatant challenge assay technique is an effective *in vitro* method for screening bacterial isolates for strong *Bd*-inhibitory activity. It contributes to the expanding field of bioaugmentation research, which could play a significant role in mitigating the effects of chytridiomycosis on amphibians around the world.

Introduction

One of the many causes of worldwide amphibian declines and associated biodiversity loss is the emergence of chytridiomycosis, an infectious disease caused by the pathogenic fungus *Batrachochytrium dendrobatidis* (*Bd* ; Berger et al. 1998; Berger et al. 1999). Amphibians have well-developed immune systems, with both innate and adaptive components (Rollins-Smith and Woodhams 2011; Voyles et al. 2011). Research into the role of the adaptive immune system in defending against *Bd* is in its infancy (Richmond et al. 2009; Ramsey et al. 2010; Savage and Zamudio 2011). In contrast, innate immune defences are known to be effective against *Bd* in many species (Rollins-Smith 2009). Anti-microbial peptides (AMPs), produced in skin glands, may be an important part of this innate immune defence (Rollins-Smith et al. 2002a; Rollins-Smith et al. 2002b; Rollins-Smith and Conlon 2005; Woodhams et al. 2006a; Woodhams et al. 2006b; Woodhams et al. 2007a). Microbes that colonise amphibian skin can also confer disease resistance on their hosts (Bettin and Greven 1986; Harris et al. 2006; Lauer et al. 2007; Woodhams et al. 2007c; Banning et al. 2008; Lauer et al. 2008). Symbiotic microbes can therefore be regarded as another component of amphibians' innate immune defence mechanisms (Woodhams et al. 2007c; Walke et al. 2011). Symbiotic bacteria resident on anuran skin may sometimes play a greater role than AMPs in defence against *Bd*, and AMP potency may be attenuated in species that rely heavily on their beneficial microbiota (Conlon 2011).

Harris et al. (2006) first demonstrated *in vitro* inhibition of *Bd* by a number of bacterial species isolated from the skin of salamanders, and suggested that *Bd*-inhibiting bacteria could be used as a bioaugmentation tool to confer disease resistance to vulnerable amphibian populations. Laboratory trials involving inoculation with a *Bd*-inhibiting bacterium, prior to pathogen exposure, have subsequently demonstrated that this is possible (Harris et al. 2009a; Harris et al. 2009b; Becker and Harris 2010).

Historically, investigations of the antimicrobial activity of microbes have used challenge assays. These *in vitro* techniques, which test the effectiveness of microbial metabolites against a particular microorganism, are typically undertaken using an agar or broth medium. Agar-based methods include disk diffusion, well diffusion and direct inoculation, while broth-based techniques use a liquid medium and are often coupled with spectrophotometry to quantitatively measure microbial growth (Jenkins et al. 1998). Agar diffusion techniques are usually non- or semi-quantitative screening methods that aim to detect the formation of a zone of inhibition, often around an antibiotic-impregnated disk or well. This zone can be

measured, enabling ordinal comparisons of results among different test organisms. However, antibiotic compounds can vary in their rate of diffusion through agar (Barry 1980; du Toit and Rautenbach 2000). Broth microdilution techniques are more time-consuming to perform and typically require some knowledge of the test compound concentration. Challenging the target organism with a range of dilutions of the test compound can facilitate determination of a minimal inhibitory concentration (Rollins-Smith et al. 2002b).

Published studies investigating bacterial antagonism against *Bd* have used agar plate assay methods (Harris et al. 2006; Woodhams et al. 2007c; Brucker et al. 2008a; Brucker et al. 2008b). In these, *Bd* zoospores are spread evenly across an agar plate surface, which is subsequently streaked directly with a bacterial isolate. The quantities of *Bd* and bacteria inoculated onto plates are not standardised, and live cultures of both of the organisms interact directly. Although this method can be used to screen large numbers of bacterial isolates over a relatively short time, it has potential disadvantages. Moisture requirements can vary between *Bd* strains, and these are not always compatible with those of the test bacteria, which can easily overgrow the *Bd*. In addition, because a distinct zone of inhibition must be observed, agar plate assays may fail to detect isolates that produce antibiotic compounds that diffuse slowly through agar. Finally, they could be subject to priority effects (Kennedy and Bruns 2005; Peay et al. 2012); *Bd* is established first, and may itself inhibit the growth of some bacteria, including isolates that would otherwise show strong activity against it.

To address the problems identified above, I developed a semi-quantitative broth-based assay technique. The method combines attributes of a fully-quantitative assay used to screen anuran AMPs for effectiveness against *Bd* (Rollins-Smith et al. 2002b), where both the concentration of *Bd* and AMPs are known, and a non-quantitative broth-based assay used to detect antifungal compounds produced by cutaneous bacteria from salamanders (Walters 2007). In my technique the concentration of *Bd* zoospores is known, while the concentration of the substance being assayed is unknown. The technique allows me to visually distinguish between fungistatic and fungicidal effects, and eliminates the possibility of direct competitive or priority effects.

In this chapter, I describe the challenge assay methodology, including techniques and analysis of results. I then apply this new challenge assay method to bacteria collected from wild frogs and report results that demonstrate the potential benefits of this technique.

Materials and Methods

Field site and species

I collected bacterial samples from the skin of green-eyed tree frogs, *Litoria serrata,* found on rocks and vegetation bordering Frenchman's Creek, in Wooroonooran National Park, northern Queensland, Australia. I captured four adult frogs, held them briefly for sampling, and then released them at the point of capture.

Sampling resident bacteria

Detailed methods of sample collection, and purification of bacterial isolates are described in Chapter Two. As a result of the data discussed in Chapter Three, I made an attempt to over-select bacterial isolates to capture the maximum culturable species richness from each agar plate.

Preparation of Batrachochytrium dendrobatidis cultures

I selected *Bd* isolate "Gibbo River, L. Les, 06-LB-1" as the strain to be challenged for its known virulence in frog infection trials (Berger et al. 2005). The isolate was maintained in sterile TGhL broth medium (8 g tryptone, 1 g hydrolysed gelatin, 2 g lactose, to 1 L deionised water, autoclaved), passed to TGhL agar plates (as above with 10 g bacteriological agar) after seven days growth, and incubated at 23^{o}C. On day three after inoculation, plates were flooded with 3 ml sterile TGhL broth to create a suspension of zoospores, which was collected and vacuum filtered through a sterile 20 µm nylon filter (Spectra Mesh, Spectrum Laboratories Inc, California, US) to remove sporangia. The filtered zoospores were counted on a haemocytometer and resuspended in sterile TGhL to a concentration of 2 x 10^{6} ml^{-1}.

Preparation of bacterial cell-free supernatant

Axenic isolates were each inoculated into 1 ml sterile TGhL medium in 24-well plates (Costar 3524, Corning, New York, US) and incubated at 23^{o}C for 48 hours. I then transferred each culture to a sterile 1.5 ml microtube and centrifuged at 7500 x g for five minutes to pellet the cells. The supernatant was then filtered through a sterile 0.22 µm syringe filter (Millex GV, Millipore, Massachusetts, US) to remove all cells, leaving bacterially produced metabolites in TGhL medium.

Challenge assay

I set up challenge assays in 96-well microplates (Costar 3595, Corning, New York, US). Experimental wells contained 50 µl of *Bd* zoospores at a concentration of 2 x 10^{6} ml^{-1} and 50 µl of a bacterial cell-free supernatant sample. Positive control wells contained 50 µl *Bd* and 50 µl TGhL medium. Negative controls contained 50 µl heat killed *Bd* (60^{o}C for 60 minutes) and

50 µl TGhL. An additional (medium-only) control consisted of wells containing 100 µl replicates of TGhL alone. A complete plate contained ten replicates of each positive control, five replicates of each negative control, nineteen wells containing the medium-only control, and five replicate experimental wells for each of twelve bacterial isolates.

Microplates were incubated at 23^{o}C until maximum growth in the positive controls was observed (typically after 6 - 8 days growth); readings were taken using a spectrophotometer (Multiskan Ascent, Thermo Scientific, Scoresby, Australia) with a 492 nm filter immediately after plate set-up was complete (day 0) and every 24 hours thereafter. After a minimum of three days growth, I examined the plate visually using an inverted microscope, to provide an independent record of the culture appearance and to identify and exclude wells where bacterial or fungal contamination developed.

Nutrient depletion in cell-free supernatants

Experimental wells in challenge assays contained 50 µl of cell-free supernatants in which bacteria had previously been cultured, while controls contained 50 µl of previously unused medium. Because of this the growth of *Bd* in experimental wells could have been affected by depletion of nutrients used by the bacteria, as well as by bacterial metabolites. To determine the range of effects that could be produced by nutrient depletion alone, I grew *Bd* in 96-well microplates with various concentrations of TGhL medium to simulate nutrient depletion in cell-free supernatants. Experimental wells contained 50 µl of *Bd* zoospores at a concentration of 2 x 10^{6} ml^{-1} and 50 µl of TGhL broth at 11 different concentrations from 100% to 0%, where 100% was undiluted TGhL medium and 0% was sterile distilled water. Five replicates of each dilution had live *Bd* zoospores and three replicates had heat-killed *Bd* controls. Four replicate microplates, with independent zoospore counts, were incubated and read on a spectrophotometer as described above.

Data analysis

Challenge assay

For each day's data I first calculated the mean daily absorbance values of all replicates of each type of control and experimental sample. Then, to remove baseline absorbance, I subtracted the mean negative control value at each day from each of the mean sample and positive control values on that day, to obtain corrected absorbance values. To adjust the dataset further for background colour, which was present in some samples, I subtracted the corrected mean sample values at day 0 from each daily mean sample value. I repeated this process by subtracting the positive control corrected mean absorbance value at day 0 from each daily

mean positive control value. I then plotted growth curves for each sample and positive control. If positive control growth curves did not follow the standard growth curve shape with a lag, log and stationary phase, the assay was repeated. The proportion of inhibition or enhancement of each sample relative to the positive control was then calculated by dividing the corrected absorbance value for each sample on its maximum growth day by the corrected absorbance value of the positive control on its maximum growth day. A value of 1 was subtracted from all proportional values so that positive values represented growth above that of the positive control and negative values represented potential growth inhibition. Data adjustments are summarized in Figure 4.1.

Nutrient depletion

I treated each dilution of TGhL as a different sample, and heat-killed replicates were used as negative controls for that sample. The 100% TGhL live *Bd* sample was considered the positive control. I performed data corrections as described above, except that colour corrections were not necessary as no cell-free supernatants were used, and negative control data for each sample for each day were subtracted from the corresponding sample containing live *Bd*. For each microplate, I calculated the proportion of growth relative to the positive control for each dilution.

Calculations	**Definitions**
$PA_j = P_j - N_j$ $SA_{ij} = S_{ij} - N_j$ $PAC_j = PA_j - PA_0$ $SAC_{ij} = SA_{ij} - SA_{i0}$ $X_i = (SAC_{imax}/PAC_{max}) - 1$	*i* = individual isolate *j* = individual day from 0 to *n* P_j = mean measured absorbance of all positive controls on day *j* N_j = mean measured absorbance of negative controls on day *j* S_{ij} = mean measured absorbance of samples of isolate *i* on day *j* PA_j = baseline absorbance corrected positive controls on day *j* SA_{ij} = baseline absorbance corrected samples of isolate *i* on day *j* PAC_j = colour corrected positive controls on day *j* SAC_{ij} = colour corrected samples of isolate *i* on day *j* PAC_{max} = corrected positive control value at maximum growth day SAC_{imax} = corrected sample value at maximum growth day X_i = proportion inhibition or enhancement relative to positive control

Figure 4.1. Summary and details of calculations performed on raw spectrophotometric absorbance data.

Results

I isolated and tested a total of 94 isolates from the four *L. serrata* sampled in this study. As a result of visual observation of the replicate microplate wells (Figure 4.4), together with the calculated proportion of inhibition and examination of each growth curve (Figure 4.3B), cell-free supernatants were considered totally inhibitory if no growth was observed. Totally inhibitory isolates occurred on all four frogs. Of all isolates tested, 23% were totally inhibitory to *Bd.*

Nutrient depletion of medium by bacterial isolates can account for some of the partial inhibition observed in challenge assays. Results from the four replicate microplates showed that a progressively greater proportion of apparent *Bd* inhibition, relative to the positive control, occurs with decreasing nutrient concentrations. The proportion of inhibition for the four 100% nutrient depleted samples was 54.6, 63.5, 44.7 and 31.7%. This range of results is due to unavoidable slight variation in zoospore counts among assays. I therefore chose to use the maximum inhibition observed (63.5%) as my cut-off value; isolates that produce inhibition beyond this value must exhibit true inhibition against *Bd*. This figure is conservative because it is unlikely that complete nutrient depletion during bacterial culture always (or even frequently) occurs; if it did, there should be a peak in the frequency distribution at 50 - 60% inhibition (Figure 4.2); in fact there is a local minimum at that point.

Once the cut-off value of 63.5% inhibition was applied to the data to account for nutrient depletion effects, an additional eight percent of cell-free supernatants were partially inhibitory to *Bd*, while 70% were not-inhibitory. Of those non-inhibitory isolates, 34% enhanced the growth of *Bd* by more than 25%. Cell-free supernatant effects on *Bd* growth show a bimodal distribution with one peak of inhibitory isolates and one of non-inhibitory isolates (Figure 4.2).

Figure 4.3 presents a set of challenge assay growth curves that demonstrate the range of possible outcomes for each isolate tested. Uncorrected mean absorbance values are plotted in Figure 4.3A and the corrected absorbance values in Figure 4.3B. The corrections align the negative control along the x-axis and remove the effect of colour, which could in some cases mask inhibition in the spectrophotometric assays. I present error bars indicating +/- 1 standard deviation; these should be a standard part of the presentation of outcomes for this assay. They indicate the degree of dispersion of replicates of each treatment or control on each microplate. Excessive variance would indicate contamination or inaccurate pipetting. I also suggest that comparisons of mean positive control values among plates set up at the same time should be carried out to identify and eliminate plates that did not grow as expected. In

addition, comparison of mean positive control values among batches of plates set up at different times should be conducted to check accuracy of zoospore cell counts. My mean maximum positive control absorbance value across all plates in this study was 0.17 (SD = 0.024, n=18).

Visual inspection of microplates revealed a range of cell-free supernatant effects on *Bd,* from inhibitory to enhancing. The photos of positive (Figure 4.4A) and negative control wells (Figure 4.4B) are typical of those treatments and provide a reference for visual categorization of other effects. Visual inspection is important as it demonstrates that the effects I observed result from a variety of mechanisms. Total inhibition can result from a simple lack of growth, as seen in the negative control, and can also result from *Bd* cell lysis, as shown in Figure 4.4F. Partially inhibitory effects range from the very low-level growth shown in Figure 4.4C to the formation of sporangia without zoospore development (Figure 4.4D). Growth enhancement results from increased numbers of zoospores and sporangia as shown in Figure 4.4E.

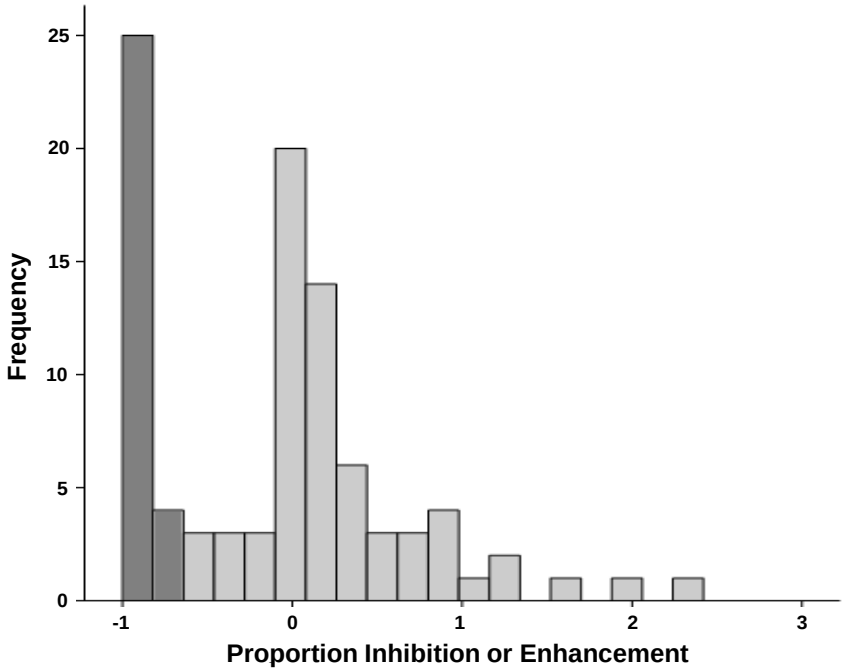

Figure 4.2. The bimodal distribution of cell-free supernatant effects upon *Batrachochytrium dendrobatidis* growth. Negative values indicate growth below that of the positive control and positive vales indicate growth greater than that of the positive control. The dark grey shading represents all cell-free supernatants with inhibitory effects greater than 63.5% and less than or equal to 100%, and the pale grey shading represents the non-inhibitory cell-free supernatants.

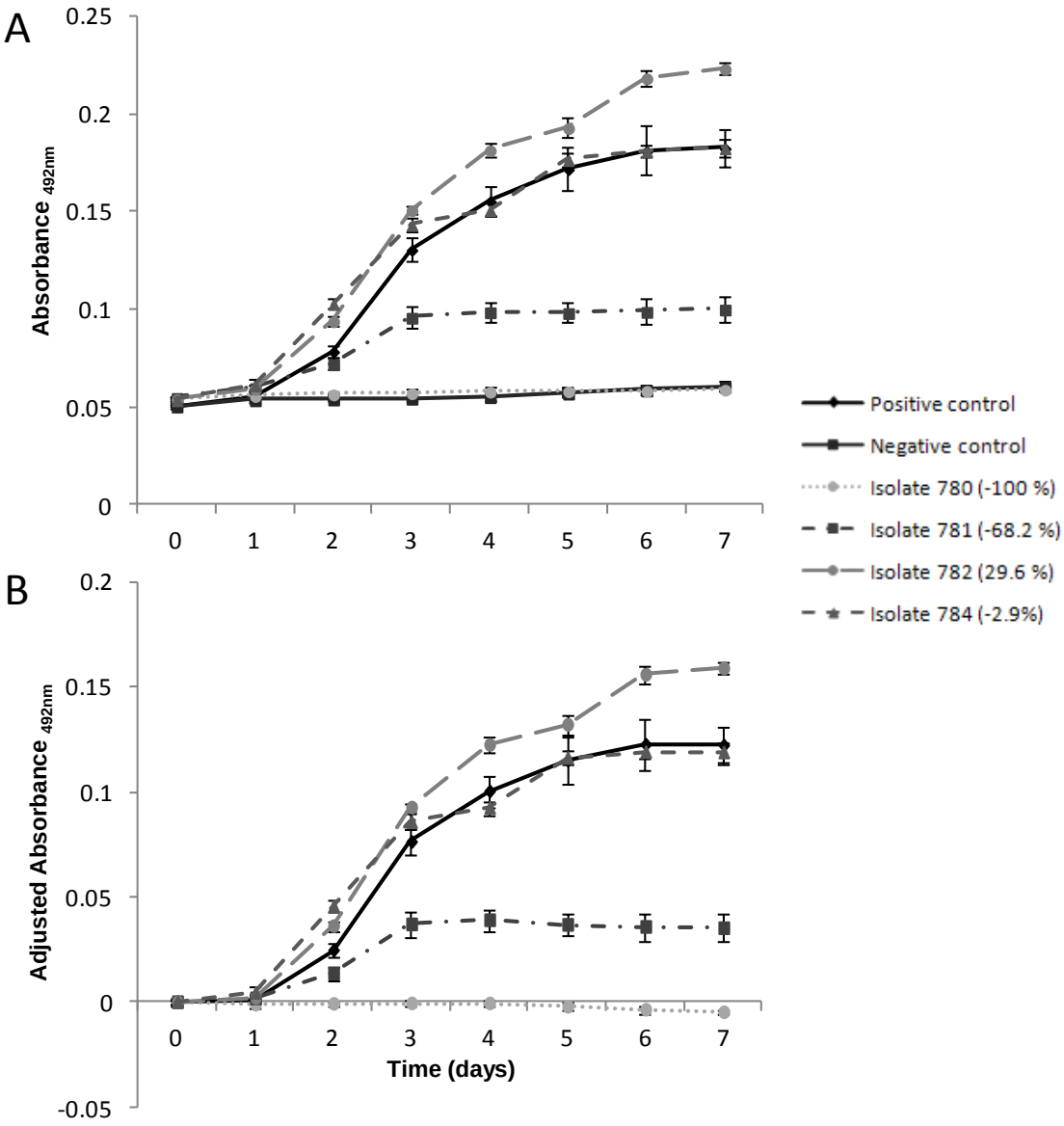

Figure 4.3. Batrachochytrium dendrobatidis growth curves showing spectrophotometric absorbance values for selected isolates demonstrating a range of effects on *Bd* growth, along with positive and negative controls. Selected isolate ID numbers are accompanied by percentage values in brackets showing growth relative to the positive control, calculated as described in Figure 4.1. **A** Uncorrected mean daily absorbance values (error bars represent ±1 standard deviation from the mean). **B** After corrections to subtract background absorbance and remove the effect of colour from the sample.

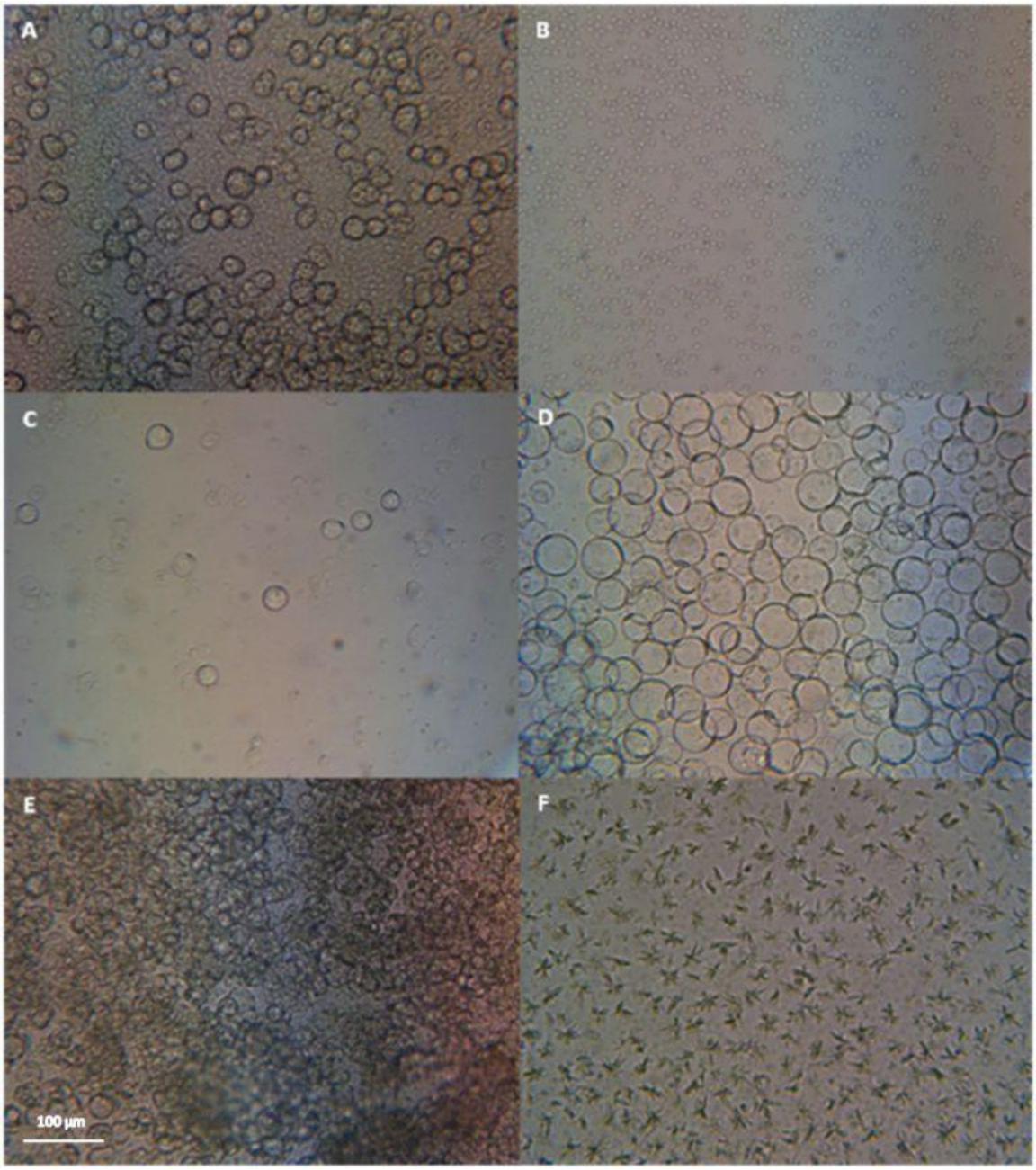

Figure 4.4. Light microscope photographs (x250) showing growth of Batrachochytrium dendrobatidis cultures 7 days post-inoculation. **A** Positive control. **B** Negative control. **C** Partial inhibition. **D** Partial inhibition where large sporangia develop but no zoospores form. **E** Enhanced growth. **F** Total inhibition showing cell lysis.

Discussion

I have developed a semi-quantitative challenge assay for testing bacterial cell-free supernatants for activity against *Bd* that reliably distinguishes totally inhibitory bacterial isolates. My technique improves on the previous method used to screen bacteria for activity against *Bd* as it removes some potential confounding effects, and allows trials to be run in a more controlled manner.

Previous studies using agar plates, have scored challenge assay results only as either inhibitory or non-inhibitory (Harris et al. 2006). This study demonstrates that bacterial cell-free supernatants can have a number of different effects on growing *Bd* cultures, from lysis of cells to growth enhancement (Figure 4.4). The mode of inhibitory action is most probably through antibiotic production, but other components such as bacteriocins (peptides produced by bacteria), bacterial degradation products, organic acids, lysozymes and proteases may also play a role (see Verschuere et al. 2000). Enhancement of *Bd* growth may occur when bacterial metabolites serve as nutrients, or through hormetic effects, where very low concentrations of antibiotics promote growth that is inhibited by higher concentrations (Southam and Ehrlich 1943; Stebbing 1982). Both of these possibilities may be potentially important in nature, and are worthy of further investigation. However, the analysis of this effect is beyond the scope of this study, which is primarily designed to screen for isolates that can exhibit strong inhibition against *Bd*. Therefore, until further investigation is undertaken, enhancing effects should probably be best considered as non-inhibitory.

Benefits and limitations

My challenge assay offers a number of advantages over the agar plate technique used to date. The use of a fixed quantity of *Bd* zoospores, instead of the non-standardised quantity used in the agar plate method, removes one source of variation from the resulting data, making my technique semi-quantitative. The agar plate assay can also be difficult to set up. For example, I found that the strain of *Bd* I used would not grow well on agar unless the plate was left slightly damp, and under those conditions bacteria always overgrew the *Bd*. In addition, antibiotic compounds can diffuse at a variety of rates through agar due to the nature of the agar matrix (Kunin and Edmondson 1968; du Toit and Rautenbach 2000), which may mean that some strongly inhibitory compounds produce only a narrow zone of inhibition, leading to inaccurate classification. My broth-based assay eliminates these problems. The agar-plate technique used to date allows direct interaction between the bacterium and *Bd* and does not account for the possibility that priority effects (sensu Kennedy and Bruns 2005; Peay et al. 2012) may occur.

Finally, interaction between growing cultures of test organisms also allows direct competition for space and nutrients, which could result in apparent zones of inhibition that are not due to inhibitory compound production (Jenkins et al. 1998).

This technique allows visual differentiation between fungicidal and fungistatic effects. Fungistatic effects block growth at different stages of *Bd* development as seen in Figures 4.4C and 4.4D, while fungicidal effects completely destroy the fungal cells (Figure 4.4E). The agar plate assay allows rapid screening of large numbers of bacterial isolates, while my spectrophotometric technique is slightly more labour intensive to set up. However, it still facilitates the screening of reasonably large numbers of isolates at a time, and I feel that the benefits my technique offers are worth the additional time required to set up and run the assays.

Obtaining reliable results from this assay technique on isolates that exhibit total inhibition against *Bd* is based on the satisfaction of three assumptions: First, that the *Bd* culture is growing optimally when the assay is set up; secondly, that haemocytometer counts of *Bd* are conducted carefully and therefore reasonably reliably replicated; and third, that 48 hours of bacterial culture growth are likely to be sufficient for production of antimicrobial compounds to have occurred. I believe that the first two assumptions have been satisfied while developing this assay, and subsequent investigation has shown that the third assumption is highly likely to be correct when assaying for isolates that show total inhibition against *Bd* as this effect appears reliably repeatable (K. Yasumiba, unpublished).

In this assay, isolates were grown and tested individually with the aim of detecting those that could exhibit strong inhibition against *Bd*. However, the interaction of two or more different bacteria, or the interaction of a bacterium with *Bd*, may initiate antibiotic production that is not observed in single isolates. In nature, it is probable that metabolite production may have a signalling role (see Yim et al. 2006) and this may only be triggered in response to the presence of other microorganisms. For example, one study demonstrated that competitive interaction between different soil bacteria in culture resulted in stronger fungal inhibition than demonstrated by each bacterium alone (De Boer et al. 2007). Therefore, in this assay, some isolates that can produce metabolites active against *Bd* only in the presence of other microorganisms may not have been detected.

Cell-free supernatants exhibit genuine inhibitory results when inhibition greater than the maximum possible value for nutrient depletion occurs. Lesser levels of inhibition might result

from nutrient depletion alone or from partial inhibition with or without nutrient depletion. The growth enhancement I observed for some isolates cannot be caused by nutrient depletion and appears likely to be genuine.

Because nutrient depletion in cell-free supernatants could contribute to partially inhibitory results, I recommend that a minimum level of inhibition for genuine partially inhibitory effects be determined for each medium and concentration of *Bd* zoospores tested, as outlined in the Materials and Methods section above. Alternatively, 100% nutrient depleted controls could be included on each 96-well microplate.

An additional simple improvement to this assay method would be to allow growth of bacterial cultures until a minimum, empirically-determined, optical density is reached before preparing the cell-free supernatant for assay. This improvement would reduce variation between isolates in terms of starting material available, almost certainly eliminate hormetic effects, and ensure that any genuine partially inhibitory effect was not simply due to low bacterial cell numbers. However, I recognize that optical density is not solely dependent on cell number but also on cell motility, cell size and cell membrane composition.

Future directions

The development of this technique has provided numerous opportunities for further research. Alternative broth media can be substituted for TGhL if desired, and comparisons can now be easily made among isolates tested at different temperatures and with mixed microbial communities rather than in isolation. Further research to determine whether hormetic effects are responsible for the growth promotion phenomenon observed can be easily conducted using this technique. The range of effects observed also provides an opportunity to study gene expression in *Bd* to identify the genes involved in growth and development of the pathogen. Furthermore, with large datasets derived from wild animals, categorized data could also be used to statistically test for differences among frogs grouped by species, site, elevation, sex, age class, or *Bd* infection status. There are many possibilities for experiments that will contribute knowledge on the diversity and interaction of microbial species found on amphibian skin.

Summary

As the field of bioaugmentation research progresses, it will become increasingly important to relate observed *in vitro* effects to the interactions occurring on amphibian skin, possibly through assessment of the contribution of the most dominant microbial community members

(Harris et al. 2006; Woodhams et al. 2007c). Characterisation of bioactive compounds will also be useful (Brucker et al. 2008a; Brucker et al. 2008b) and, coupled with development of techniques for detection of ambient levels of antibiotics on amphibian skin without sacrifice of the animal, should aid in elucidating a fuller picture of the contribution of microorganisms to immune defence against this widespread and serious pathogen. In Chapter Five, I use this challenge assay technique to investigate differences in the ability of the cutaneous bacterial microbiota to inhibit *Bd* among five species of frogs at five sites within two bioregions in northern Queensland, Australia.

CHAPTER FIVE : BACTERIAL INHIBITION OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN AUSTRALIAN WET TROPICS FROGS

Chapter Overview

In Chapter Four, I developed a challenge assay technique to investigate the presence of *Bd*-inhibitory metabolites in bacterial cell-free supernatants. In this chapter, I use this challenge assay technique to investigate differences in the ability of the cutaneous bacterial microbiota to inhibit *Bd* among five species of frogs at five sites within two bioregions in northern Queensland, Australia.

Abstract

Bacterial symbionts can protect their hosts against disease. The emergence of the pathogen *Batrachochytrium dendrobatidis* (*Bd*) caused declines and disappearances of frogs in the Australian Wet Tropics. The severity of its effects varied among sites and species, and some species have since recovered or recolonised, although *Bd* is now enzootic in these populations. The pathogen does not appear to have colonised the adjoining Cape York region; frogs there appear to be free from *Bd* infection and have not suffered population declines. This wide range of variation in history among species and sites provides an opportunity to investigate the role of anti-fungal cutaneous bacteria in protecting frogs against *Bd* infection. I collected cutaneous swab samples from five species of frogs at two upland and two lowland sites in the Wet Tropics and at one upland site in the Cape York bioregion. I used *in vitro* challenge assays to determine the capacity of bacteria isolated from each frog to inhibit *Bd*. I tested the hypotheses that there were no differences in the bacterial isolate richness or *in vitro* capacity to inhibit *Bd* among frogs grouped by bioregion, species and site, and *Bd* infection status. The number of cultured bacteria and the proportion of inhibitory bacteria did not differ between uninfected frogs from both bioregions, suggesting that, on average, frogs in populations subject to *Bd* infection have not acquired large numbers of novel *Bd*-inhibitory bacteria. However, at one of the upland Wet Tropics sites the proportion of frogs that had bacterial isolates with *Bd*-inhibitory activity was significantly higher than at any other site. Frogs at this site also had greater cultured bacterial isolate richness and a higher proportion of inhibitory isolates than their lowland conspecifics. Therefore, bacteria capable of *Bd* inhibition might have played a role in the recolonisation of upland frogs at this site, while alternative mechanisms for decreasing susceptibility are likely to have been involved at the other upland site.

Introduction

Bacterial symbionts can protect their hosts from disease (Currie et al. 1999; Ritchie 2006; Scott et al. 2008). For example, a high proportion of bacteria isolated from healthy coral mucus could produce antibiotic compounds active against pathogens (Ritchie 2006). Similar mutualisms exist between insects and their bacterial symbionts. Fungus-farming ants house antibiotic-producing bacteria in specialised cuticular crypts to control a fungal pathogen that parasitises their farmed food supply (Currie et al. 1999; Currie et al. 2006), and pine beetles use antibiotic-producing bacteria to help reduce fungal pathogen attacks on their larval food supply (Scott et al. 2008). Variation in the bacterial strain present can cause differential morbidity in fungus-farming ants (Poulsen et al. 2010), demonstrating that antibiotic production is important in this symbiosis.

Patterns of frog decline and recovery in the Australian Wet Tropics, following the arrival of the pathogen *Bd* in the late 1980s (Laurance et al. 1996; Berger et al. 1999; McDonald and Alford 1999), differed among species and sites. Frog populations of all species at lowland sites (below 400m) did not appear to undergo severe declines, while several species, including *Litoria nannotis* and *L. rheocola,* suffered declines to local extirpation at all upland sites (above 400m; Richards et al. 1993; McDonald and Alford 1999). At upland sites *L. serrata* appeared to suffer temporary declines; reduced numbers of frogs were observed on some surveys during the years from 1989 to 1993 when declines of other species reached their peaks (Richards et al. 1993; McDonald and Alford 1999; Richards and Alford 2005). Sites in the Kirrama and Wooroonooran upland areas were included in these surveys. Exact dates of initial declines at these sites are not available, however *L. nannotis* had disappeared at both sites prior to December 1991, while *L. rheocola* disappeared in the Kirrama uplands during 1990 and was last seen in the vicinity of the Wooroonooran upland site in 1992 (Richards et al. 1993).

The timing of reappearance of frogs at upland sites has varied among species and sites. Sites in the Kirrama and Wooroonooran uplands were regularly surveyed between 1999 and 2007 (S.Bell, unpublished; Woodhams 2003; Woodhams and Alford 2005). Records demonstrating reappearance show that *L. nannotis* had recolonised the Kirrama upland site by 2000 (Woodhams 2003), while just two individuals were reported in the vicinity of the Wooroonooran site in 1999 (Retallick 2002). *L. rheocola* has not yet recolonised the Kirrama upland site (pers. obs.), but a number of individuals were present in the vicinity of the Wooroonooran upland site during surveys in 1999 (Retallick 2002). Further anecdotal records

suggest that frogs that have reappeared were probably only absent for two to three years (K. McDonald, pers. comm.).

The mechanisms allowing species' recovery are likely to vary among species and sites (Jackson and Tinsley 2005). At some sites, recolonisation may occur from lowland populations within the same catchment, while at others, a small remnant population may increase over time until it reaches a detectable level. Physical factors such as distance to travel from the source population or barriers such as unsuitable habitat will affect the time it takes species to recolonise sites, while levels of resistance to mortality from chytridiomycosis are likely to affect rates of population growth. Population dynamics and responses to selection are likely to be independent among many sites in the Wet Tropics, since there is substantial genetic separation among Wet Tropics frog populations (Schneider et al. 1998; Cunningham 2001; Hoskin et al. 2005; Richards et al. 2010; Bell et al. 2012). Variation among populations in both innate and acquired immune defences due to selection pressures imposed by the pathogen may affect host resistance to *Bd* and ultimately survival (Rollins-Smith et al. 2002a; Rollins-Smith et al. 2011; Savage and Zamudio 2011). Pathogen virulence may also vary among regions (Berger et al. 2005; Voyles 2011).

Several studies in the Wet Tropics have investigated a number of the factors allowing frog populations at higher elevations to coexist with enzootic *Bd*. One radio-tracking study revealed that *L. nannotis* in rainforest sites were never found outside the thermal optimum for *Bd* (Rowley 2006), while other studies showed that *L. nannotis* present in open canopy environments had more opportunity to elevate their body temperatures potentially reducing pathogen load (Daskin et al. 2011; Puschendorf et al. 2011). This suggests that appropriate habitat selection may allow coexistence with *Bd* and that downstream open forest refugia might provide source populations from which to recolonise upstream rainforest sites following declines (Puschendorf 2009). Kenyon (2008) showed that the proportion of *L. serrata* with anti-microbial peptides (AMPs) effective against *Bd* was higher in an upland population than it was in a lowland population. Both upland and lowland populations had higher levels of AMPs in winter suggesting that selection pressures from *Bd* may have acted to increase AMP production at upland sites and in winter when infection prevalence and intensity is often highest (Kenyon 2008).

If *Bd* has only recently emerged as a pathogen, it is possible that there has been little time for adaptation of host defences against it. However, amphibians carry a suite of cutaneous bacteria (Bettin and Greven 1986; Lauer et al. 2007; Woodhams et al. 2007c; Lauer et al. 2008;

Walke et al. 2011), that have evolved broad antimicrobial activity against invading pathogens and which can evolve much faster than the host's innate and acquired defences (Orr 2000). Some of these bacteria produce compounds capable of preventing *Bd* growth (Chapters Four and Five; Harris et al. 2006; Woodhams et al. 2007b; Lam et al. 2010; Walke et al. 2011) and these bacteria can confer disease resistance on their hosts (Harris et al. 2009a). Therefore it is possible that at least some of the recolonised frogs in the Wet Tropics uplands have developed resistance to *Bd* through acquisition or evolution of more effective cutaneous bacterial symbionts.

The different patterns of frog reappearances in the Wet Tropics uplands provide an opportunity to investigate whether the anti-fungal cutaneous bacterial microbiota have played a role in the ability of frogs to recolonise upland sites. In this chapter, I investigate the *in vitro* effects of cell-free supernatants from cultured cutaneous bacteria on *Bd.* I test the hypotheses that there are no differences in bacterial isolate richness or *in vitro* capacity to inhibit *Bd* among frogs grouped by bioregion, species and site, and *Bd* infection status. Patterns are discussed in the context of population reappearances at the Kirrama and Wooroonooran upland field sites.

Materials and Methods

Field sites and species

I collected cutaneous swab samples from the stream-dwelling rainforest frogs *L. serrata, L. nannotis*, *L. rheocola*, *L. eucnemis* and *L. longirostris* (Table 5.1). Sampling was conducted in winter to maximise the chance of finding infected animals; *Bd* prevalence and intensity of infection are highest at this time of year (Berger et al. 2004; Woodhams and Alford 2005; Kriger and Hero 2007). Two upland and lowland pairs of sites were sampled within the Wet Tropics Bioregion in Queensland, Australia where enzootic *Bd* infection exists. I also sampled an additional upland site in the Cape York Peninsula Bioregion, which is thought to be free from *Bd* (Skerratt et al. 2010). Field sites and species are described in detail in Chapter Two.

Collection of bacterial samples and detection of inhibitory isolates

Detailed methods of sample collection, purification of bacterial isolates, and challenge assay technique and analysis are described in Chapters Two, Three and Four. When initial agar plates generated from swabs plated in the field did not yield any viable bacterial colonies, I excluded samples from those frogs from further analysis. In contrast, R2A agar plates originating from *L. serrata* at the upland and lowland sites in Wooroonooran National Park produced very large numbers of viable bacterial colonies. I therefore only selected agar plates from a total of four

frogs to represent that species at each of those two sites (Table 5.1). As a result of the data discussed in Chapter Three, I made an attempt to over-select bacterial isolates to capture the maximum culturable species richness from each agar plate. The data collected for this chapter therefore assume that over-selection of bacterial isolates occurred, in a similar unbiased way, for all cultured isolates present for all frogs at every field site. Quantitative PCR was used to diagnose *Bd* infection status following the protocol of Boyle et al. (2004). I considered samples positive when more than zero zoospore equivalents were detected in at least two of three replicates.

Table 5.1. Locations, species and numbers of frogs sampled for each survey site. Numbers indicate both the number of individuals sampled and the number of individuals from which at least some isolates were obtained; the remaining samples failed to grow after initial plating on R2A agar plates. Species are *Litoria serrata* (LS), *L. nannotis* (LN), *L. rheocola* (LR), *L. eucnemis* (LE), and *L. longirostris* (LL).

Site	Species				
	LS	LN	LR	LE	LL
Windin Creek North (Upland), Wooroonooran National Park	10 (4)	10 (7)	10 (8)	-	-
Frenchmans Creek (Lowland), Wooroonooran National Park	10 (4)	10 (9)	10 (9)	-	-
Kirrama Bridge 11 Creek (Upland), Murray Upper National Park	10 (10)	10 (10)	-	-	-
Kirrama Bridge 8 Creek (Lowland), Murray Upper National Park	10 (10)	10 (10)	10 (8)	-	-
Peach Creek (Upland), McIlwraith Range/Kulla National Park	-	-	-	10 (10)	10 (9)

Data manipulation and analysis

I categorised isolates as totally inhibitory, partially inhibitory, or non-inhibitory according to their effects on *Bd* growth in challenge assays, as described in detail in Chapter Four. In order to balance the dataset for analysis, I excluded all frogs from the Cape York site and *L. rheocola* from the Kirrama lowland site (because it did not occur at the Kirrama upland site) from analyses involving Wet Tropics frogs alone. The initial experimental design was factorial, and although data lend themselves to presentation that way (see Figures 5.2, 5.4, 5.5, 5.7 and 5.8) data were missing from some categories (e.g. no *L. rheocola* in Kirrama uplands and no uninfected *L. nannotis* in Wooroonooran uplands) and had inadequate replication in others, making a generalised linear model unfeasible. Therefore, for some analyses, I combined each

species at each site that it occurred into “species-site” treatments, and similarly, I combined the infection status of the frog at each site into “infection status-site” treatments.

As data were not normally distributed, I used non-parametric statistical tests. I used Kruskal-Wallis tests (PAST v.2.09; Hammer et al. 2001), with pre-planned Kolmogorov-Smirnov post-hoc pairwise comparisons on biologically relevant groupings to examine which groups differed significantly, to compare 1) the total number of cultured bacterial isolates obtained from each frog (isolate richness) among combined “species-site” treatments, and 2) the proportion of inhibitory isolates among combined “infection status-site” treatments. I relied on the initial Kruskal-Wallis tests to maintain the experimentwise error rate at $\alpha = 0.05$, and carried out preplanned post-hoc comparisons at comparisonwise $\alpha = 0.05$. Attempting to maintain the experimentwise error rate at 0.05, for example by carrying out a Bonferroni correction, would have greatly reduced my power to detect genuine differences and would very likely have produced numerous Type II errors due to the relatively large number of post-hoc comparisons that were carried out (Moran 2003; Nakagawa 2004).

I used Mann-Whitney U (MWU) tests (PAST v.2.09; Hammer et al. 2001) to compare 1) isolate richness between uninfected frogs from each bioregion, 2) isolate richness between uninfected and infected frogs of each species, and 3) proportion of inhibitory isolates between infected and uninfected frogs.

To determine whether the ability of bacterial cell-free supernatants to inhibit *Bd* differed between bioregions, infected and uninfected frogs, or among species-site treatments, I ran non-parametric multivariate analysis of variance tests (NPMANOVAs, PAST v.2.09; Hammer et al. 2001) on the proportion of isolates from each frog that were in each of the three challenge assay result categories; totally inhibitory, partially inhibitory and non-inhibitory. Tests were performed with 100000 permutations using Euclidean distance as the distance measure. When results were statistically significant, I used pre-planned pairwise comparisons to determine where differences occurred. I set the comparisonwise error rate at 0.05 and did not use Bonferroni corrections, as described above.

I used Fisher’s Exact tests (StatXact4;Gajjar et al. 1998) to compare the proportions of uninfected frogs with and without inhibitory bacteria between bioregions, and to compare the proportions of uninfected or infected frogs with and without inhibitory bacteria among Wet Tropics sites.

Results

I sampled a total of 118 frogs. Ten frogs failed to produce any culturable bacterial isolates and were excluded from further analyses. A total of 1146 isolates were obtained, with a mean of 10.6 cultured bacterial isolates per frog (Range = 1-36, SD = 7.9). The effects of cell-free supernatants on *Bd* growth followed a bimodal distribution, which is summarised in Figure 5.1. Challenge assay results showed that 64% of frogs had one or more isolates that produced cell-free supernatants active against *Bd*; 56% had totally inhibitory isolates and 38% had partially inhibitory isolates (those that inhibited the growth of *Bd* by more than 63.5% but less than 100%). Sixty-five percent of frogs had isolates that enhanced the growth of *Bd* by 25% or more relative to the positive control (Table 5.2). A greater proportion of inhibitory isolates came from frogs at the Kirrama upland site than from frogs at any other site (Figure 5.2).

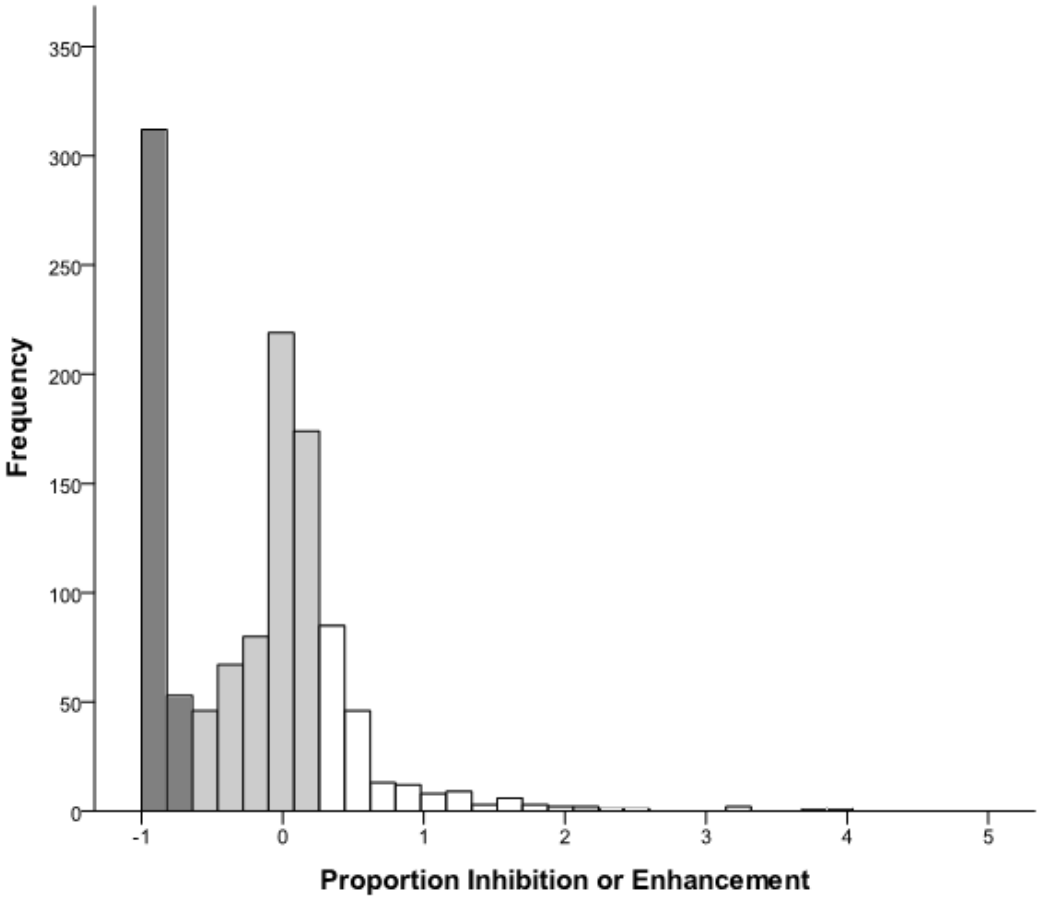

Figure 5.1. Effects of cell-free supernatant on growth of *Batrachochytrium dendrobatidis*. Negative values indicate growth below that of the positive control and positive vales indicate growth greater than that of the positive control. Data are standardised so that the maximum possible negative value is -1 (Chapter Four). The dark grey shading represents all cell-free supernatants with inhibitory effects greater than 63.5% and less than or equal to 100% (Chapter Four), and the pale grey shading represents the non-inhibitory cell-free supernatants. The white shading represents those cell-free supernatants that enhanced *Batrachochytrium dendrobatidis* growth by more than 25% relative to the positive control.

Table 5.2. Number of culturable isolates obtained from each frog by challenge assay result. Frogs that generated no cultured isolates have been excluded. Species are *Litoria serrata* (LS), *L. rheocola* (LR), *L. nannotis* (LN), *L. eucnemis* (LE) and *L. longirostris* (LL), and sites are Kirrama upland (KU), Kirrama lowland (KL), Wooroonooran upland (WU), Wooroonooran lowlands (WL) and McIlwraith upland (MU). *Bd* infection intensity scores (zoospore equivalents) greater than that of the highest standard in the qPCR assay are reported as >16667 zoospores.

					Number of Culturable Isolates		
Frog ID	Species	Site	Sex/Age	*Bd* Infection Intensity	Totally Inhibitory	Partially Inhibitory	Not Inhibitory
1	LS	KL	M	0	0	0	3
2	LS	KL	M	0	0	0	5
3	LS	KL	M	25	0	0	3
4	LS	KL	M	0	0	0	3
5	LS	KL	M	0	0	0	3
6	LS	KL	F	0	2	0	7
7	LS	KL	M	0	1	0	7
8	LS	KL	M	0	4	1	10
9	LS	KL	M	0	1	4	7
10	LS	KL	M	0	0	0	4
11	LN	KL	M	0	0	2	8
12	LN	KL	M	8	6	1	10
13	LN	KL	M	0	2	2	8
14	LN	KL	M	10	2	2	10
15	LN	KL	M	0	3	1	14
16	LN	KL	M	5	0	0	1
17	LN	KL	M	0	2	2	5
18	LN	KL	M	>16667	1	1	8
19	LN	KL	M	53	1	0	1
20	LN	KL	M	0	0	1	2
21	LR	KL	M	0	0	0	2
22	LR	KL	F	0	0	0	19
23	LR	KL	F	0	0	0	2
24	LR	KL	M	0	0	0	8
25	LR	KL	SA	0	2	0	13
26	LR	KL	M	0	0	0	3
27	LR	KL	F	0	0	1	11
28	LR	KL	SA	0	3	0	9
29	LS	KU	M	0	0	1	5
30	LS	KU	M	0	0	0	10
31	LS	KU	M	0	7	0	1
32	LS	KU	M	0	8	1	9
33	LS	KU	M	3	0	3	4
34	LS	KU	M	0	5	1	2
35	LS	KU	M	0	1	2	6
36	LS	KU	M	0	8	5	10
37	LS	KU	M	0	2	4	10
38	LS	KU	M	0	3	3	7
39	LN	KU	SA	0	6	10	11
40	LN	KU	SA	0	3	2	1
41	LN	KU	SA	2	4	4	3
42	LN	KU	F	0	7	0	10
43	LN	KU	SA	0	4	9	8
44	LN	KU	M	0	6	14	6
45	LN	KU	F	0	6	4	14
46	LN	KU	M	0	6	4	8
47	LN	KU	M	0	10	7	7
48	LN	KU	M	0	7	7	5
49	LS	WL	M	0	1	2	24
50	LS	WL	M	7	12	3	16

Table 5.2 Contd.

Frog ID	Species	Site	Sex/Age	*Bd* Infection Intensity	Number of Culturable Isolates		
					Totally Inhibitory	Partially Inhibitory	Not Inhibitory
51	LS	WL	M	0	6	1	14
52	LS	WL	M	7	3	1	11
53	LN	WL	SA	0	0	0	1
54	LN	WL	F	7	0	0	4
55	LN	WL	F	0	1	0	15
56	LN	WL	F	0	0	0	3
57	LN	WL	F	0	0	1	7
58	LN	WL	F	3	2	0	12
59	LN	WL	F	2	0	1	4
60	LN	WL	F	0	0	0	3
61	LN	WL	F	3	0	0	18
62	LR	WL	M	0	1	0	6
63	LR	WL	M	8	0	0	5
64	LR	WL	M	102	0	0	1
65	LR	WL	M	0	4	0	9
66	LR	WL	M	0	2	0	28
67	LR	WL	M	0	0	0	5
68	LR	WL	M	18	0	0	9
69	LR	WL	M	0	12	2	22
70	LR	WL	M	3685	0	1	5
71	LS	WU	M	7	0	0	12
72	LS	WU	M	0	5	0	15
73	LS	WU	SA	0	3	2	17
74	LS	WU	M	32	4	0	5
75	LN	WU	F	3	0	0	2
76	LN	WU	SA	20	0	0	1
77	LN	WU	F	57	0	0	2
78	LN	WU	F	52	0	0	6
79	LN	WU	M	45	4	0	0
80	LN	WU	F	98	8	0	10
81	LN	WU	M	2	0	0	1
82	LR	WU	M	0	0	0	1
83	LR	WU	M	0	0	0	4
84	LR	WU	F	7	14	2	13
85	LR	WU	F	295	0	0	2
86	LR	WU	F	0	0	0	6
87	LR	WU	SA	337	3	3	7
88	LR	WU	M	2898	7	0	6
89	LR	WU	SA	>16667	2	0	15
90	LE	MU	M	0	9	0	9
91	LE	MU	M	0	1	0	8
92	LE	MU	M	0	4	0	4
93	LE	MU	M	0	1	0	6
94	LE	MU	M	0	0	0	8
95	LE	MU	M	0	0	0	7
96	LE	MU	M	0	7	0	11
97	LE	MU	M	0	1	0	6
98	LE	MU	M	0	0	1	11
99	LE	MU	M	0	0	0	9
100	LL	MU	F	0	2	0	0
101	LL	MU	F	0	1	0	0
102	LL	MU	F	0	0	0	6
103	LL	MU	F	0	0	0	7
104	LL	MU	M	0	2	1	4
105	LL	MU	M	0	0	0	3
106	LL	MU	F	0	1	0	0
107	LL	MU	F	0	2	0	1
108	LL	MU	M	0	0	0	8
				Total	248	120	778

Table 5.3. Prevalence of *Batrachochytrium dendrobatidis* infection in each species at each field site.

	Litoria serrata (n=40)	*Litoria nannotis* (n=36)	*Litoria rheocola* (n=25)	Overall prevalence
Kirrama upland	0.1	0.1	-	0.1
Kirrama lowland	0.1	0.5	0	0.21
Wooroonooran upland	0.2	1	0.63	0.56
Wooroonooran lowland	0.2	0.44	0.44	0.36

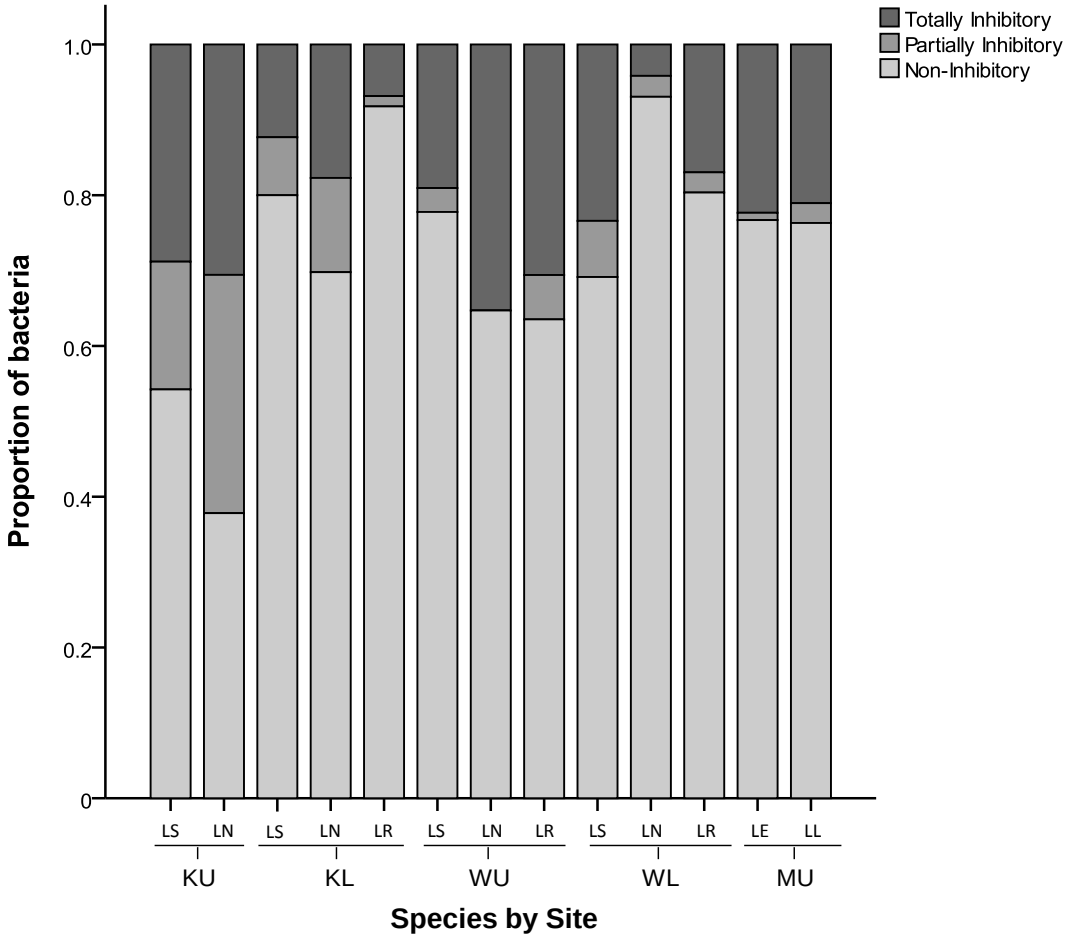

Figure 5.2. The proportion of culturable isolates categorized by challenge assay results for each frog species at each site. Species are *Litoria serrata* (LS), *L. nannotis* (LN), *L. rheocola* (LR), *L. eucnemis* (LE), and *L. longirostris* (LL). Sites are Kirrama upland (KU), Kirrama lowland (KL), Wooroonooran upland (WU), Wooroonooran lowland (WL), and McIlwraith upland (MU).

No *Bd* infection was found in frogs from the Cape York Peninsula site while 32 of the 89 frogs (36%) sampled at Wet Tropics sites were infected with *Bd* (Table 5.2, Table 5.3). A significantly higher proportion of frogs were infected with *Bd* in the Wooroonooran region than in the Kirrama region (Fisher's Exact Test, p = 0.0012); only 8 of 50 frogs (16%) were infected with *Bd* at the Kirrama sites, while 27 of 61 frogs (44%) were infected at the Wooroonooran sites.

I could not statistically compare intensity of infection among species within each site due to the low numbers of infected frogs. However, of the 32 infected frogs, 16 had infections of 10 zoospore equivalents or less indicating relatively light infection levels (Table 5.2, Figure 5.3). Higher infection intensities occurred almost exclusively in *L. rheocola* at the Wooroonooran sites.

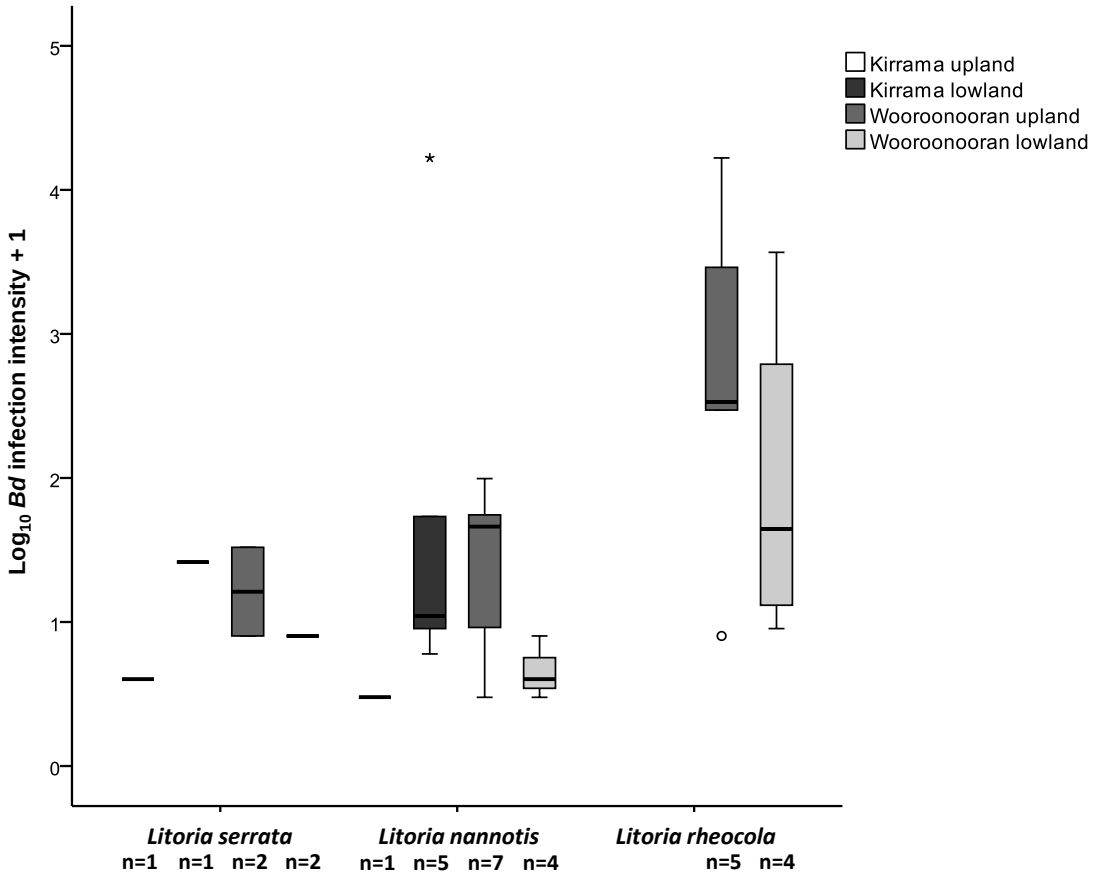

Figure 5.3. Distributions of *Batrachochytrium dendrobatidis* infection intensity for each species of frog at each Wet Tropics site. Boxes represent the interquartile range and bold bars, the median. Whiskers represent the minimum and maximum. Outliers are marked with a circle and are cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box, while extreme values are marked with a star and represent cases with values greater than 3 box lengths from the upper edge of the box.

Comparisons among frog species at each site

Isolate richness of cultured bacteria

The cultured isolate richness per frog differed significantly among species-site treatments (Figure 5.4; Kruskal-Wallis; $H = 28.28$, $df = 9$, $p < 0.00086$). When Kolmogorov-Smirnov pairwise comparisons were examined to determine which pairs of values differed significantly, the Kirrama sites showed a clear species by elevation interaction that was absent at the Wooroonooran sites. Both species of Kirrama upland frogs had significantly greater bacterial isolate richness than their lowland conspecifics; $p = 0.047$ for *L. serrata* and $p = 0.0334$ for *L. nannotis*. *L. nannotis* had a significantly greater number of isolates than *L. serrata* at the Kirrama upland site ($p = 0.0306$). At the Wooroonooran sites, *L. nannotis* had significantly fewer isolates than *L. serrata* at both upland and lowland sites ($p = 0.0026$ and $p = 0.0282$ respectively). *L. rheocola* also had significantly fewer isolates than *L. serrata* at the Wooroonooran lowland site ($p = 0.0261$).

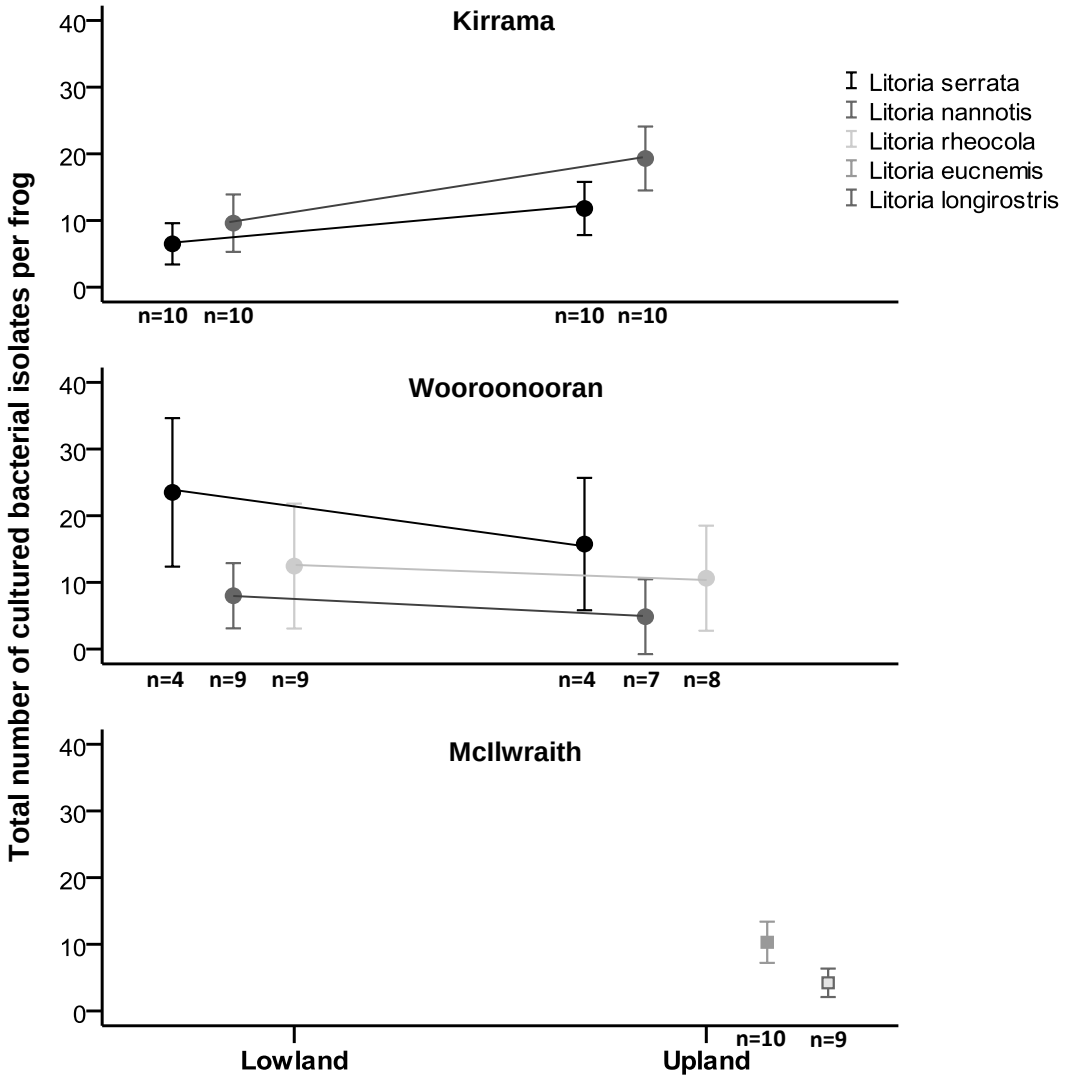

Figure 5.4. The mean number of cultured bacterial isolates per frog by species, elevation and site. Error bars represent the 95% confidence intervals of the means. The lines between each species at each elevation are solely to aid in visualization of patterns.

Proportion of inhibitory bacteria

The proportion of isolates in each category of inhibition differed significantly among species-site treatments (Figure 5.5; NPMANOVA, $F = 4.97$, $p < 0.00001$). Pre-planned comparisions were used to determine differences among relevant treatments. Upland and lowland frogs at the Wooroonooran sites did not differ in their bacterial capacity to inhibit *Bd* but, at the lowland site, *L. serrata* had significantly greater inhibitory capacity than *L. nannotis* ($p = 0.0042$). However, at Kirrama upland both *L. nannotis* and *L. serrata* had a significantly higher proportion of inhibitory isolates than their lowland conspecifics ($p = 0.00015$ and $p = 0.041$ respectively), and Kirrama lowland *L. nannotis* had a higher proportion of inhibitory isolates than *L. serrata* ($p = 0.02315$).

To compare the proportions of frogs with and without inhibitory bacteria among Wet Tropics sites, I used a Fisher's Exact Test. The proportions of frogs with and without inhibitory bacteria differed significantly among sites when the Kirrama upland site was included in the analysis (Fisher's Exact Test; $p = 0.011$), but not when it was excluded (Fisher's Exact Test; $p = 0.799$).

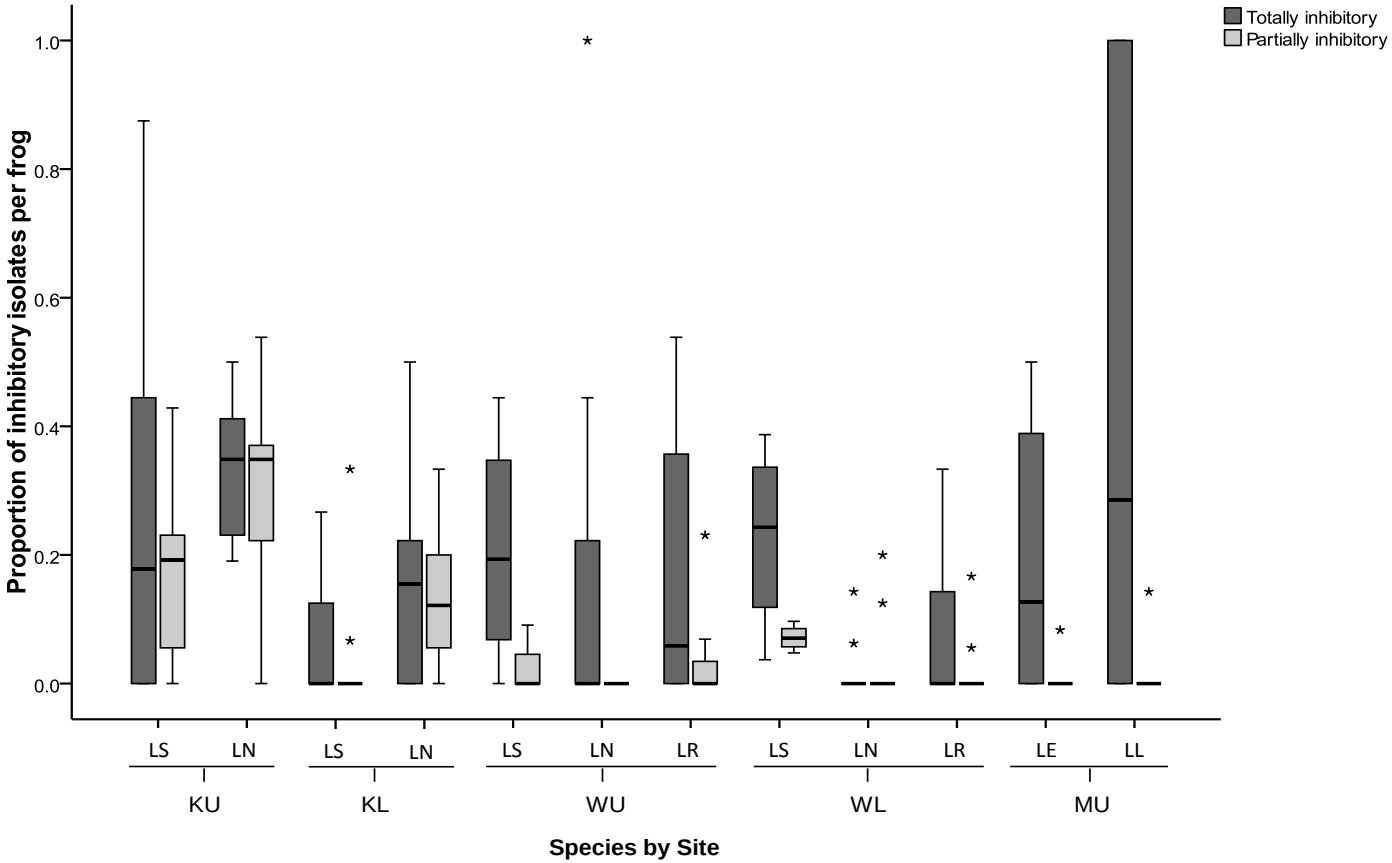

Figure 5.5. The proportion of isolates per frog with effects on *Bd* that fell into the totally and partially inhibitory categories. Species are *Litoria serrata* (LS), *L. nannotis* (LN), *L. rheocola* (LR), *L. eucnemis* (LE), and *L. longirostris* (LL).Sites are Kirrama upland (KU), Kirrama lowland (KL), Wooroonooran upland (WU), Wooroonooran lowland (WL), and McIlwraith upland (MU).Boxes represent the interquartile range and bold bars, the median. Whiskers represent the minimum and maximum data points. Outliers are marked with a circle and are cases with values between 1.5 and 3 box lengths from the upper edge of the box, while extreme values are marked with a star and represent cases with values greater than 3 box lengths from the upper edge of the box.

Inhibitory activity of isolates in *Bd*-naïve frogs

Uninfected frogs from the two bioregions were not significantly different in terms of overall number of bacterial isolates (Figure 5.6; Mann-Whitney U test; $U_{77} = 637.5$, $Z = -1.677$, $p = 0.0936$) or proportion of bacteria in each inhibition category (Figure 5.5; NPMANOVA, $F = 2.9$, $p = 0.0766$). In addition, the proportion of uninfected frogs with and without inhibitory bacteria did not differ between bioregions (Fisher's Exact Test, $p = 0.44$).

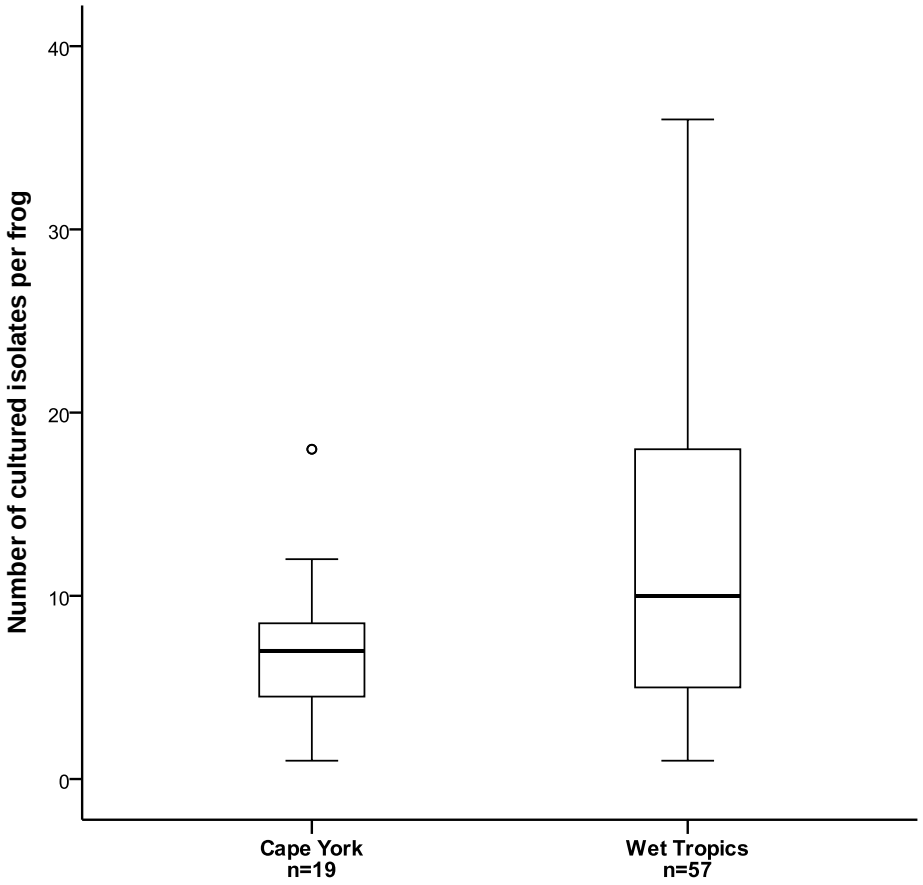

Figure 5.6. The number of isolates from uninfected frogs in the Wet Tropics and Cape York bioregions. Boxes represent the interquartile range and bold bars, the median. Outliers are marked with a circle and are cases with values between 1.5 and 3 box lengths from the upper edge of the box.

Proportion of inhibitory bacteria

There were no differences in the proportion of isolates that inhibited *Bd* (Mann-Whitney U test; U_{80} = 663.5, z = -1.182, p = 0.237) or in the proportion of isolates in each category of inhibition (NPMANOVA, F = 1.05, p = 0.319) between infected and uninfected individuals. However, the proportion of inhibitory isolates was significantly different among infection status-site treatments (Kruskal-Wallis; H = 27.57, df = 7, p < 0.0003). Figure 5.7 shows that, at Kirrama, uninfected upland frogs had a greater proportion of inhibitory isolates than uninfected lowland frogs (Kolmogorov-Smirnov pairwise comparison, p < 0.0001). A similar pattern can be observed in infected frogs at the Kirrama upland and lowland sites; however, due to the small number of infected frogs at the Kirrama upland site, the effect of elevation is not significant (Kolmogorov-Smirnov pairwise comparison, p = 0.149).

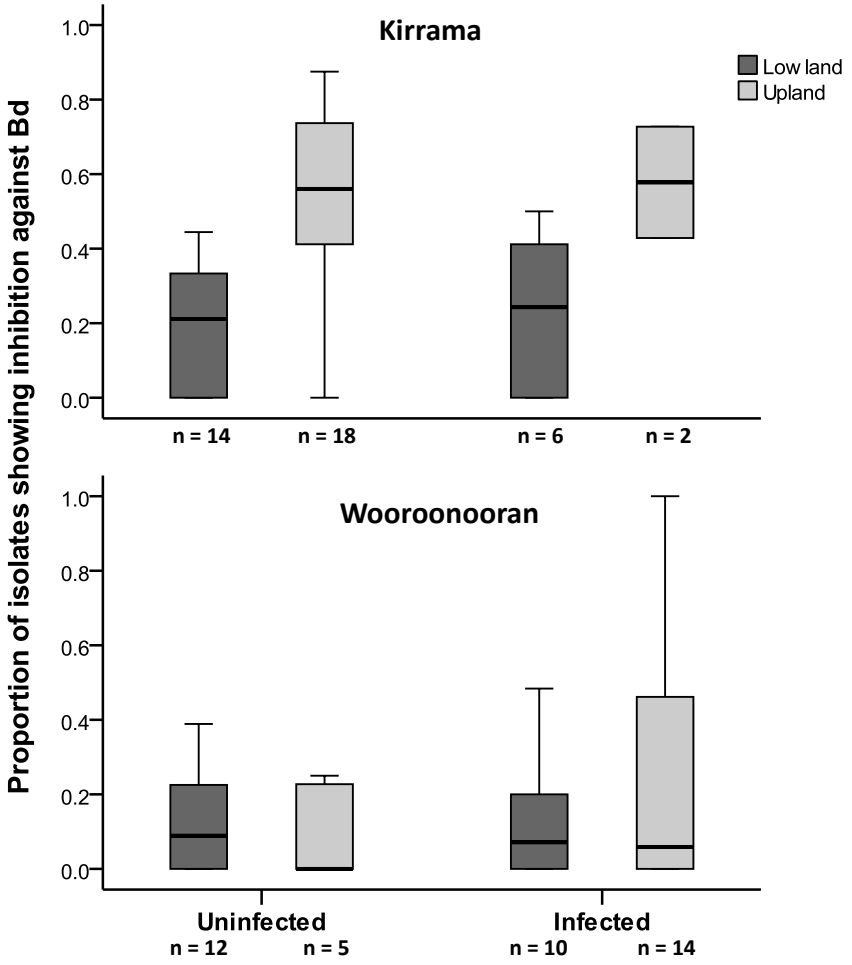

Figure 5.7. The proportion of bacterial isolates with inhibitory effects against *Batrachochytrium dendrobatidis* in infected and uninfected upland and lowland frogs. Boxes represent the interquartile range and bold bars, the median. Whiskers represent the minimum and maximum data points.

Cultured bacterial isolate richness

Uninfected *L. nannotis* had significantly more bacterial isolates than infected *L. nannotis* (Mann-Whitney U test; $U_{34} = 81$, Z = -2.34, p = 0.0192), but uninfected and infected *L. serrata* and *L. rheocola* did not significantly differ in isolate numbers (MWU tests; *L. serrata,* $U_{28} = 75.5$, Z = -0.05, p = 0.959; *L. rheocola*, $U_{16} = 35.5$, Z = 0, p = 1). However, the boxplot in Figure 5.8 shows that these results are not likely to be consistent among field sites. No attempt was made to compare species among sites due to the low numbers of animals in each category.

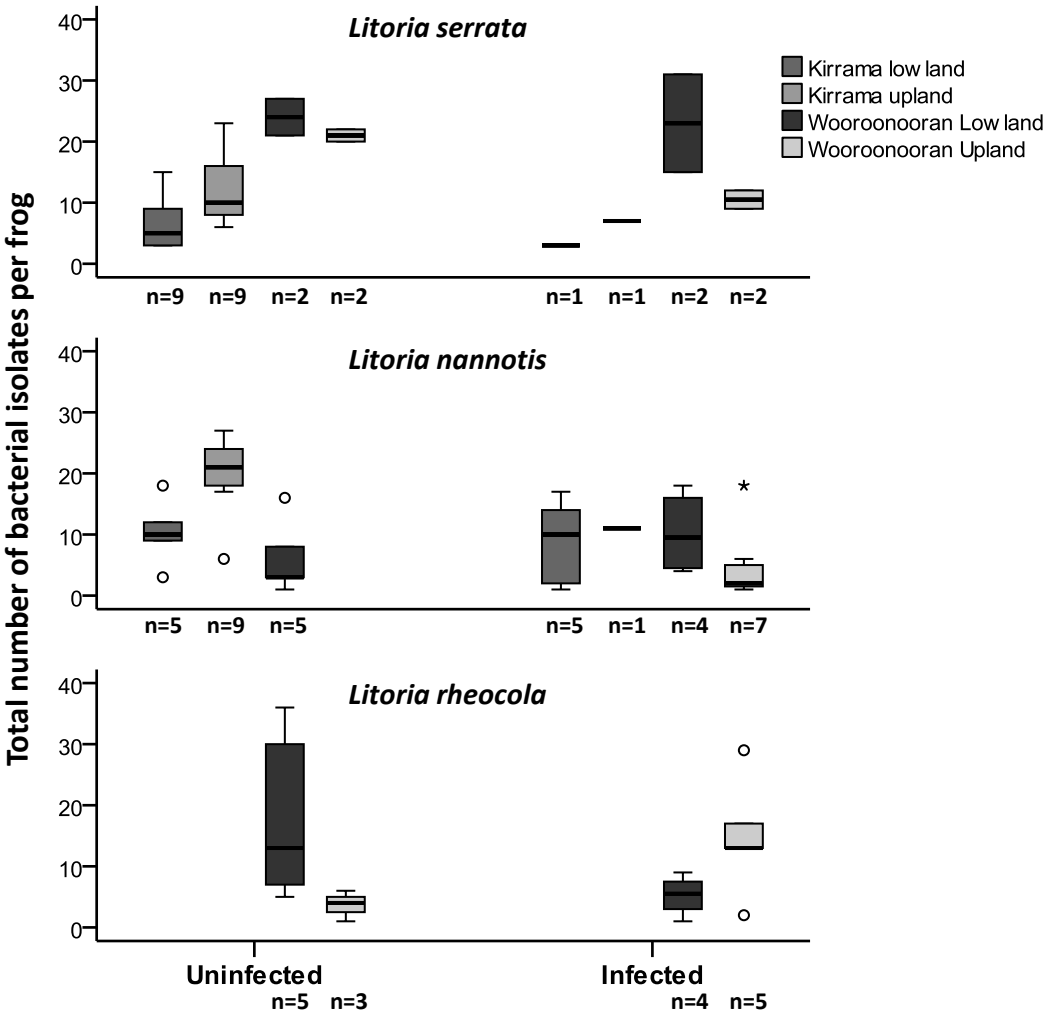

Figure 5.8. The total number of bacterial isolates from infected and uninfected frogs for the species sampled at each Wet Tropics site. Boxes represent the interquartile range and bold bars, the median. Whiskers represent the minimum and maximum data points. Outliers are marked with a circle and are cases with values between 1.5 and 3 box lengths from the upper edge of the box, while extreme values are marked with a star and represent cases with values greater than 3 box lengths from the upper edge of the box.

Discussion

This study had two major goals. The first was to provide baseline data on the capacity of the cutaneous bacterial microbiota of Australian Wet Tropics frogs to provide antimicrobial defences against *Bd*; and the second, to investigate the potential role that cutaneous bacterial microbiota may have played in the reappearance of upland frog populations following the population declines in the early 1990s. We now know that the majority of Wet Tropics frogs carry bacteria that can produce anti-fungal metabolites that are active against *Bd*, and that this capacity can vary among species and sites.

A mean of 10.6 bacterial isolates were cultured from each frog. This is approximately in line with two previous studies on *Rana muscosa* populations in the USA that reported 8.03 and 8.62 bacterial isolates per frog (Woodhams et al. 2007c), but twice that reported for *Hyalinobatrachium colymbiphyllum* in Panama (Walke et al. 2011). In my study, I made thorough attempts to select all morphologically different bacterial colonies for culture and, in addition, to select extra colonies that were morphologically similar to those already selected, as morphology alone is not a reliable indicator of species (see Chapter Three). This could have accounted for the slightly higher numbers of cultured bacterial isolates in my study.

All frogs from the Cape York Peninsula were free of *Bd*, in agreement with results from previous surveys (Skerratt et al. 2010), while *Bd* was enzootic in frogs at all Wet Tropics sites. Frogs in the Cape York bioregion are unlikely to have been exposed to *Bd* and it is probable that the environmental conditions at McIlwraith Range are not favorable for proliferation of the pathogen. The tendency for Cape York frogs to have fewer overall bacterial isolates than Wet Tropics frogs, although not statistically significant, is possibly due to the low number of isolates derived from *L. longirostris* alone, rather than an effect of exposure to *Bd*. Similarly, observed differences in the proportion of inhibitory isolates in each category of inhibition, between frogs from each bioregion, was due to the lack of partially inhibitory isolates on frogs in the McIlwraith uplands (see Figure 5.5). The proportion of uninfected frogs with inhibitory bacteria did not differ significantly between the two bioregions. This suggests that, on average, frogs in populations subject to *Bd* infection have not acquired large numbers of novel *Bd*-inhibitory bacteria. The resident bacterial microbiota may supplement the host innate immune response against a broad range of potentially pathogenic environmental microorganisms.

Frogs at the Kirrama upland site were responsible for most of the significant effects observed. The highest proportion of frogs with bacterial isolates that were capable of producing *Bd*-inhibitory metabolites occurred at the Kirrama uplands site. Frogs of both species at this site

had greater cultured bacterial isolate richness and a higher proportion of inhibitory isolates than their lowland conspecifics. In addition, upland *L. nannotis* had more cultured bacteria overall than upland *L. serrata*, while lowland *L. nannotis* had a higher proportion of inhibitory isolates than lowland *L. serrata*.

Compared with the Kirrama sites, few significant patterns emerged at the Wooroonooran sites. Any patterns observed may have arisen as a result of the conservative over-selection of bacterial colonies from *L. serrata* agar plates, given the high number of bacterial colonies produced by this species at both upland and lowland sites.

***Bd* infection**

Infection status did not significantly affect the proportion of inhibitory isolates present on frogs. However, uninfected *L. nannotis* had more cultured bacterial isolates than infected *L. nannotis*, although the boxplot in Figure 5.8 suggests that this effect might be site-specific and driven entirely by frogs at the Kirrama upland site. Unfortunately, due to the low number of infected frogs, statistical comparisons among sites for each species were not possible. Given that phylogeographic isolation has occurred among many of these populations (Richards et al. 2010; Bell et al. 2012), it is quite possible that different mechanisms have evolved in the Kirrama and Wooroonooran regions to facilitate survival with *Bd* infection.

Frogs can gain and lose *Bd* infection multiple times throughout their lives (Kriger and Hero 2006; Briggs et al. 2010), and it is not yet known how cutaneous bacteria change with the changing infection status of the frog (however, see Chapter Seven). It is entirely possible that frogs that have recently lost *Bd* infection have a bacterial microbiota more similar to that of infected frogs than to frogs that have been uninfected for some time. Conversely, frogs that have recently gained *Bd* infection might have a microbiota more similar to uninfected frogs than to frogs that have been infected for longer. Therefore, the lack of any detected effect related to *Bd* infection could simply be an artifact of a single time-point sample that does not consider the changing infection status of the frog.

The low intensities of infection observed in the frogs of the Kirrama region during this study are not necessarily representative of those found in this area in previous years. Quantitative PCR results, from a study conducted during the winter of 2007, indicated the presence of many high intensity infections in *L. nannotis*, while *L. serrata* generally had lower levels of infection (S. Bell, unpublished). This pattern was opposite from that found in winter 2005, where *L. serrata* had higher infection intensities than *L. nannotis* (S. Bell, unpublished). Infection

intensities are related to minimum and maximum winter temperatures and duration of the winter season (Puschendorf 2009), which can vary from year to year. It is not yet known whether differences in infection intensities among years are associated with changes in the number, composition, or *Bd*-inhibitory capacity of bacterial species present on frog skin. It would be necessary to collect samples throughout the winter season over several years to determine whether any correlation exists.

Of the three species in this study, *L. nannotis* are the most, and *L. serrata* the least, strongly associated with water, and *L. serrata* are the least susceptible to population fluctuations as a result of *Bd* infection. At the Kirrama upland site, a higher proportion of *L. nannotis* spend more time out of water, in contact with vegetation and stream-side substrates, than at other sites where wet rock is the favoured habitat (pers. obs.; B. Roznik, pers. comm.). This difference in behaviour could account for the lower infection prevalence observed, because vegetation offers a drier habitat that may be less favourable for *Bd* growth. In addition, *L. nannotis* that use vegetation as preferred habitat may be exposed to a greater diversity of environmental bacteria that are not present in water or on wet rocks. This could account for the higher number of cultured bacteria present and consequently the higher proportion of inhibitory bacteria in this species at the Kirrama upland site.

Mechanisms of population recovery in the Wet Tropics

It is entirely possible that different mechanisms are involved in the recovery of each species at each site as there are a number of species- and site-specific differences that may contribute to the different patterns observed. A previous study indicated that Wooroonooran upland *L. nannotis* have more effective AMPs than Kirrama upland *L. nannotis* (S. Bell, unpublished) suggesting that AMPs may be more important than *Bd*-inhibitory bacteria at the Wooroonooran upland site. However, Wooroonooran upland AMP sample sizes were small compared with those at the Kirrama sites, so larger sample sizes will be needed to confirm this observation. The documented genetic separation between the Wooroonooran and Kirrama populations of *L. nannotis* (Cunningham 2001; Bell et al. 2012) and *L. serrata* (Richards et al. 2010) adds weight to the suggestion that the mechanisms for population coexistence with *Bd* infection are likely to have evolved independently in these two regions.

Summary

There are many reasons why species- and site-specific differences in frog population reappearances exist, and there is no reason why the mechanisms responsible should be the same for each species or site. There are physical and genetic barriers that may affect recolonisation progress, as well as differences in host tolerance to *Bd* (which include immune defence and behavioural adaptation), and potential differences in pathogen virulence. However, it was beyond the scope of this study to test other factors that may interact with the microbiota to facilitate population reappearance. This study investigated the possibility that amphibian cutaneous bacteria play a role in maintaining upland populations that have reappeared through the production of inhibitory compounds that can kill *Bd*. Kirrama upland frogs had a higher proportion of inhibitory isolates compared with Wooroonooran upland frogs. This provides some indication that bacteria able to inhibit *Bd* might have played a role in the reappearance of frogs at this site. However, no such patterns were evident in frogs at the Wooroonooran sites where greater prevalence of infection and a higher intensity of infection were present, and yet *L. nannotis* and *L. rheocola* have been able to recolonise this area too. Wooroonooran upland *L. nannotis* have more effective AMPs than Kirrama upland *L. nannotis* (S. Bell, unpublished) indicating that AMPs may be more important to frogs at the Wooroonooran upland site, while bacterial inhibition of *Bd* may be more important to Kirrama upland frogs.

In this chapter, I assessed all cultured bacteria for inhibitory effects against *Bd*. However, it is possible that the dataset of bacterial isolates from each frog could have contained duplicates selected during the effort to pick all unique bacteria present on the agar plate. While it is reasonable to assume that any duplicate isolates were selected with equal frequency from each sample, it is only possible to confirm the effects of unique inhibitory isolates if DNA is sequenced and duplicate DNA sequences are removed. Therefore, in my next chapter, I identify all totally inhibitory isolates by DNA sequencing of the 16S ribosomal DNA gene, and remove sequences that occur more than once on each frog. I then investigate the ways in which inhibitory bacteria could mitigate the effects of *Bd* infection.

CHAPTER SIX : BACTERIAL SPECIES RICHNESS AND INFECTION: ARE BACTERIA HELPING TO MITIGATE THE EFFECTS OF *BATRACHOCHYTRIUM DENDROBATIDIS* IN WILD FROG POPULATIONS?

Chapter Overview

In Chapter Five, I investigated differences in the ability of the cutaneous bacterial microbiota to inhibit *Batrachochytrium dendrobatidis* (*Bd*) among five species of frogs at five sites within two bioregions in northern Queensland, Australia. However, there was a high probability of duplicate bacterial isolates within the dataset. In this chapter, I identify all totally inhibitory isolates by DNA sequencing of the 16S ribosomal DNA gene, and remove sequences that occur more than once on each frog. I then investigate the ways in which inhibitory bacteria could mitigate the effects of *Bd* infection.

Abstract

Symbiotic bacterial communities resident on amphibian skin are likely to perform functions that benefit their hosts. For example, antibiotic production by these communities could control pathogens such as *Batrachochytrium dendrobatidis* (*Bd*). One aspect of community ecology theory predicts that complex communities, with higher numbers of taxa and hence more potential interactions, are more resistant to invasion than simple communities with fewer taxa. With these hypotheses in mind, I used DNA sequencing to identify 121 *Bd*-inhibitory bacteria from five species of rainforest frogs at five sites in northern Queensland, Australia to determine whether cutaneous bacterial taxa were associated with particular frog species, sites, infection status of frogs, or intensity of infection. Ninety-four percent of isolates were from the Proteobacteria phylum and were distributed across three families; Pseudomonadaceae, Enterobacteriaceae and Xanthomonadaceae. *Bd* infection intensity was negatively correlated with number of genera present per frog suggesting that increased diversity of *Bd*-inhibitory taxa may play a role in reducing the intensity of *Bd* infections, hence facilitating frog coexistence with enzootic *Bd*. One upland site had a significantly higher proportion of frogs with one or more *Bd*-inhibitory bacteria, a greater number of inhibitory bacterial genera present per frog, and statistically significant clustering of individual frogs with similar *Bd*-inhibitory genera signatures compared to all other sites. This suggests that *Bd*-inhibitory taxa are likely to be particularly important to frogs at this site and may have played a role in their ability to recolonise following population declines in the early 1990s.

Introduction

One aspect of community ecology theory predicts that complex communities, with higher numbers of species and hence more potential interactions, are generally more resistant to invasion than simple communities with fewer species (Robinson and Valentine 1979). Frog skin with its mucus layer can be viewed as a substrate for bacterial communities that may play a role in preventing invasion of the pathogen *Bd*. To date, we know very little about the complexity of the microbial communities that inhabit this niche.

A number of studies have revealed that amphibian skin is colonised by bacteria (Bettin and Greven 1986; Barra et al. 1998; Austin 2000; Boman 2000; Potter and Norman 2006; Culp et al. 2007), and some have proposed that these colonists may confer disease resistance on their hosts (Bettin and Greven 1986; Austin 2000; Harris et al. 2006; Lauer et al. 2007; Woodhams et al. 2007c; Banning et al. 2008; Lauer et al. 2008; Becker et al. 2009; Harris et al. 2009a; Harris et al. 2009b; Becker and Harris 2010). Four studies to date have described the anti-*Bd* cutaneous bacterial microbiota of amphibians. Harris et al. (2006) described *Bd*-inhibitory bacteria from three phyla, Proteobacteria, Bacteriodetes and Firmicutes, isolated from the salamanders *Plethodon cinereus* and *Hemidactylium scutatum*. They proposed that the mode of action of *Bd* inhibition was likely to be through production of inhibitory compounds. Both Woodhams et al. (2007c) and Lam et al. (2010) described the *Bd*-inhibitory bacteria of the sister species of frogs, *Rana muscosa* and *R. sierra*, and their findings showed that the same three phyla described by Harris et al. (2006) were present on frogs at each of three field sites sampled. Walke et al. (2011) were the first to report the *Bd*-inhibitory bacterial community on a tropical rainforest frog, *Hyalinobatrachium colymbiphyllum,* finding representatives of the Proteobacteria and the Bacteriodetes phyla present. Antibiotic compounds are known to be produced by many of the bacterial genera found in these four studies. Some of the inhibitory compounds described to date are provided in Appendix A.

In theory, if a sufficiently high proportion of a population has resistance to a pathogen, the population as a whole may be resistant to epizootic outbreaks because of reduced likelihood of contact between susceptible and infectious individuals (Anderson and May 1985). Therefore, amphibian populations with a high proportion of individuals that have *Bd*-inhibitory bacteria may be able to survive with enzootic *Bd* infection (Lam et al. 2010). Frogs with at least one *Bd*-inhibitory cutaneous bacterial species may be able to resist *Bd* infection better than frogs with none (Woodhams et al. 2007c). In one *R. muscosa* population in the Sierra Nevadas, 62% of frogs had one or more inhibitory bacteria; this population declined to extinction

following exposure to *Bd* while, in a second population from the same region, 85% of frogs had one or more inhibitory bacteria, and these frogs survived with *Bd* infection enzootic within the population. Lam et al. (2010) revisited the population that Woodhams et al. (2007c) found surviving with enzootic *Bd* infection and once again found that a high proportion (80%) had bacteria active against *Bd*. They also sampled a second, *Bd*-naïve population, and found that 79% of frogs carried inhibitory bacteria. When *Bd* subsequently arrived in this population, it declined in numbers but persisted in coexistence with *Bd*.

This reinforces the concept that artificial augmentation of a high proportion of members of vulnerable populations with bacteria effective against *Bd* could allow them to persist through initial colonization by *Bd* (Harris et al. 2006), providing time for selection to produce behavioural or immune defences against the pathogen. In a laboratory bioaugmentation trial, Harris et al. (2009a) showed that inoculated bacteria could be detected on frogs 20 weeks after the initial application, demonstrating that there is potential for the applied bacteria to be incorporated into the frogs' resident microbiota over at least a relatively extended period.

Very little information is available on how amphibians acquire their skin microbiota. Some bacteria are able to colonise from the environment (Austin 2000; Culp et al. 2007). Unfortunately, little information is available on environmental assemblages of bacteria in rainforest environments. There has been some characterization of rainforest soil microbiota in the Peruvian Amazon (Fierer and Jackson 2006), but microbial diversity in Australian Wet Tropics rainforest soils may not be comparable. However, the frog species in this study rarely contact soil, preferring arboreal, rock and water environments with occasional contact with superficial leaf litter; the bacterial microbiota of these substrates have not been characterised. They rarely contact one another and do not brood their young, ruling out horizontal or vertical transmission of bacterial symbionts as a primary means of symbiont acquisition. Therefore, it seems plausible that selection for environmental bacteria that perform a functional role within the mucosal microbial community may occur.

This chapter describes the anti-*Bd* cutaneous bacterial microbiota of five species of rainforest frogs from five sites in northern Queensland, Australia, and assesses how its composition may contribute to the health and recovery of frogs at sites with different histories of pathogen-related population declines.

Materials and Methods

Field sites and species

I collected cutaneous swab samples from five species of stream-dwelling rainforest frogs at five sites in northern Queensland, Australia, as previously described in Chapter Five.

Collection of bacterial samples and detection of inhibitory isolates

Methods used for sample collection, purification of isolates, challenge assays and analysis are described in Chapters Two and Three. I selected pure bacterial isolates, whose cell-free supernatants exhibited total inhibition against *Bd* (see Chapter Five data), for identification by DNA sequencing. Quantitative PCR (qPCR) was used to diagnose and quantify *Bd* infection status following the protocol of Boyle et al. (2004).

DNA sequencing

I relied on DNA sequencing to identify bacteria. DNA was extracted from pure isolates and 16S rRNA was amplified by PCR as described in Chapter Two. PCR product purification and DNA sequencing were conducted by Macrogen Inc. (South Korea). I aligned forward and reverse nucleotide sequences in Geneious Pro (Biomatters Inc.; Drummond et al. 2010) to create a consensus sequence of approximately 1400bp. All consensus sequence editing and alignments were performed within Geneious Pro using the ClustalW algorithm (Larkin et al. 2007). Nucleotide sequences were subject to National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) search against those in the GenBank database (Benson et al. 2011) and identification assigned based on highest sequence similarity as sequences all had similarities of greater than 98%.

Data manipulation and analyses

As a basis for analysis, I considered each unique 16S rDNA sequence to be a strain-level operational taxonomic unit (OTU). As there are no set criteria to define strain or species-level identification (Janda and Abbott 2007) there were several reasons for this decision: First, each DNA band on a DGGE gel is normally considered a different species, and band separation can occur with as little as 1 - 2 bp difference in 16S rDNA sequence (Muyzer et al. 1993); second, sequences with high 16S rDNA similarity can still constitute different species as differences elsewhere in the genome can still be large (Fox et al. 1992); and third, it is possible that strain-level mutations control the ability to produce antibiotic compounds (Fravel 1988). I removed replicate sequences from each frog's dataset on the assumption that they were most likely to be of the same bacterial strain. It is possible that this either under- or overestimates OTU diversity, since bacterial species and strains can have more than one 16S rDNA gene (Kang et

al. 2010) or, less commonly, a 16S rDNA gene in common with other species (Muyzer and Smalla 1998).

As data were not normal, I used Kruskal-Wallis tests with pre-planned Kolmogorov-Smirnov pairwise comparisons, as described in Chapter Five, to examine site-specific differences in the number of *Bd*-inhibitory cutaneous bacterial genera present on frogs. For this, I excluded all data from Cape York frogs and from *L. rheocola* in the Wet Tropics, to ensure that species were balanced across sites. To examine the relationship between taxonomic diversity and prevalence of *Bd* infection on each stream (calculated as detailed in Chapter Five), I used Spearman's rank correlations.

When totally inhibitory isolates were present on *Bd*-infected frogs, I used quantile regression (Blossom; Cade and Richards 2005) at the 90^{th} percentile level to examine the relationship present between the intensity of *Bd* infection and the number of unique inhibitory bacterial taxa present on each frog. This produces a test statistic (T_{obs}), which is analogous to R^2. Frogs that did not carry any totally inhibitory isolates were excluded from analyses because in estimating the influence of number of inhibitory bacterial OTUs on the limits of infection intensity, individuals with no inhibitory bacterial OTUs are not simply individuals with one fewer than one, they are in a different class, not comparable to those with at least one.

To determine if taxonomic clustering existed among species, sites, or with *Bd* infection status, I used one-way analysis of similarity (ANOSIM) tests (PAST; Hammer et al. 2001), with Bray-Curtis (Bray and Curtis 1957) as the distance measure run with 100000 permutations. When results were statistically significant, I used pre-planned pairwise comparisons to determine where differences occurred. I set the comparisonwise error rate at 0.05 and did not use Bonferroni corrections, as described in Chapter Five. I constructed a dendrogram to demonstrate clustering of frogs among sites using the unweighted pair group method with arithmetic averages (Michener and Sokal 1957) with Bray-Curtis as the distance measure (UPGMA; PAST; Hammer et al. 2001). Frogs from the McIlwraith upland site were excluded from this analysis as all were uninfected, different species were present, and the site was outside the Wet Tropics bioregion.

I used Fisher's Exact tests (StatXact-4; Gajjar et al. 1998) to examine the association between the number of frogs with totally inhibitory OTUs or genera and field site.

Results

One hundred and twenty-one unique *Bd*-inhibitory OTUs were identified from 60 frogs across all five species (Table 6.1). Of these OTUs, 107 were found on Wet Tropics frogs and 17 on Cape York frogs with three occurring on frogs in both bioregions. Twenty-four occurred on more than one frog. The percentage sequence similarity to closest matches in GenBank was high (Table 6.2). Eighty-one percent of OTUs had 99.5% or greater sequence similarity with those in Genbank and the remaining 19% had between 98.2 and 99.5% similarity. Given the high number of similar sequences in Genbank, this indicates that identifications are likely to be accurate at genus level with a possible indication of species level classification.

Table 6.1. Numbers of frogs yielding totally inhibitory bacteria from the total frogs sampled at each site, shown by infection category. The figures represent the number of frogs without totally inhibitory bacteria / number of frogs with totally inhibitory bacteria, with the range of unique OTU numbers on each frog indicated in brackets. "-" indicates absence of samples in a given category.

Site & Species	Uninfected frogs (n=83)	Infected frogs (n=35)				Total frogs (n=118)
		Low intensity 1-10 ZSE	Medium intensity 11-100 ZSE	High intensity 101-1000 ZSE	Very high intensity 1001+ ZSE	
Kirrama Upland - Bridge 11 Creek						
L. serrata	2 / 7 (1 - 7)	1 / -	- / -	- / -	- / -	10
L. nannotis	- / 9 (3-6)	- / 1 (4)	- / -	- / -	- / -	10
Kirrama Lowland - Bridge 8 Creek						
L. serrata	5 / 4 (1 - 3)	- / -	1 / -	- / -	- / -	10
L. nannotis	2 / 3 (1 - 2)	1 / 2 (2 - 6)	- / 1 (1)	- / -	- / 1 (1)	10
L. rheocola	8 / 2 (1 - 3)	- / -	- / -	- / -	- / -	10
Wooroonooran Upland - Windin N Creek						
L. serrata	- / 2 (2 - 4)	1 / -	- / 1 (1)	- / -	- / -	4
L. nannotis	1 / -	2 / -	4 / 2 (2 - 5)	- / -	1 / -	10
L. rheocola	4 / -	- / 1 (6)	1 / -	1 / 1 (3)	- / 2 (2)	10
Wooroonooran Lowland - Frenchmans Creek						
L. serrata	- / 2 (1 - 4)	- / 2 (2 - 8)	- / -	- / -	- / -	4
L. nannotis	5 / 1 (1)	3 / 1 (1)	- / -	- / -	- / -	10
L. rheocola	2 / 4 (1 - 8)	1 / -	1 / -	1 / -	1 / -	10
McIlwraith Upland - Peach Creek						
L. eucnemis	4 / 6 (1 - 6)	- / -	- / -	- / -	- / -	10
L. longirostris	5 / 5 (1 - 2)	- / -	- / -	- / -	- / -	10

The Proteobacteria dominate the bacterial phyla present with 114 unique sequences with only five in the Bacteroidetes. The Firmicutes and Actinobacteria had one unique sequence each. The Gammaproteobacteria class dominated the Proteobacteria phyla with 99 OTUs with only two and 13 in the Alpha- and Beta-proteobacteria classes respectively. The Pseudomonadaceae, Enterobacteriaceae and Xanthomonadaceae families dominated the Gammaproteobacteria with 45%, 29% and 19% of OTUs respectively (Table 6.2). Multiple sequences, differing by as little as one nucleotide, identified with high similarity as *Pseudomonas fluorescens* strain 1408 (GenBank accession number GU726880; Pf1408). The most abundant of the Pf1408 sequences occurred on 12 individual frogs across all species and field sites. The next most commonly occurring sequence identified as *P. fluorescens* strain HXQ-N33 (GenBank accession number HM439651; PfHXQ) and was found on 10 frogs of two species at one site. Pf1408 was originally isolated from sea water from the South China Sea, Vietnam, and PfHXQ was isolated from garden soil in Anhu province, China.

Negative correlations existed between infection prevalence for each site and the number of inhibitory OTUs (Figure 6.1; Spearman's rho = -0.384, p = 0.016), genera (Spearman's rho = -0.518, p = 0.001) and families (Spearman's rho = -0.414, p = 0.009) per frog. In addition quantile regression, at the 90th percentile level, demonstrated a significant negative relationship between the number of *Bd*-inhibitory genera present per frog and the upper limits of *Bd* infection intensity (Figure 6.2B; n = 15, T_{obs} = 0.643, p = 0.043) and was suggestive of a relationship at OTU (Figure 6.2A; n = 15, T_{obs} = 0.379, p = 0.074) and family (Figure 6.2C; n = 15, T_{obs} = 0.585, p = 0.075) levels. No frogs with strong *Bd* infections and high numbers of inhibitory bacteria were found during the course of this study.

Table 6.2. Closest taxonomic affiliation from GenBank for all unique 16S rDNA sequences. Numbers of frogs possessing each unique sequence are shown by species: *Litoria serrata* (LS), *L. rheocola* (LR), *L. nannotis* (LN), *L. eucnemis* (LE) and *L. longirostris* (LL), and site: Kirrama upland (KU), Kirrama lowland (KL), Wooroonooran upland (WU), Wooroonooran lowland (WL), McIlwraith upland (MU).

			Number of frogs with each unique sequence by species and site												
			KU		KL			WU			WL			MU	
Taxonomy/GenBank closest match	Accession No.	% Similarity	LS	LN	LS	LR	LN	LS	LR	LN	LS	LR	LN	LE	LL
Actinobacteria															
Curtobacterium flaccumfaciens	EU977762	99.5	-	-	-	-	-	-	-	-	1	-	-	-	-
Bacteroidetes															
Flavobacteria															
Chryseobacterium sp.	EF442766	99.1	-	1	-	-	-	-	-	-	-	-	-	-	-
Chryseobacterium sp.	EF608166	99.1	-	-	-	-	-	-	-	-	-	-	-	1	-
Chryseobacterium sp.	GU353126	99.1	-	-	-	-	-	-	-	1	-	-	-	-	-
Chryseobacterium sp.	HQ154575	99.8	-	-	-	-	-	-	-	-	-	-	-	1	-
Sphingobacteria															
Sphingobacterium sp.	EF426437	98.8	-	1	-	-	-	-	-	-	-	-	-	-	-
Firmicutes															
Bacilli															
Bacillus cereus	EU741074	100	-	-	-	-	-	-	-	-	-	-	-	1	-
Proteobacteria															
Alphaproteobacteria															
Pseudochrobactrum glaciei	AB369864	98.7	-	-	-	-	1	-	-	-	-	-	-	-	-
Sphingomonadaceae bacterium	AB461727	99.8	-	-	-	1	-	-	-	-	-	-	-	-	-
Betaproteobacteria															
Burkholderia sp.	EU998634	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Chromobacterium sp.	AY117572	99.8	-	-	-	-	-	-	-	-	-	-	-	1	-
Chromobacterium violaceum	EU372837	99.2	-	-	1	-	-	-	-	-	-	-	-	-	-
Chromobacterium violaceum	EU372837	100	-	-	-	-	-	-	-	-	-	-	-	-	1
Chromobacterium violaceum	HM449690	99.2	-	-	-	-	2	-	-	-	-	-	-	-	-
Chromobacterium sp.	EF633687	99.6	-	-	-	-	-	-	-	-	-	1	-	-	-
Duganella sp.	HQ829836	99.4	-	2	-	-	-	-	-	-	-	-	-	-	-
Duganella sp.	HQ829836	99.4	-	1	-	-	-	-	-	-	-	-	-	-	-
Iodobacter sp.	FJ872386	98.8	-	-	-	-	-	-	1	1	-	-	-	-	-
Iodobacter sp.	FJ872386	98.8	-	-	-	-	-	-	-	-	-	1	-	-	-
Iodobacter sp.	FJ872386	98.8	-	-	-	-	-	-	-	1	-	-	-	-	-
Iodobacter sp.	FJ872386	98.9	-	-	-	-	2	-	-	-	-	-	-	-	-

Table 6.2 Contd.

Taxonomy/GenBank closest match	Accession No.	% Similarity	Number of frogs with each unique sequence by species and site												
			KU		KL			WU			WL			MU	
			LS	LN	LS	LR	LN	LS	LR	LN	LS	LR	LN	LE	LL
Gammaproteobacteria															
Acinetobacter sp.	FJ984618	99.9	-	1	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	HM626417	100	1	-	-	-	-	-	-	-	-	-	-	-	-
Acinetobacter sp.	DQ366092	99.9	1	4	-	-	-	-	-	-	-	-	-	-	-
Aeromonas hydrophila	AB473005	99.9	-	-	-	-	-	-	-	-	-	-	-	-	1
Aeromonas hydrophila	AB473005	100	-	-	-	-	-	-	-	-	-	-	-	-	1
Averyella dalhousiensis	DQ158204	99.6	-	-	-	-	-	-	-	-	1	-	-	-	-
Averyella dalhousiensis	DQ158205	99.2	-	-	-	-	-	-	-	-	1	-	-	-	-
Citrobacter sp.	EU704230	98.2	-	-	-	-	-	-	-	-	-	-	-	1	-
Enterobacter sp.	AM184265	99.6	-	2	-	-	-	-	-	-	-	-	-	-	-
Enterobacter sp.	AM184268	99.4	1	-	-	-	-	-	-	-	-	-	-	-	-
Enterobacter sp.	AM184268	99.5	1	1	-	-	-	-	-	-	-	-	-	-	-
Enterobacter sp.	GQ395336	99.8	-	-	1	-	-	-	-	-	-	-	-	-	-
Enterobacter sp.	FJ897483	99.5	1	1	-	-	-	-	-	-	-	-	-	-	-
Enterobacter sp.	GU201549	99.9	-	1	-	-	-	-	-	-	-	-	-	-	-
Hafnia alvei	AB519795	99.9	-	-	-	-	-	-	-	1	-	-	-	-	-
Hafnia sp.	HM489947	99.7	-	-	-	-	1	-	-	-	-	-	-	-	-
Klebsiella sp.	EU870375	99.9	-	-	1	-	-	-	-	-	-	-	-	-	-
Klebsiella sp.	HQ836365	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Luteibacter rhizovicinus	EU022023	99.7	-	-	-	-	-	-	-	-	1	-	-	-	-
Pseudomonas beteli	DQ299947	99.7	-	-	1	-	-	-	-	-	-	-	-	-	-
Pseudomonas entomophila	CT573326	100	-	-	-	1	-	-	-	-	-	-	-	-	-
Pseudomonas entomophila	CT573326	99.9	-	-	-	-	-	-	-	-	-	1	-	-	-
Pseudomonas entomophila	CT573326	99.9	-	-	-	1	1	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	AB266613	98.9	-	-	-	-	-	-	1	-	-	-	-	-	-
Pseudomonas fluorescens	CP000094	99.9	-	-	-	-	-	-	-	-	-	2	-	-	-
Pseudomonas fluorescens	CP000094	99.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	CP000094	100	-	-	-	-	-	-	-	-	-	1	-	-	-
Pseudomonas fluorescens	FJ608707	99.7	-	-	-	-	-	-	-	-	1	-	-	-	-
Pseudomonas fluorescens	GU726880	100	1	1	-	-	2	-	1	-	-	2	-	3	2
Pseudomonas fluorescens	GU726880	99.9	-	-	1	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	-	1	-	-	-	-	-	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	-	-	1	1	1	-	-	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	1	-	-	-	-	-	-	-

Table 6.2 Contd.

Taxonomy/GenBank closest match	Accession No.	% Similarity	Number of frogs with each unique sequence by species and site												
			KU		KL			WU			WL			MU	
			LS	LN	LS	LR	LN	LS	LR	LN	LS	LR	LN	LE	LL
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	-	-	1	-	-	-	1	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	-	-	-	2	-	-	-	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	-	-	-	1	-	-	-	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	1	2	-	1	1	-	-	-
Pseudomonas fluorescens	GU726880	99.9	-	-	-	-	-	1	-	-	-	-	-	-	-
Pseudomonas fluorescens	HM439651	99.9	3	7	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	HM439651	99.9	-	1	-	-	-	-	-	-	-	-	-	1	-
Pseudomonas fluorescens	HM439651	99.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	HM439651	99.9	-	1	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	HM439651	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	HQ143617	99.9	-	-	1	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	HQ166099	99.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	HQ166099	99.9	-	-	-	-	-	-	-	-	-	-	-	1	-
Pseudomonas graminis	AB109886	99.2	-	-	-	-	-	-	-	-	-	1	-	-	-
Pseudomonas koreensis	NR025228	99.9	-	-	-	-	-	-	1	-	-	-	-	-	-
Pseudomonas mosselii	DQ837709	99.9	-	-	-	-	1	-	-	-	-	-	-	-	-
Pseudomonas putida	EU043325	99.7	-	-	-	-	-	-	1	-	-	-	-	-	-
Pseudomonas sp.	EF427863	99.5	-	-	-	-	1	-	-	-	-	-	-	-	-
Pseudomonas sp.	EF427863	99.6	-	-	-	-	-	-	-	1	-	-	-	-	-
Pseudomonas sp.	EU693553	100	-	-	-	-	-	-	-	-	-	-	-	1	-
Pseudomonas sp.	EU912472	99.4	-	-	-	1	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	FJ652623	99.9	-	-	-	-	1	-	-	-	-	-	1	-	-
Pseudomonas sp.	HM047297	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	HM996802	99.4	-	-	-	-	-	-	1	-	1	-	-	-	-
Pseudomonas teessidea	HQ003439	99.9	-	-	-	-	-	-	-	-	-	-	-	-	1
Pseudomonas tolaasii	AF320990	99.9	1	1	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas tolaasii	AF320990	99.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas tolaasii	AF320990	99.8	-	1	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas tolaasii	AF320990	99.9	-	-	-	-	-	1	-	-	-	-	-	-	-
Pseudomonas tolaasii	AF320990	100	-	-	-	-	-	-	1	-	-	-	-	-	-
Pseudomonas tolaasii	HQ529376	100	1	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas vranovensis	HQ202851	98.8	1	-	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	FJ608006	100	1	-	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	FJ608708	99.9	-	-	-	-	-	-	-	-	-	1	-	-	-

Table 6.2 Contd.

Taxonomy/GenBank closest match	Accession No.	% Similarity	Number of frogs with each unique sequence by species and site												
			KU		KL			WU			WL			MU	
			LS	LN	LS	LR	LN	LS	LR	LN	LS	LR	LN	LE	LL
Serratia marcescens strain	GU220796	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	GU220796	99.7	-	1	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	GU220796	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	GU220796	99.6	-	1	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	GU220796	99.9	3	2	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	GU220796	99.9	1	-	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens strain	GU220796	99.9	4	-	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens subsp. *marcescens*	AB594756	99.5	-	1	-	-	-	-	-	-	-	-	-	-	-
Serratia marcescens subsp. *sakuensis*	NR036886	99.8	-	-	-	-	-	-	-	-	-	-	-	-	1
Serratia sp.	EF195352	99.7	1	-	-	-	-	-	-	-	-	-	-	-	-
Serratia sp.	GU120657	99.7	-	-	-	-	-	-	-	-	-	-	-	1	-
Stenotrophomonas maltophilia	AY445079	99.7	-	-	-	-	-	-	-	-	1	-	-	-	-
Stenotrophomonas maltophilia	AY445079	99.6	-	-	-	-	-	1	-	-	-	-	-	-	-
Stenotrophomonas maltophilia	AY445079	100	-	-	-	-	-	-	1	-	-	-	-	-	-
Stenotrophomonas maltophilia	CP001111	100	-	-	-	-	-	-	-	-	1	-	-	-	-
Stenotrophomonas maltophilia	CP001111	99.9	-	-	-	-	-	-	-	-	-	1	-	-	-
Stenotrophomonas maltophilia	EU022689	99.9	-	-	-	-	-	1	-	-	-	-	-	-	-
Stenotrophomonas maltophilia	EU022689	99.6	-	-	-	-	-	1	-	-	-	-	-	-	-
Stenotrophomonas maltophilia	EU034540	100	-	-	-	-	1	-	-	-	-	-	-	-	-
Stenotrophomonas maltophilia	EU741084	100	1	2	-	-	-	-	-	-	-	-	-	-	-
Stenotrophomonas maltophilia	GQ268318	99.6	-	-	1	-	-	-	-	-	-	-	-	-	-
Stenotrophomonas maltophilia	GU391032	99.9	-	-	-	-	-	-	-	-	-	-	-	1	-
Stenotrophomonas maltophilia	GU391032	99.6	-	-	-	-	-	-	-	-	-	-	-	1	-
Stenotrophomonas maltophilia	JF271856	100	-	1	-	-	-	-	-	-	-	-	-	-	-
Stenotrophomonas sp.	AY179327	100	-	3	-	-	-	-	-	-	-	-	-	-	-
Stenotrophomonas sp.	EU054384	99.6	-	-	-	-	-	-	1	-	-	-	-	-	-
Stenotrophomonas sp.	FJ193149	99.8	-	-	-	-	-	-	-	-	1	-	-	-	-
Stenotrophomonas sp.	GQ416168	100	-	-	-	-	-	-	-	-	-	1	-	-	-
Stenotrophomonas sp.	GQ478276	99.8	-	-	-	-	-	-	1	-	-	-	-	-	-
Uncultured bacterium	FR667252	99.9	-	-	-	-	1	-	-	-	-	-	-	-	-
Uncultured bacterium	FR667257	99.9	-	-	-	-	1	-	-	-	-	-	-	-	-
Yokenella regensburgei	AB519796	99.9	-	-	-	-	-	-	-	-	-	1	-	-	-
Yokenella regensburgei	AB519796	99.6	-	-	-	-	-	-	-	-	1	-	-	-	-
		Total	27	44	7	4	15	7	13	7	14	15	1	15	7

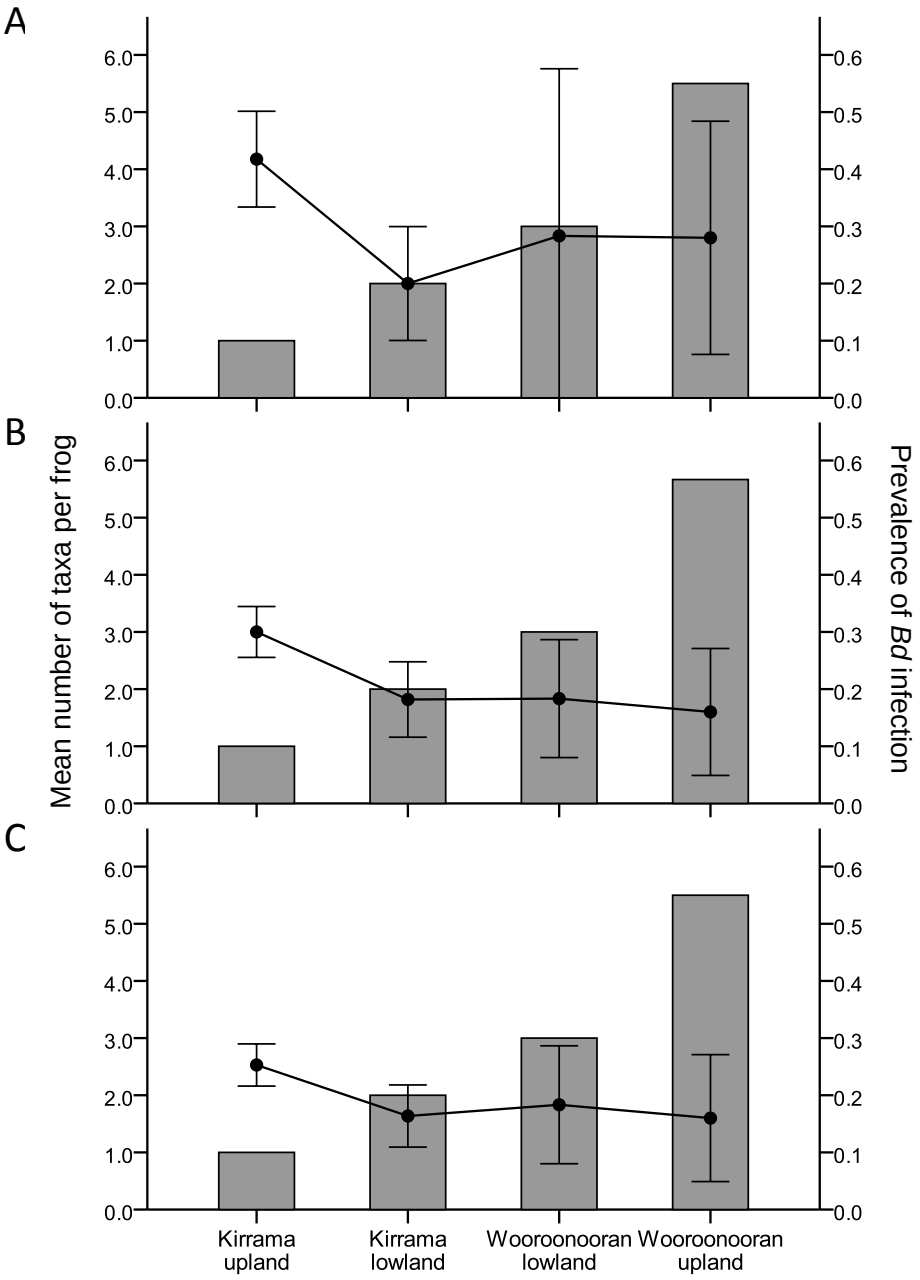

Figure 6.1. Points show mean number of *Bd*-inhibitory **A** OTUs; **B** genera; and **C** families present per frog. Error bars represent 95% confidence intervals of the means. Bars represent prevalence of *Bd* infection at each stream during the sampling period.

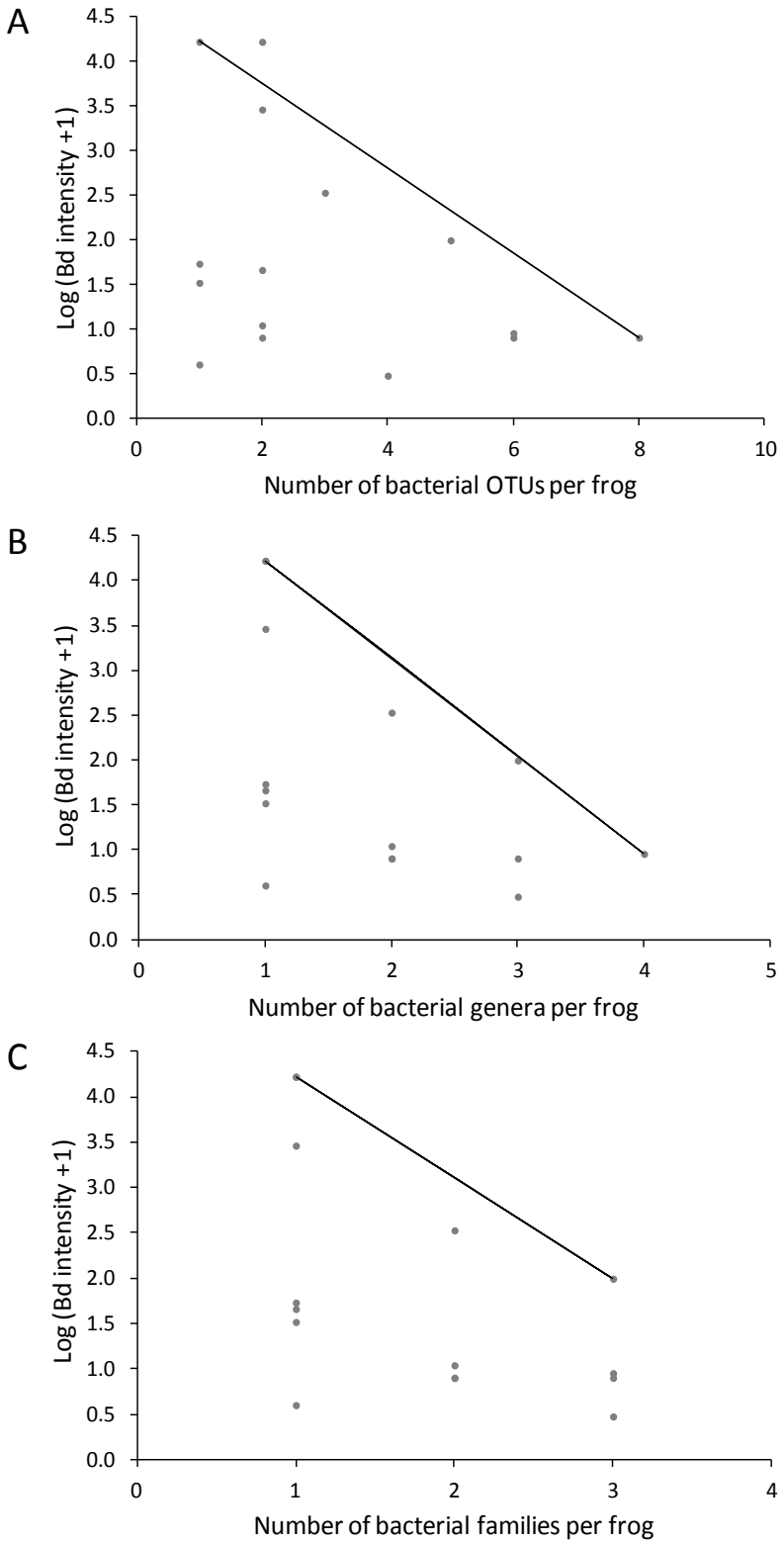

Figure 6.2. Intensity of *Bd* infection decreased as the number of bacterial taxa increased. Ninetieth percentile regression lines show the negative relationship between the number of unique OTUs (**A**; n=15, Tobs=0.379, p=0.074), genera (**B**; n=15, Tobs=0.643, p=0.043) and families (**C**; n=15, T_{obs}=0.585, p=0.075) per infected frog and *Bd* infection intensity.

No taxonomic clustering of bacterial genera was found when frogs were grouped by *Bd* infection status (one-way ANOSIM; p = 0.518), bioregion (one-way ANOSIM; p = 0.312) or frog species (one-way ANOSIM; p = 0.6903). However, significant taxonomic clustering was evident when frogs were grouped by site (Figure 6.3; one-way ANOSIM; R = 0.13, p = 0.00074). Pre-planned pairwise post-hoc comparisons revealed that the Kirrama upland site was significantly different from all other sites (p < 0.004 in all cases).

Significant differences were present in the number of totally inhibitory OTUs, genera and families present on frogs among Wet Tropics sites (Kruskal-Wallis tests; OTU, H = 10.36, df = 3 p = 0.016; genus, H = 11.71, df = 3, p = 0.0085; family, H = 8.02, df =3, p = 0.046). Pre-planned Kolmogorov-Smirnov pairwise comparisons revealed that these differences were driven by the frogs of the Kirrama upland site (Figure 6.1). Frogs from the Kirrama upland site had significantly greater numbers of *Bd*-inhibitory bacterial OTUs than frogs at the Kirrama lowland site (p < 0.0001), significantly greater numbers of genera than frogs at all other sites (Kirrama lowland, p = 0.0018; Wooroonooran upland, p = 0.0208; Wooroonooran lowland, p = 0.0213), and significantly greater numbers of families than frogs at the Kirrama lowland (p < 0.007) and Wooroonooran upland sites (p = 0.019). The genera *Acinetobacter*, *Enterobacter* and *Duganella* were unique to frogs at this site (Figure 6.4).

Because Kirrama upland frogs were responsible for overall patterns observed and only one infected frog was present at this site, it was not possible to compare taxa numbers on infected and uninfected frogs for each site individually. When the Kirrama upland site was excluded from analysis, taxa numbers were not significantly different between infected (n = 15) and uninfected (n = 16) frogs (Mann-Whitney U Test; OTU, U_{30} = 100, z = -0.799, p = 0.424; genus, U_{30} = 113, z = -0.277, p = 0.782; family, U_{30} = 108.5, z = -0.475, p = 0.635). However, uninfected Kirrama upland frogs (n = 16) had significantly more totally inhibitory taxa than uninfected frogs at all other sites (n = 16; Mann-Whitney U Test; OTU, U_{31} = 53, z = -2.849, p = 0.004; genus, U_{31} = 47.5, z = -3.115, p = 0.002; family, U_{31} = 46, z = -3.207, p = 0.001).

As well as having significantly more inhibitory bacterial taxa per frog, the Kirrama upland site also had a significantly higher proportion of frogs with one or more *Bd*-inhibitory OTU than other sites. This pattern was the same whether OTU or genus was selected as the taxonomic unit (Table 6.3; Figure 5.5; Fisher's Exact Tests; OTU p = 0.0156, genus p = 0.0002). All other sites did not differ significantly from each other (Fisher's Exact Test; OTU p = 0.351, genus p = 0.627).

Table 6.3. Number of frogs with 0 to 8 totally inhibitory genera/OTUs by site.

Site	Number of frogs with 0-8 genera/OTUs									Total frogs
	0	1	2	3	4	5	6	7	8	
Kirrama upland	3	1 / 1	3 / 1	8 / 4	5 / 4	0 / 4	0 / 1	0 / 2	0 / 0	20
Kirrama lowland	15	6 / 6	5 / 4	1 / 2	1 / 0	0 / 0	0 / 1	0 / 0	0 / 0	28
Wooroonooran upland	10	6 / 1	2 / 4	1 / 1	0 / 1	0 / 1	0 / 1	0 / 0	0 / 0	19
Wooroonooran lowland	12	5 / 4	2 / 2	2 / 0	0 / 2	0 / 0	1 / 0	0 / 0	0 / 2	22
McIlwraith upland	8	9 / 6	0 / 3	1 / 1	1 / 0	0 / 0	0 / 1	0 / 0	0 / 0	19

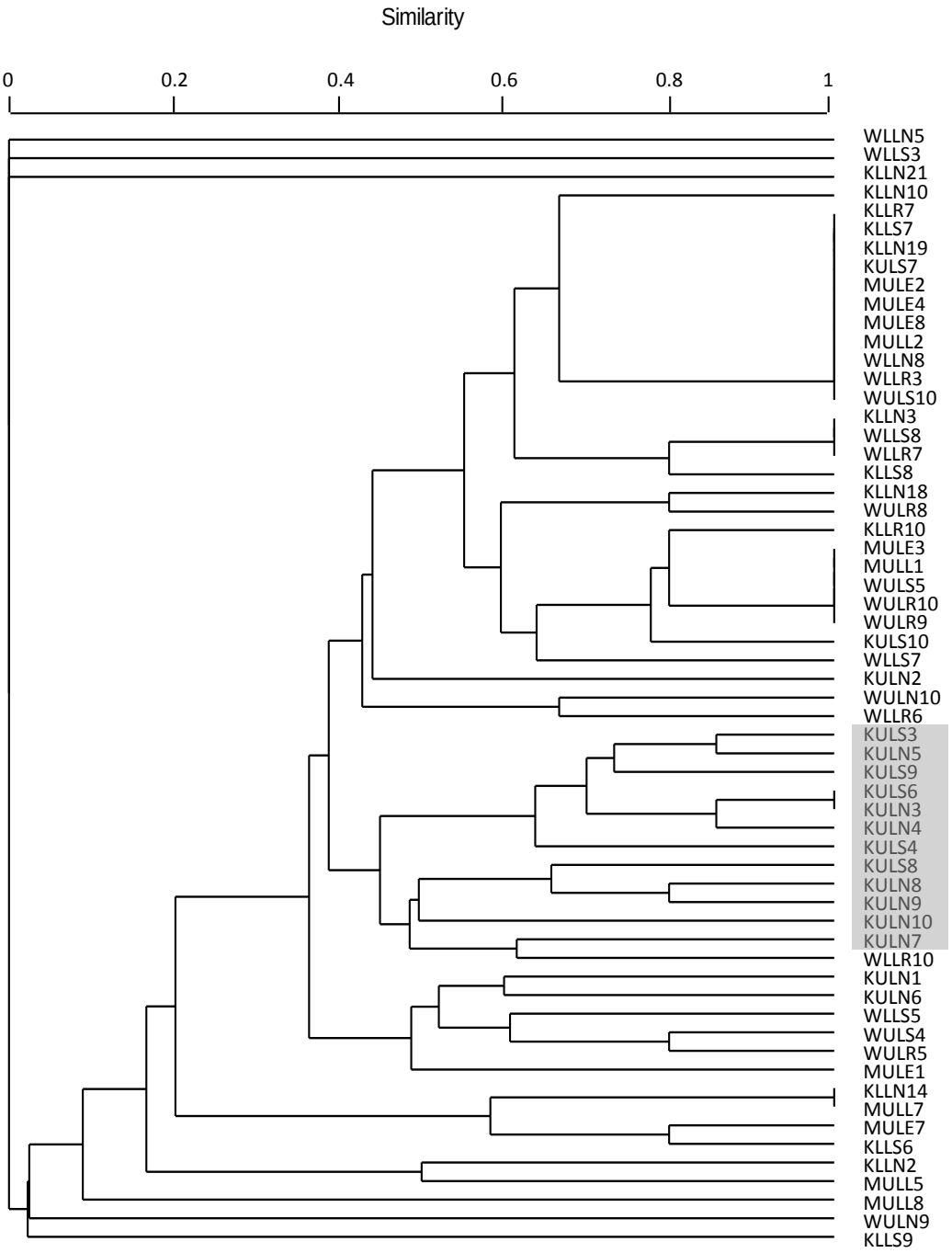

Figure 6.3. Dendrogram (Unweighted Pair Group Method with Arithmetic Mean) showing similarity of genera on individual frogs at each site. Identification numbers are unique to each frog and denote site, species and frog number. The grey box highlights the clustering present in the majority of Kirrama upland frogs.

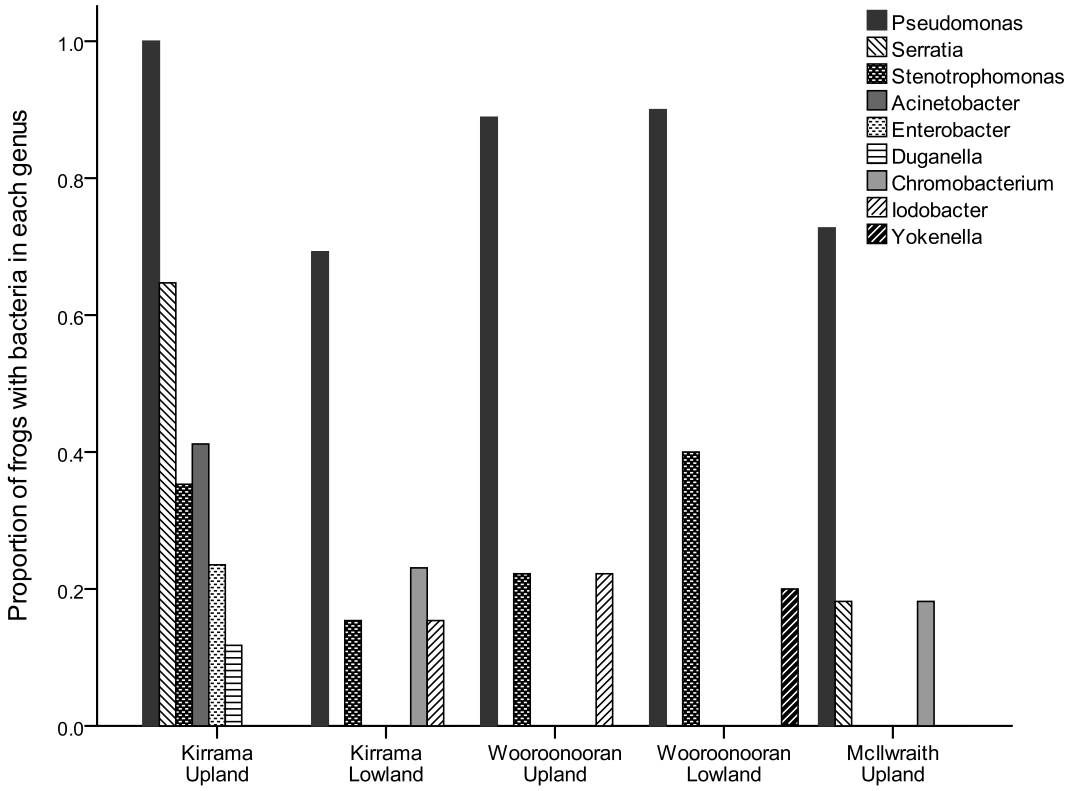

Figure 6.4. Proportion of frogs with inhibitory genera by site. For ease of visualization, genera that occurred on fewer than two frogs at each site have been excluded.

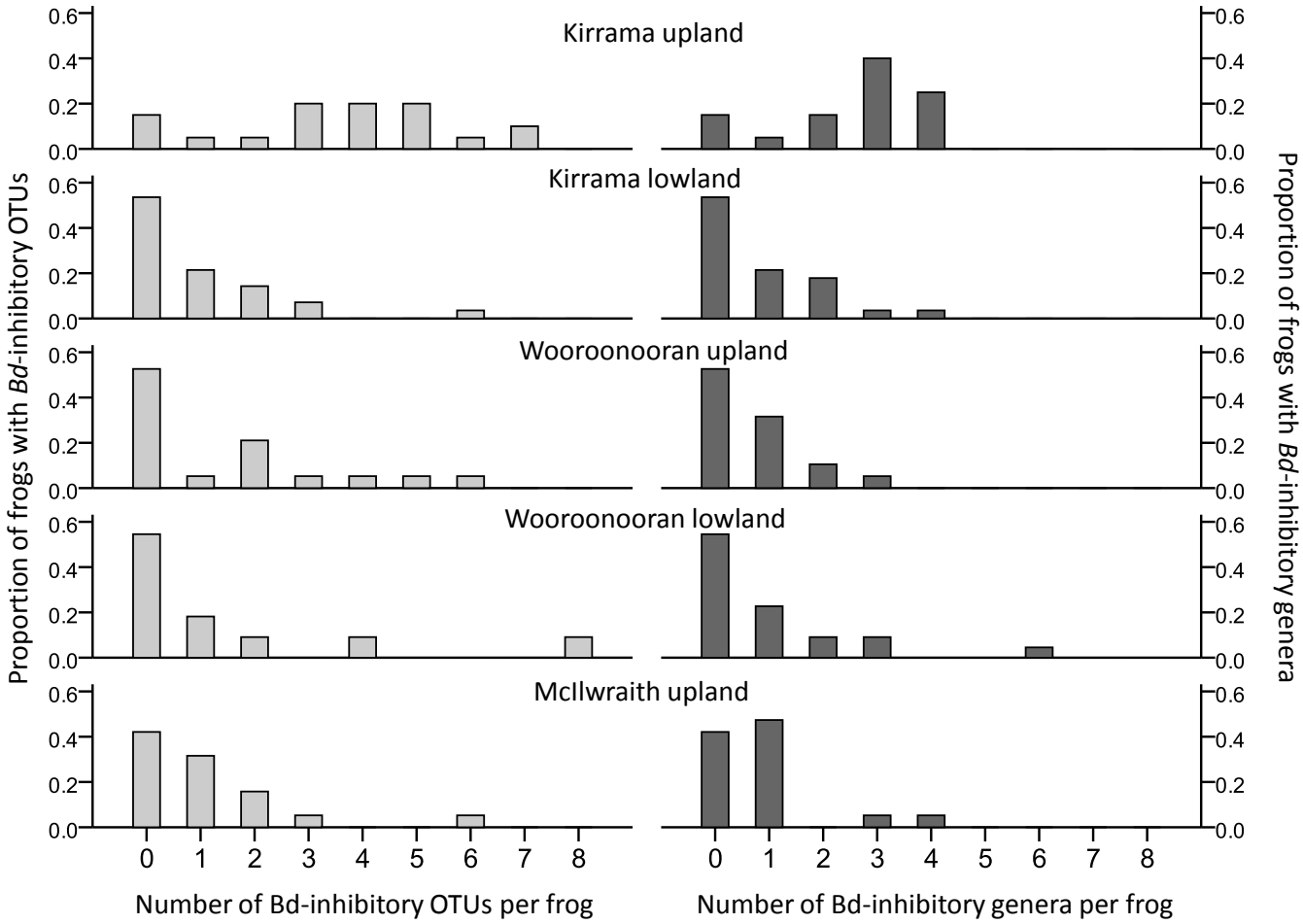

Figure 6.5. The proportion of frogs with *Bd*-inhibitory bacteria numbers classified by OTU and genera for each field site.

Discussion

Sixty frogs from five species at five sites collectively had 121 *Bd*-inhibitory OTUs. The majority (94%) were from the phylum Proteobacteria with a small contribution from the phyla Bacteriodetes, Actinobacteria and Firmicutes, indicating that these latter groups may play only a minor role in the cutaneous bacterial communities of tropical Australian rainforest frogs.

More detailed investigation regarding possible reasons for the dominance of the class Gammaproteobacteria is needed, as 88% of frogs in this study carried members of this group. The Gammaproteobacteria have also dominated microbial assemblages in other studies of bacteria from anuran skin, with the genus *Pseudomonas* driving this pattern (Woodhams et al. 2007c; Lam et al. 2010; Walke et al. 2011). In this study 83% of frogs with *Bd*-inhibitory bacteria had one or more *Pseudomonas* species. Walke et al. (2011, Online Supplementary Information) suggest that culturing biases could be responsible for the dominance of this genus, or that it may out-compete others on amphibian skin through production of siderophores. However, they propose that the most likely reason for its observed dominance in cutaneous microbiota samples is that it is selected for by the host as it offers antimicrobial protection via the well-documented wide range of compounds it can produce (Walke et al. 2011; Online Supplementary Information). In addition, *Pseudomonas* spp. are ubiquitous in soil, water and on plant surfaces (Paulsen et al. 2005) and therefore may just be present in greater numbers in habitat frequented by amphibians, leading to greater uptake by frogs.

A functional role has been shown for the dominant bacteria in a number of microbial communities such as human skin (Grice et al. 2008), gut (Rawls et al. 2006) and vagina (Zhou et al. 2004) and in agricultural soils (Haas and Défago 2005). It is likely that the dominance of *Pseudomona*s in the cutaneous bacterial microbiota of frogs also has a functional role, possibly in combination with other co-occurring bacterial groups. In this study, three families within the Gammaproteobacteria (Pseudomonadaceae, Xanthomonadaceae and Enterobacteriaceae; Table 6.2) were consistently found at all field sites and on all but one frog species. It is possible that co-occurrence of particular groups has evolved during acquisition, by the host, of assemblages with the most diverse antimicrobial potential against pathogens. However, in order to reveal the mechanisms that microbes use to coexist, and the functional capacity within and among communities, a genomic approach will be necessary (Dethlefsen et al. 2006; Whitaker and Banfield 2006).

Many bacteria produce compounds inhibitory to other microbes. For some of the genera described in this study, production of inhibitory compounds is well-described (Appendix A),

while for others there is little information available on antimicrobial activity. The diverse compounds that can be produced by the principal genera in this study might perform complementary roles. For example, one compound may act on the pathogen cell membrane while another interferes with transcription of DNA and therefore cell replication. Production of a diverse range of inhibitory compounds will enable the resident bacteria to resist a wide range of invaders, including environmental pathogens. The ability to acquire and control the composition of symbiotic microbiota, and therefore consequent antimicrobial potential, is likely to contribute to host survival (Poulsen et al. 2010).

Despite the low numbers of infected frogs in this study, the significant negative relationship between the intensity of the *Bd* infection and the number of unique *Bd*-inhibitory bacterial genera on infected frogs (Figure 6.2) lends further support to the idea that greater inhibitory potential is linked to a reduction in the level of *Bd* infection. Frogs with more intense *Bd* infections tended to have fewer *Bd*-inhibitory bacterial taxa while those with weaker infections tended to have more. No frogs were found with high *Bd* infections and high numbers of inhibitory bacterial taxa. Therefore, greater *Bd*-inhibitory bacterial taxonomic richness may contribute to host survival with enzootic *Bd* infection.

There are two possible reasons for the observed patterns. Frogs with naturally lower bacterial taxonomic diversity could be more susceptible to *Bd*. Conversely, as *Bd* infection progresses a reduction in bacterial taxonomic diversity may result. A laboratory experiment could be designed to determine whether increasing the diversity of *Bd*-inhibitory bacterial symbionts on frog skin alters susceptibility to *Bd*, or intensity of *Bd* infections. Such experiments have been conducted on plants and locusts. Matos et al. (2005) exposed wheat plants to different densities of bacterial symbionts and Dillon et al. (2005) exposed axenic locusts to different combinations of three species of gut bacteria prior to pathogen exposure. In both cases the density of the pathogen was lower in animals with greater diversity of bacterial symbionts (Dillon et al. 2005; Matos et al. 2005). The inverse hypothesis, that infection reduces bacterial diversity, could be tested by collecting and analyzing cutaneous bacterial skin swabs from frogs before and after a *Bd*-infection laboratory trial.

In Chapter Five, my data showed that frogs at the Kirrama upland site had significantly greater bacterial isolate richness than their lowland conspecifics but reasons for this difference were not clear. In this chapter, my data demonstrate that the Kirrama upland site differs from all other sites in a number of ways. There are a greater number of inhibitory bacterial genera present (Figures 6.1 and 6.4), a lower infection prevalence, a significantly higher proportion of

frogs with more inhibitory bacterial OTUs or genera (Figure 6.5), and statistically significant clustering of individual frogs with similar *Bd*-inhibitory genera signatures (Figure 6.3). A higher proportion of Kirrama upland frogs have bacteria from the genera *Pseudomonas* and *Serratia* than at any other site, and the genera *Actinobacter*, *Enterobacter* and *Duganella* are unique to frogs from this site. This suggests that Kirrama upland frogs have been able to acquire greater *Bd*-inhibitory genera richness through active or passive selection than frogs elsewhere. This may result in production of a greater diversity of antimicrobial compounds and lead to a reduction in the number of *Bd*-infected individuals at this site. Indeed, Kirrama upland frogs were the driver of the significant negative correlation between taxonomic richness and infection prevalence.

All Wet Tropics frog populations in this study have persisted for many years with *Bd* infection. It has been suggested that populations in which a high proportion of frogs possess inhibitory bacteria are more likely to coexist with *Bd* (Lam et al. 2010). In this study, 85% of frogs overall (*L. serrata* – 70%; *L. nannotis* – 100%) at the Kirrama upland site had *Bd*-inhibitory bacteria (Figure 6.5), significantly more than at any other site. This may have contributed to their ability to recover following population declines in the early 1990s, although *L. rheocola* has not yet reappeared at any upland site in the Kirrama region. However, all three species in this study have been able to recolonise the Wooroonooran upland site despite only 47% of frogs with inhibitory bacteria (*L. serrata* – 75%; *L. nannotis* – 29%; *L. rheocola* – 50%; Figure 6.5). It is therefore possible that the high proportion of frogs with inhibitory bacteria contribute to survival of the Kirrama upland population, but that other reasons, such as host immune defence, behaviour, and environmental factors contribute to survival with enzootic *Bd* infection at the Wooroonooran upland site (Daskin et al. 2011; Rollins-Smith et al. 2011; Savage and Zamudio 2011). While no *Bd* infection was present in Cape York frogs, only 58% had bacteria capable of producing *Bd*-inhibitory compounds, suggesting that if *Bd* was to emerge at this site, bacteria alone would be unlikely to facilitate population survival.

Comparison of the bacterial taxonomic richness of totally inhibitory isolates between infected and uninfected frogs revealed no significant differences among sites when Kirrama upland was excluded from analysis. However, uninfected Kirrama upland frogs had significantly more taxa than uninfected frogs other sites suggesting that, in this study, this effect may be site-specific and possibly linked to the low level of infection prevalence observed. Given that phylogeographic isolation among many of these populations has occurred (Bell et al. 2012), it is quite possible that selection has led to different mechanisms for coping with *Bd* infection.

Summary

In this chapter, I have shown that increased taxon richness of *Bd*-inhibitory bacteria may play a role in host survival with enzootic *Bd* infection, possibly through the functional redundancy offered by the greater number of inhibitory compounds available. Therefore, when selecting bacteria for bioaugmentation trials, cocktails of several bacteria from different genera may be more effective against *Bd* than a single bacterial isolate. The bacterial microbiota on frog skin is likely to be constantly shaped by availability of suitable colonists, host adaptation and exposure to pathogens in addition to environmental variables; a microscopic arms-race. The ability of frogs to recolonise sites where they existed prior to *Bd* invasion is likely to depend on host ability to acquire suitable *Bd*-inhibitory candidates.

This study was conducted entirely in winter to maximize the chances of encountering infected individuals that are more likely to be present within a population at this time of year (Berger et al. 2004; Woodhams and Alford 2005; Kriger and Hero 2007; Puschendorf 2009). However, seasonality may also affect the cutaneous microbial diversity. In Chapter Seven, I follow individual wild frogs sampled over the course of a year to explore natural variation of the cutaneous microbiota.

CHAPTER SEVEN : ASSESSING THE STABILITY OF THE CUTANEOUS BACTERIAL MICROBIOTA OF THE COMMON MIST FROG, *LITORIA RHEOCOLA:* CHANGES WITH TIME AND *BATRACHOCHYTRIUM DENDROBATIDIS* INFECTION

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Chapter Overview

In Chapter Six, I demonstrated that the number of *Bd*-inhibitory bacterial genera present on frog skin is negatively correlated with both the intensity and prevalence of *Bd* infection, and suggested that frogs with a greater number of *Bd*-inhibitory bacterial genera are likely to be better equipped to survive with enzootic *Bd* infection. In this chapter, I investigate natural changes in uncultured cutaneous bacterial microbiota in individual wild frogs tracked over the course of a year, to examine possible changes in species richness and assemblage structure.

Abstract

No preventative treatments are currently available to protect wild frogs against infection by the pathogenic fungus, *Batrachochytrium dendrobatidis* (*Bd*); however, the possibility of bioaugmentation, by adding bacteria with antifungal properties to the natural assemblage of skin bacteria, offers hope. However, the stability of the symbiotic microbial assemblage is likely to affect its susceptibility to manipulation. A microbial assemblage that changes little with time may have highly stable species interactions and therefore be difficult to supplement, while a dynamic microbial assemblage may be more amenable to manipulation. As yet, there is no information on the stability of the cutaneous microbial assemblages of wild amphibians. In this study, skin swabs were collected from 14 individual *Litoria rheocola* (common mist frogs) that were captured, marked and subsequently recaptured on Windin North Creek, northern Queensland, Australia, from February 2010 to February 2011. Mixed bacterial assemblage DNA was extracted, a small fragment of the 16S rDNA gene was amplified, and the concentrated, purified PCR product was run on denaturing gradient gels. The bacterial assemblage profile generated for each swab sample was compared among frogs grouped by date sampled and by pattern of *Bd* infection. Twenty-five of 114 unique bacterial strains found eight times or more constituted the dominant assemblage members, with five core strains occurring in greater than 65% of samples. The proportion of core bacterial strains differed significantly between frogs that were not infected with *Bd* prior to sampling and frogs that were infected or had recently lost infection. The taxonomic composition of the dominant microbial assemblage also differed significantly between these two groups of frogs. Bacterial species richness decreased over the year, with a corresponding statistically significant change in assemblage composition.

The temporal changes observed suggest that the microbiota would be amenable to manipulation through bioaugmentation using *Bd*-inhibitory bacteria. However, turnover of core strains is low, suggesting that bioaugmentation success could be enhanced by application of one or more of these dominant assemblage members.

Introduction

All animals are colonized by microbes, and some symbionts have evolved very specific functional roles (Moran 2006). In many cases, associations between symbiotic microbes and their hosts are co-evolved and have stable species interactions. For example, the sepiolid squids house a single bacterial symbiont in a specialised light organ (Kimbell and McFall-Ngai 2003), and fungus farming ants show a similar co-evolved relationship with their symbionts, housing antibiotic-producing bacteria in specialised cuticular crypts (Currie et al. 2006). The human gut contains many different bacterial symbionts; these can vary greatly among individuals due to the lack of opportunity for migration between hosts, strong interaction with the host and host genetic differences (Dethlefsen et al. 2007). However, samples of the microbial assemblage collected over time from a sample of individuals showed little change (Zoetendal et al. 1998), demonstrating that the species composition of individuals' microbial assemblages may remain stable for many months.

Temporal stability has been noted in many symbiotic host-microbe interactions, although the time scale can vary from a few weeks to many years. For example, *Hydra* spp. maintained their microbial assemblage over a 30 year period in both the laboratory and natural environment (Fraune and Bosch 2007), while corals hosted species-specific bacterial communities that remained stable over a year despite spatial separation by as much as 3000km (Rohwer et al. 2002).

Fluctuating environmental conditions contribute to the breakdown of temporal stability in some symbioses. For example, a mean water temperature change from 24^{o}C to 30^{o}C was responsible for a shift towards dominance of pathogenic bacteria in coral systems (Ritchie 2006; Sharon and Rosenberg 2008), and bacterial assemblage shifts in alpine soil (Lipson et al. 2002) and lake water (Yannarell et al. 2003) coincide with seasonal temperature changes. Clearly, the specificity and stability of host-microbe associations varies with the host and the nature of the symbiotic relationship.

Disease and exposure to pathogens can also be linked to changes in the composition of the resident microbial assemblage. Several coral disease studies have demonstrated that bacterial assemblages differ in characteristic ways between healthy and diseased corals, with the recurrent presence of *Vibrio* spp. in diseased coral tissue in warmer water conditions (Ritchie 2006; Bally and Garrabou 2007; Sharon and Rosenberg 2008; Sunagawa et al. 2009; Wilson et al. 2012). Similarly, assemblages of skin bacteria on human patients with atopic dermatitis can differ from those on healthy individuals (Kong et al. 2012).

Some authors have suggested that the site-specific microbiota of vertebrates represent climax communities that may be difficult to alter (Tagg and Dierksen 2003), while others perceive them as dynamic assemblages influenced by their history (Dethlefsen et al. 2006). It seems likely that vertebrates present a wide range of habitats, some more stable than others, that are occupied by a diverse array of microbial assemblages, some more and some less stable. The stability of symbiotic microbial assemblages may affect their susceptibility to manipulation by bioaugmentation. For example, a microbial assemblage that changes little with time is likely to have highly stable species interactions and be difficult to supplement, while a dynamic microbial assemblage that is continuously changing may be more amenable to manipulation.

The amphibian cutaneous microbiota inhabits a relatively unstable physical environment, because amphibians are ectotherms that typically move between terrestrial and aquatic habitats. The cutaneous bacterial assemblage is subject to immigration and potentially replacement as it is in intimate physical contact with the external environment (Rowley and Alford 2007b; Muletz et al. 2012). Studies in North America and Australasia (Austin 2000; Potter and Norman 2006; Culp et al. 2007; Banning et al. 2008) have shown that many bacteria found on amphibian skin can also be found in soil and on other non-amphibian substrates, suggesting that the association between amphibians and their skin microbiota may not be highly specialised. In environments inhabited by amphibians, factors such as air and water temperature, rainfall, water flow rate, substrate pH, UV radiation and presence of contaminants could alter the composition of bacterial assemblages by shifting conditions to favour one symbiont over another. In addition, host processes such as habitat selection, behaviour and rate of skin shedding are likely to influence exposure to, and retention of potential bacterial colonists. Hormonal changes associated with breeding and changes in diet may make the mucosal surface more or less favourable to certain bacterial species (Porat et al. 1991; Kleessen et al. 2003). This could affect the ability of symbionts to produce antimicrobial compounds, which can be suppressed in the absence of a key nutrient (Sánchez et al. 2010;

pers. obs.), or that may be produced only at a certain symbiont density (K. Yasumiba, unpublished). In addition, changes in innate immune defences such as rate of anti-microbial peptide (AMP) production might also affect the microbial assemblage (Conlon 2011). These factors probably all interact to shape the symbiotic cutaneous microbiota.

In Chapter Six, I demonstrated that the number of *Bd*-inhibitory bacterial genera on frogs is negatively correlated with both the intensity and prevalence of *Bd* infection. This suggested that frogs with a greater number of *Bd*-inhibitory genera are likely to be better equipped to survive with enzootic *Bd* infection. Increasing species richness by encouraging the colonisation of multiple bacterial taxa that have activity against *Bd* is one potential approach to bioaugmentation for increased disease resistance. However, intentional manipulation of bacterial communities should be based upon sound knowledge of the targeted host-microbial symbioses. At present there is no information on the temporal stability of the cutaneous microbial assemblage on the skins of rainforest frogs; research efforts to date have focused almost entirely on determining whether, and how many, bacteria can produce compounds effective against *Bd*. In this chapter, I investigate changes in the cutaneous microbial assemblage over the course of a year on the common mist frog, *Litoria rheocola*. I test the hypotheses that there are no differences in the number of bacterial strains or bacterial assemblage composition among frogs grouped by date sampled, overall *Bd*-infection status or patterns of *Bd* infection.

Materials and Methods

Study species and site

I sampled the cutaneous bacterial microbiota on common mist frogs, *L. rheocola*, on four occasions, in February 2010, June 2010, October 2010 and February 2011, along a 400 m transect at Windin North Creek, Wooroonooran National Park, northern Queensland, Australia.

Temperature dataloggers

I suspended five temperature dataloggers (Thermochron iButton – DS1921G; Dallas Semiconductor, Sunnyvale, CA. USA) coated in PlastiDip (PlastiDip International, Blaine, MN, USA), as described by Roznik & Alford (2012), approximately 1.5 m above the ground from trees, at 100 m intervals along the stream to record air temperature during sampling. I calculated mean air temperature from hourly recordings over a 24 hour period.

Swabbing and marking frogs

Frogs were captured in clean plastic bags and cutaneous swab samples collected as described in Chapter Two. Briefly, frogs were rinsed and then swabbed five times on their dorsal and ventral surfaces from knee to neck with a moist rayon swab (MW112, MW&E, Bath UK). The swab was immediately placed in a sterile, labelled 1.5 ml microtube and frozen in dry ice. A second swab was collected for analysis of *Bd* infection (MW100, MW&E, Bath UK). Swabs were transferred to James Cook University pending analyses.

Upon first capture, frogs were marked with Visible Implant Elastomers (VIEs; Northwest Marine Technology Inc., Shaw Island, WA, USA) using a subcutaneous injection with an insulin syringe with a 29 gauge needle (Terumo Medical Corporation, Somerset, NJ, USA) on the underside of the thigh. Five colours (pink, orange, green, yellow, and blue) were used in unique combinations so that individual animals could be re-identified at subsequent capture.

Laboratory analysis of swab samples

From 135 frogs that were captured throughout the year, I identified 14 male frogs that were captured between two and four times, and processed their swab samples. DNA extraction from swabs, PCR, PCR product purification and concentration, and gel electrophoresis were performed as described in Chapter Two. I ran multiple time-point samples for each individual frog in adjacent lanes of the denaturing gradient gel to facilitate visual comparison. A marker ladder was run on the left, right and middle of the gel to enable samples to be compared among gels.

Determination of *Bd* infection

Swabs collected to determine *Bd* infection status were sent for quantitative PCR analysis at Washington State University, Pullman, USA according to Boyle et al. (2004), with DNA extraction by Qiagen DNEasy Blood and Tissue kit (Qiagen Inc., Valencia, CA, USA). I considered animals positive for *Bd* infection when zoospore DNA was detected in at least two of three sample replicates.

Data analysis

I analysed gel images using the software package QuantityOne (Bio-Rad, Hercules, CA). Each unique fluorescent gel band (ribotype) was considered to represent a different operational taxonomic unit (OTU), and given an identification number. A band set was created for each swab sample. From these band sets, I created a binary matrix for presence or absence of ribotypes. Samples were grouped in three ways to facilitate analysis. First, comparisons were made among frogs grouped by date sampled; second, by infection category as “infected”, “not

infected throughout the year", "uninfected but would later become infected", and "uninfected but had previously been infected"; and third as "infected or had lost infection" and "not yet infected". These categories represent the infection status of the frogs at each time sample and do not take into account infection history prior to the first sampling, or changes that could have occurred between sampling events as these were beyond my control. Statistical analyses were conducted in the software package PAST (Hammer et al. 2001). One-way ANOVAs were used to compare the mean number of bacterial strains among frog samples grouped by date sampled and by infection category. A t-test, assuming unequal sample sizes and equal variances, was used to compare the mean number of bacterial strains present in samples from infected and uninfected frogs.

Where ribotypes occurred fewer than eight times, they were eliminated from non-metric multi-dimensional scaling (nMDS) and one-way analysis of similarity (ANOSIM) analyses as they did not contribute meaningful data to the groups. Also, one additional sample was excluded as it contained only one ribotype and resulted in an extreme outlier during analysis. A Dice (Dice 1945) similarity matrix was analyzed using nMDS to reduce the number of dimensions in the data and create two-dimensional plots to visualize any change in the bacterial microbiota. Data were grouped in three ways; first, by date sampled, second, by the two infection categories "infected or had lost infection" and "not yet infected", and third, by individual frog. I used an ANOSIM test, run with 100,000 permutations, to determine whether samples grouped by date differed significantly in their location within the space defined by nMDS axes 1 and 2. I used a t-test, assuming equal sample sizes and unequal variance, to compare the mean distance of each nMDS coordinate from its group centroid for the two infection categories. To test for differences between group centroids for the two infection categories, and to investigate whether successive samples for individual frogs were more similar to one another than to samples from other frogs, I grouped nMDS coordinates accordingly and used Multiresponse Permutation Procedures (MRPP; Blossom; Cade and Richards 2005).

Ribotypes that occurred in more than 65% of swab samples were considered to be "core" ribotypes. I used a Fisher's Exact Test (FET; StatXAct4; Gajjar et al. 1998) to investigate the association between the number of core ribotypes in each sample and the infection categories "infected or had lost infection" and "not yet infected".

Results

I found 114 unique ribotypes on denaturing gradient gels in the 41 swab samples from 14 frogs recaptured multiple times during this study. The twenty-five most commonly occurring ribotypes each occurred in eight or more swab samples with the most common ribotype occurring in 35 of 41 (85%) samples (Figure 7.1). Five core ribotypes each occurred in more than 65% of swab samples. However, the majority of the ribotypes (52%) occurred in less than three samples. Quantitative PCR revealed that frogs were infected by *Bd* in ten of the 41 samples collected, and that some frogs gained or lost infections during the year (Table 7.1).

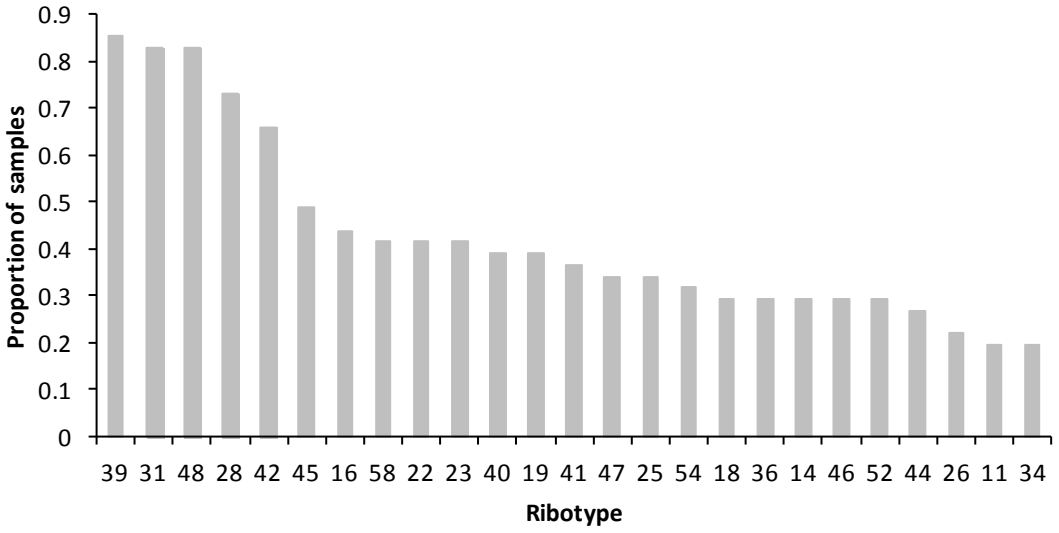

Figure 7.1. Unique bacterial ribotypes that each occurred in a total of eight or more swab samples when mixed assemblage PCR amplicons were run on denaturing gradient gels.

Table 7.1. Quantitative PCR results in zoospore equivalents per swab for samples collected from each frog between February 2010 and February 2011. "-" indicates that the frog was not recaptured and therefore no sample was collected.

Frog Number	Feb 2010	Jun 2010	Oct 2010	Feb 2011
20	3	0	0	0
29	3	-	0	0
32	0	-	0	0
38	0	-	99	0
43	0	-	0	0
64	0	0	-	0
70	0	-	0	7
72	0	-	0	7
76	0	0	0	3
81	0	-	0	0
92	5	-	0	100
131	0	-	-	0
132	5	-	-	0
135	1	-	-	0

The mean number of bacterial OTUs present across all 41 samples collected was 15.5. As data were not normally distributed, the numbers of OTUs in each sample group were log-transformed and I used parametric analyses to compare group means. The mean number of OTUs differed significantly among samples collected at different times (Figure 7.2; Repeated measures ANOVA, $F_{2,29} = 5.08$, $p = 0.0178$); there was a significant reduction in the number of OTUs per frog over time (Figure 7.2; Tukey's pairwise comparison between February 2010 and February 2011 samples, $p = 0.0224$). The mean number of OTUs did not differ significantly among frogs grouped by infection category (ANOVA, $F_{3,36} = 1.651$, $p = 0.195$). However, the median number of OTUs per frog was greatest in frogs that were never infected by *Bd*, lower in frogs that would later become infected, lowest in infected frogs, and slightly higher again in previously infected frogs (Figure 7.3), as might have been expected given the effects of bacterial assemblage richness on infection intensity that I demonstrated in Chapter Six.

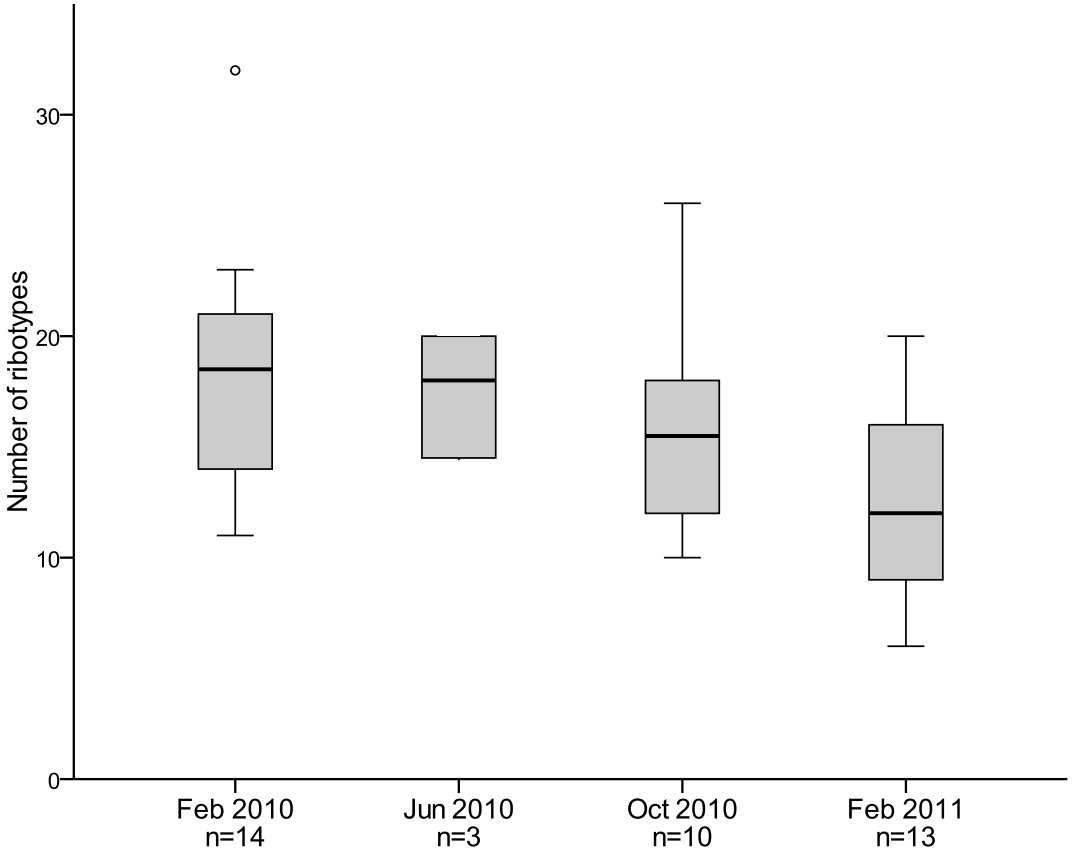

Figure 7.2. The number of OTUs present in samples collected during each sampling trip. Boxes represent the interquartile range and bold bars, the median. Whiskers represent the minimum and maximum data points while outliers are marked with a circle and represent cases with values between 1.5 and 3 box lengths from the upper edge of the box.

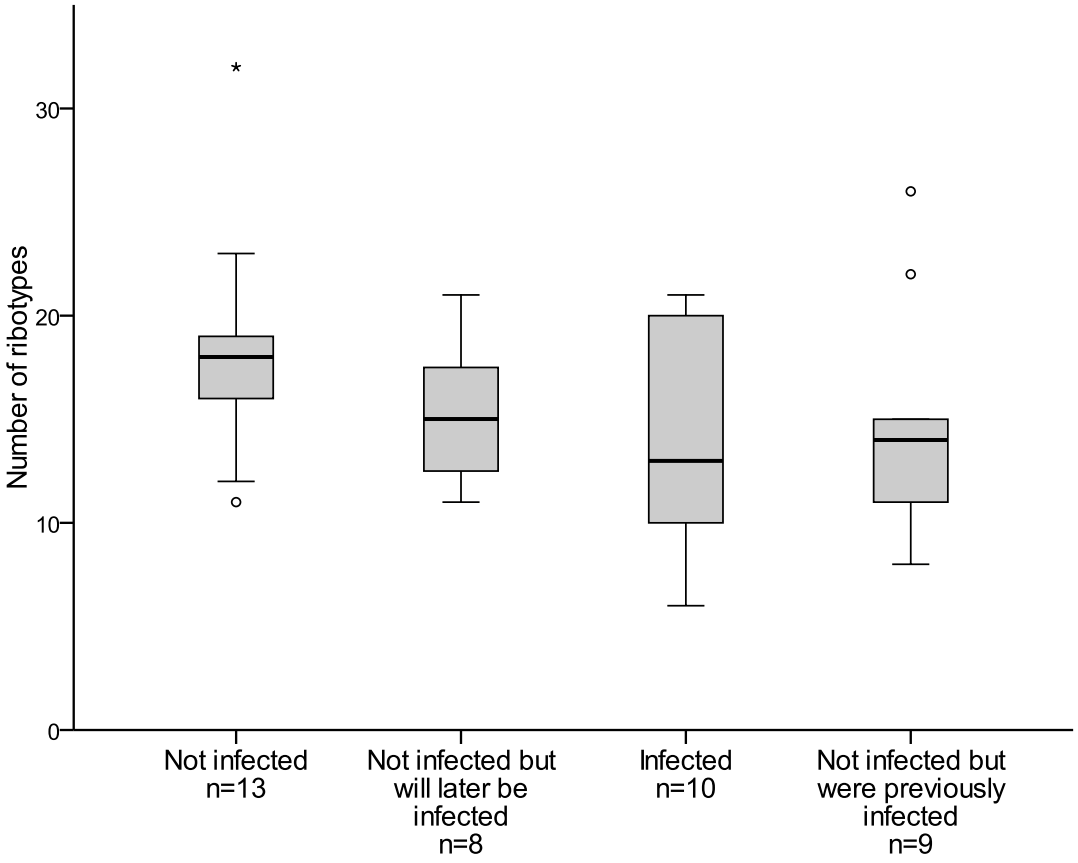

Figure 7.3. The number of OTUs present in samples taken from frogs in each infection category. Boxes represent the interquartile range and bold bars, the median. Whiskers represent the minimum and maximum data points while outliers are marked with a circle and represent cases with values between 1.5 and 3 box lengths from the upper edge of the box. Extreme outliers are marked with a star and represent values greater than 3 box lengths from the upper edge of the box.

The overall composition of the bacterial assemblage as summarized by nMDS changed significantly among sample collection dates (Figure 7.4; ANOSIM, $R = 0.165$, $p < 0.0015$). Pairwise comparisons indicate significant differences between February 2010 and October 2010 samples ($R = 0.142$, $p < 0.032$), October 2010 and February 2011 samples ($R = 0.221$, $p < 0.0048$) and February 2010 and February 2011 samples ($R = 0.232$, $p = 0.00032$), suggesting that changes in the microbiota occurred throughout the year with the majority of changes occurring between October 2010 and February 2011. Frogs that occurred towards the edges of the nMDS distribution in the February 2011 sample had fewer OTUs than frogs that were more tightly clustered (t-test, assuming unequal sample sizes with equal variances, $t_{12} = -3.924$, $p = 0.0024$).

Samples collected in February 2010 and 2011 were collected during periods of similar mean daily air temperature (20.2 and 20.8^{o}C respectively), while the frogs sampled in June and October 2010 were subject to slightly lower daily mean air temperatures; 17.2 and 19^{o}C.

The overall composition of the bacterial assemblage as summarized by nMDS also differed significantly when samples were categorized in two ways; infected frogs and frogs that had lost infection were grouped together, and uninfected frogs and frogs that had not yet become infected were jointly grouped (Figure 7.5; ANOSIM, $R=0.12$, $p<0.004$). Although there was no difference between the group centroids (Figure 7.5; MRPP; $n = 40$, $\delta = -0.85$, $p = 0.166$), the distance between each point on the nMDS plot and its category centroid was significantly different between the two groups (t-test, assuming unequal sample sizes with unequal variances, $t_{39} = 4.732$, $p=0.00006$). This result suggests that variation in bacterial composition on individual frogs was greater in the infected frog group than the uninfected frog group. The five core ribotypes were present in a higher proportion of samples from “not yet infected” frogs than samples from frogs that were “infected or had lost infection” (Figure 7.6; FET; $p = 0.0017$).

All individual frogs exhibited shifts in their microbiota during the year, with some frogs changing their microbial assemblage composition more than others. Nine of the 14 frogs showed shifts in their bacterial assemblage towards the outer edges of the nMDS distribution for samples collected in February 2011 (Figure 7.7). Overall, samples from individual frogs were more similar to one another than to those of other frogs (Figure 7.7; MRPP; $n = 40$, $\delta = -2.81$, $p = 0.0052$).

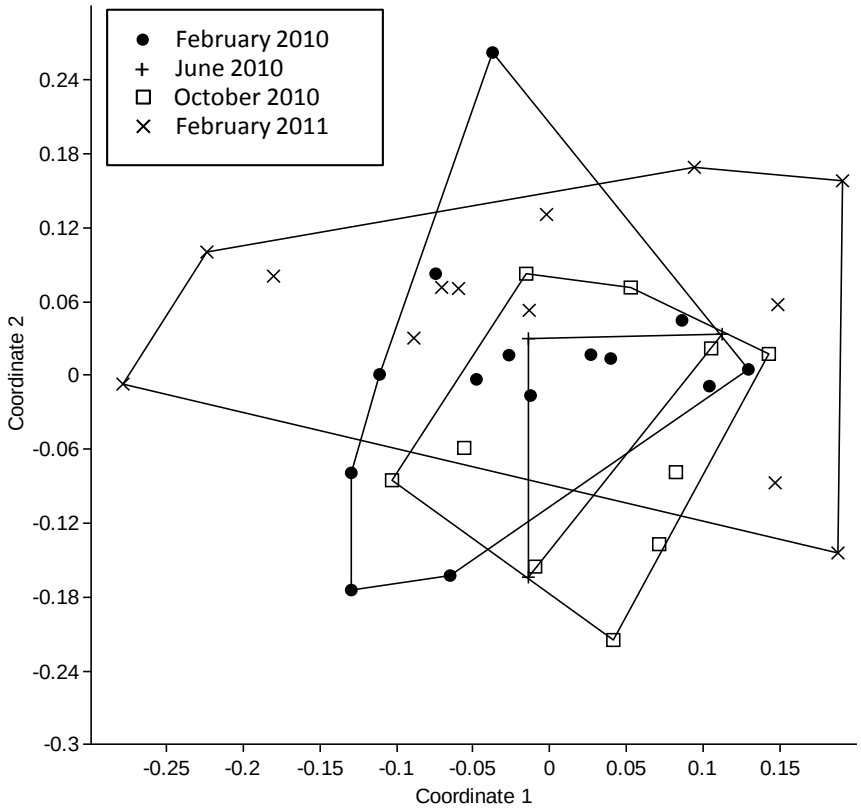

Figure 7.4. Non-metric multi-dimensional scaling ordination of the bacterial assemblage composition of individual frogs. Symbols indicate time at which samples were collected, and outer convex polygons indicate the region of the ordination space encompassing each group of samples.

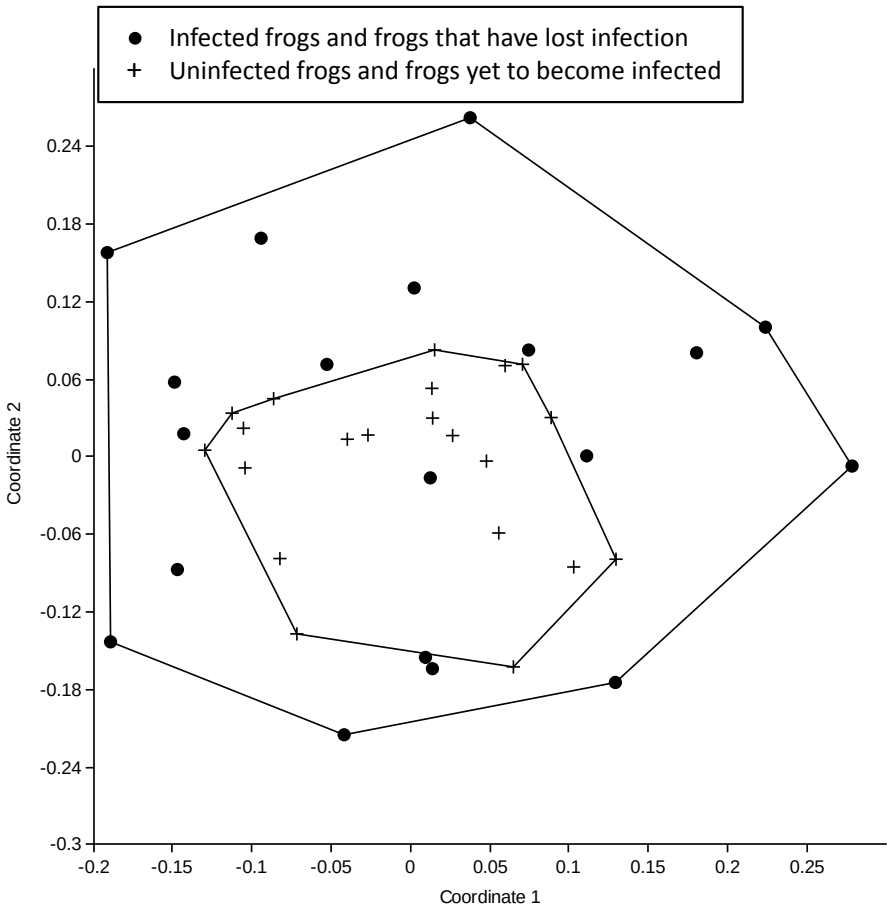

Figure 7.5. Non-metric multi-dimensional scaling ordination of the bacterial assemblage composition of individual frogs. Symbols indicate sample infection category, and outer convex polygons indicate the region of the ordination space encompassing each group of samples.

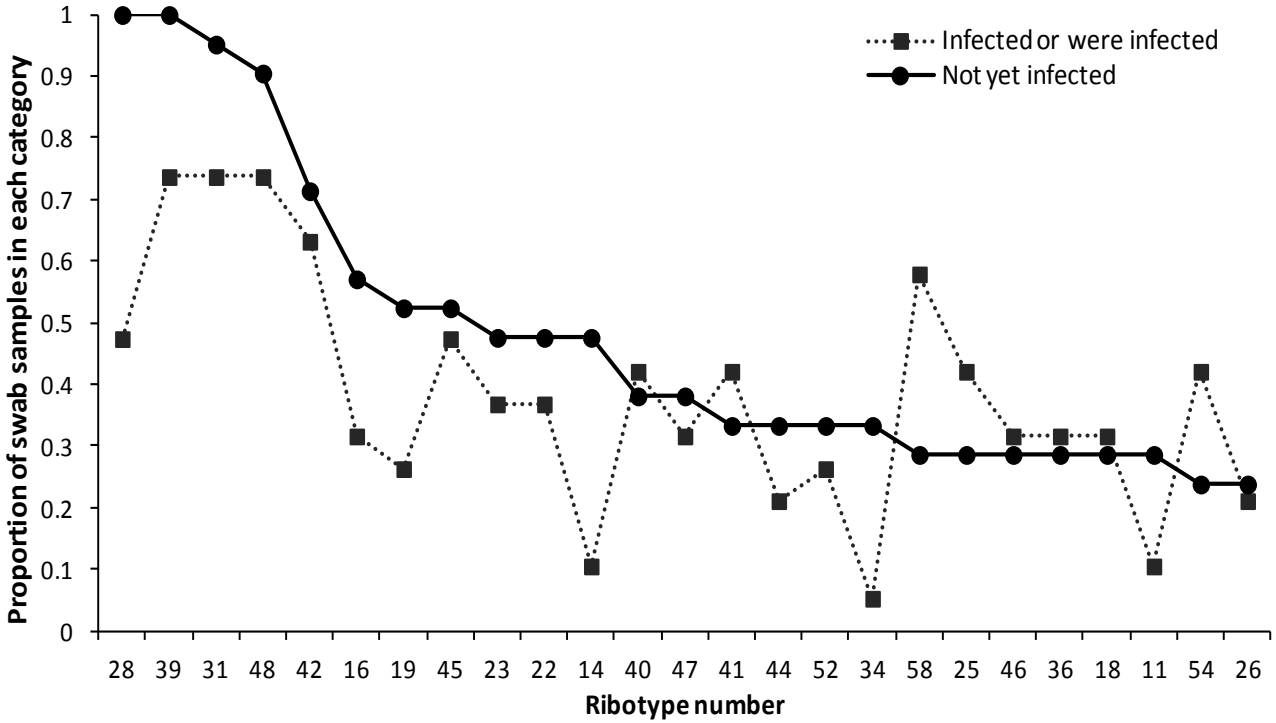

Figure 7.6. The proportion of swab samples with each of the twenty-five most common bacterial ribotypes displayed by two infection groupings; frogs that were infected or had lost infection, and frogs that were uninfected or would become infected later.

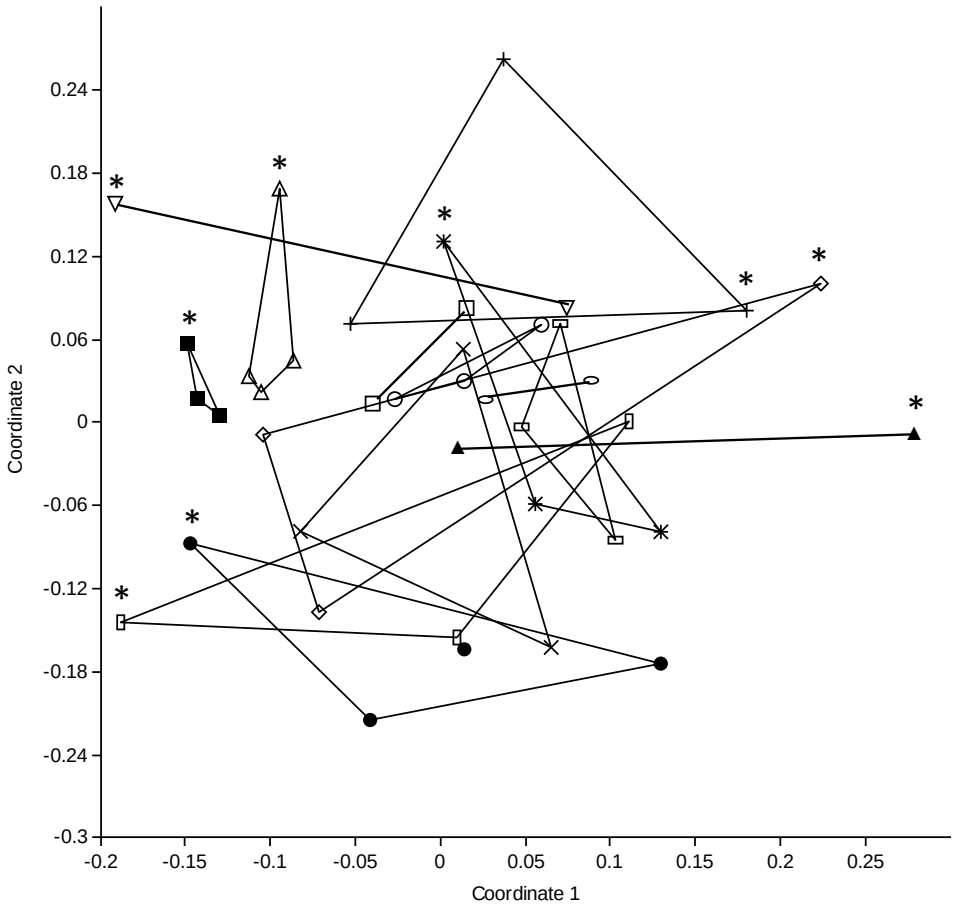

Figure 7.7. Non-metric multi-dimensional scaling ordination of the bacterial assemblage compositions of individual frogs. Unique symbols indicate samples collected from the same frog and outer convex polygons indicate the region of the ordination space encompassing the group of samples from each frog. Those samples marked by an asterisk represent the nine samples collected in February 2011 that showed shifts towards the outer edges of the plot.

Discussion

One hundred and fourteen unique bacterial OTUs were identified from 41 swab samples collected from the 14 frogs recaptured multiple times during this study. The microbial assemblage on each frog comprised up to five commonly occurring OTUs, which may be present simply due to high prevalence within the environment, or because they have evolved a specific function within the microbial assemblage, such as pathogen defence. A further 20 less commonly occurring OTUs and a large number of rare OTUs also contribute to the overall

bacterial assemblage composition. However, rare OTUs may still play an important role in the functional capacity of the bacterial assemblage and, as discussed in Chapter Six, might contribute to the production of a diverse range of antibiotic compounds that protect frogs from environmental pathogens and disease.

While the subset of data used in this study showed that higher *Bd* infection prevalence occurred in summer (February 2010 and 2011 samples) than in winter (June 2010), overall infection prevalence on the stream, calculated using the full dataset, was highest in June 2010 (S. Sapsford, pers. comm.). This is consistent with the findings of previous surveys (S. Bell, unpublished, Berger et al. 2004; Woodhams and Alford 2005; Kriger and Hero 2007). The low *Bd* prevalence observed in the subset of winter samples used in this study was therefore a result of the lower number of frogs in the sample.

Variation among samples across the year

The mean numbers of bacterial OTUs from samples collected at different time points were significantly different ($p = 0.0338$). The boxplot in Figure 7.2 shows that there was a trend for decreasing numbers of OTUs across successive samples. This could have occurred as a result of repeated swabbing of individuals. However, sampling occurred every four months, and many frogs were not recaptured at every survey, so there should have been more than adequate time for the cutaneous microbiota to recover from any swabbing effects. Swabbing was conducted by the same person throughout the year, so sampling variation among seasons is very unlikely to have occurred. Other factors, such as changes in host diet, hormonal fluctuations and environmental temperature variation, may also contribute to the observed variation.

Changes in host diet might affect the composition of mucosal secretions, leading to changes in the resident microbial populations that depend on these for nutrition (Kleessen and Blaut 2005). In the laboratory, bacterial production of inhibitory compounds active against *Bd* can vary as a result of carbon source (Sánchez et al. 2010). No published data are available on the natural diets of Australian rainforest frogs, however invertebrate abundance and therefore composition can change seasonally (Wolda 1980; Frith and Frith 1985), and seasonal changes in the composition of frogs' diets could alter their cutaneous microbiota.

In a similar way, hormonal fluctuations associated with breeding could potentially influence the cutaneous microbiota within the mucosal layer by boosting or inhibiting growth. For example, human hormones have been shown to boost the *in vitro* growth of pathogenic bacteria (Porat et al. 1991). However, unlike many amphibian species with defined breeding seasons, *L. rheocola* gravid females and calling males have been found at Windin North Creek in both winter and summer (pers. obs; S. Sapsford pers. comm.). This suggests that, as suitable conditions exist for breeding all year round, hormonal fluctuations in the male frogs in this study might not show seasonal patterns that greatly influence the microbial assemblage composition. However, at an individual level, frogs could still experience hormonal cycles related to their particular reproductive state.

Environmental temperature is another factor that could have affected the changes in bacterial assemblage composition documented in this study. Daily means of hourly air temperature readings collected every hour during periods of sample collection differed by only a few degrees among visits to the stream. Mean daily temperatures during sample collection in February 2010 and February 2011 were very similar (20.8 and 20.2^{o}C respectively), while temperatures during sample collection in June and October 2010 were somewhat cooler, at 17.2 and 19^{o}C respectively. Although these differences are small, they could have contributed to the observed shifts in bacterial assemblage composition, either via direct temperature effects on the bacteria, or through indirect effects of temperature, such as immunosuppression, on hosts. While no data exist for frogs, similar small changes in temperature among seasons can induce a shift in microbial assemblage composition in corals (Ritchie 2006; Sharon and Rosenberg 2008).

The 2-dimensional nMDS plot in Figure 7.4 shows that microbial assemblage composition changed over time. There were only three samples from recaptured frogs in June 2010 making results from this time point difficult to interpret. Samples from February 2011 are more dissimilar to one another than are samples from either February 2010 or October 2010, which are more tightly clustered, indicating that variation in OTU composition among the assemblages on individual frogs was substantially greater in February 2011 than at the other sampling times. The significant ANOSIM result suggests that changes in the average composition of the microbiota occurred throughout the year, and both the ANOSIM results and examination of Figures 7.2 and 7.4 suggest that the greatest changes probably occurred between October 2010 and February 2011. In my dataset, these changes were accompanied by a decline in the median number of bacterial OTUs per frog. One possible cause of the differences between the February 2011 and earlier samples was the disturbance created by

Cyclone Yasi. The cyclone caused substantial damage to the area, altering habitat availability for the frogs, just before the sample was collected in early February 2011. In the February 2011 sample, frogs with bacterial fingerprints that occurred towards the edges of the nMDS distribution had significantly fewer OTUs than frogs that were more tightly clustered. This suggests that they may have lost some of their microbiota as a result of the cyclone.

Overall, samples from individual frogs collected over the year were more similar to other samples from the same frog than to samples from other frogs. However, cutaneous bacterial assemblage composition changed more on some frogs than on others. The cause of the fluctuations is unknown, but could be related to skin shedding. Similar patterns of variation among individuals were described in marine sponges over a 21 month study; in addition to seasonal temperature fluctuations, competition, host physiology and nutrition were postulated as possible causes of variation (Anderson et al. 2010). The temporal loss and acquisition of bacterial strains, observed in individual frogs in this study, suggests that the bacterial assemblage would be amenable to manipulation through bioaugmentation of *Bd*-inhibitory bacteria. However, turnover of the most prevalent strains is low, suggesting that bioaugmentation success could be enhanced by application of one or more of these dominant assemblage members (assuming that they have anti-fungal properties), thus enabling antifungal effects to be long-lasting.

Variation among frogs with different *Batrochochytrium dendrobatidis* infection histories

The mean number of bacterial OTUs did not differ significantly among samples grouped by infection category. However, Figure 7.3 shows that the median number of OTUs in the sampled assemblages decreased across infection groups from uninfected to frogs that would later become infected to infected, then increased in previously-infected frogs. The trend shown in my samples towards reduction in OTU richness with increasing susceptibility to infection is consistent with my findings in Chapter Six, where frogs with more intense *Bd* infections had lower *Bd*-inhibitory bacterial taxonomic richness. However, since this trend was not statistically significant, it might just reflect noise in the data; larger sample sizes are needed.

Taxonomic richness is not the only factor that might be linked to *Bd* infection. The ribotype composition of the bacterial assemblage was significantly different between the two broad infection categories as summarized by the nMDS plot in Figure 7.5. Although the locations of the group centroids did not differ significantly, the most striking fact illustrated by Figure 7.5 is that frogs that were not yet infected are clustered in a relatively small region near the centre of the ordination space. The actual regions of that space occupied by these frogs and by frogs that

were infected or had recently lost infection are almost mutually exclusive; frogs that were not yet infected had a much more consistent microbiota than frogs that were infected or had recently lost infection that had more divergent microbiotas. Figure 7.5 strongly suggests that there is a relatively narrow set of microbial assemblage compositions that resists *Bd* infection, and that individuals with assemblages outside this set are more prone to becoming infected.

Unfortunately, it is not possible to determine whether the low species richness, high assemblage variability, or divergence from the central set of assemblages observed in this study caused increased susceptibility to infection, or whether becoming infected by *Bd* may have caused these differences. It is possible that the damage to amphibian skin caused by *Bd* infection compromises skin defences and results in colonisation by potentially harmful bacteria. A laboratory infection trial using a group of frogs with known bacterial species richness could elucidate an answer.

The majority of the 25 most common ribotypes were more prevalent in frogs that were uninfected or yet to become infected than in frogs that were infected or had lost infection. It is possible that the five most ubiquitous bacterial ribotypes, present in a significantly higher proportion of "uninfected" samples, might have a particular role in the "uninfected" microbial assemblage, such as antibiotic production, providing protection against *Bd* to these frogs. However, the majority of the ribotypes in this study occurred in very low numbers, with 52% occurring less than three times. Despite this low prevalence, the importance of these ribotypes cannot be discounted as they are likely to play a role in the complex interactions that occur among members of the microbial assemblage.

Future directions

Identification of the most commonly occurring bacterial strains would provide insight into possible core bacterial assemblage members. It was not possible in this study as the majority of the gel bands occurred too close together to enable accurate excision without contaminating non-target DNA, even after the additional refinement of the denaturant gradient to provide greater band separation. However, detailed knowledge of the composition of the entire bacterial assemblage could resolve how frogs obtain their microbiota, provide more details of the functional structure of the microbial assemblage, and could help to suggest how this is maintained over time. Future studies using next-generation DNA sequencing will allow acquisition of the necessary detail. In addition, a quantitative assessment of the abundance of the most prevalent bacterial groups would provide a more complete picture of how the microbial assemblage changes with time.

Bioaugmentation of a diverse *Bd*-inhibitory bacterial cocktail to a frog population that is highly susceptible to *Bd*, might help protect the population during initial pathogen exposure. Future research should aim to maximize the duration of temporary protection while host innate and adaptive immune systems adjust to provide longer-lasting means of defence against *Bd*.

CHAPTER EIGHT : SUMMARY, GENERAL DISCUSSION AND RECOMMENDATIONS FOR FUTURE RESEARCH

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The number of recorded emerging infectious disease events has been steadily increasing (Jones et al. 2008), with fungal diseases posing a serious threat to animal and plant health (Fisher et al. 2012). The emergence of one of these diseases, chytridiomycosis, caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) (Berger et al. 1998; Longcore et al. 1999), has caused the declines or extinctions of many amphibian species worldwide (Daszak et al. 1999; Stuart et al. 2004). Antifungal drugs have been used successfully in treating *Bd* infected frogs in the laboratory (Garner et al. 2009; Berger et al. 2010; Martel et al. 2011), but broad scale environmental application could be difficult or have unwanted ramifications. While more benign treatment methods, such as sodium chloride applications, have been proposed as a means of reducing infection loads in amphibians in pond ecosystems (Stockwell et al. 2012), they cannot provide long-term protection against disease, and are impossible in flowing water. Other proposed strategies include biocontrol with *Bd* predators such as Daphnia (Buck et al. 2011; Woodhams et al. 2011; Hamilton et al. 2012), selective breeding for resistant individuals (Woodhams et al. 2011) and immunization, which has had variable success (Stice and Briggs 2010; Murphy et al. 2011). To date, bioaugmentation of naturally occurring bacteria that are antagonistic to *Bd* offers the only potentially practical solution for long-term disease prevention (Harris et al. 2006; Harris et al. 2009a; Harris et al. 2009b). In a field setting, where large numbers of frogs are involved, it has potential for broad scale application. Bioaugmentation can increase innate immune resistance to *Bd* if the probiotic is accepted by, and integrates with, the natural microbiota. Probiotics have been used in aquaculture (Verschuere et al. 2000; Irianto and Austin 2002) and agriculture (Sanchis and Bourguet 2009) at broad scales and with considerable success.

Proof of concept trials have shown that inoculations of amphibians with antibiotic-producing bacteria can prevent mortality from *Bd* infection (Harris et al. 2009a). However, much work remains to be done to understand the mechanism behind this trial's success. My thesis contributes additional background knowledge on the role of the cutaneous bacterial microbiota and the potential for bioaugmentation. The broad aims of this thesis were, firstly, to determine the effectiveness of techniques used to date to select and test the bacteria resident on frog skin; secondly, to determine whether the amphibian cutaneous bacterial microbiota may be important in the coexistence of *Bd*; and finally, to investigate the stability of

the resident cutaneous bacterial assemblage, and factors that may affect this stability. These aims were addressed through the research presented in Chapters Three to Seven of this thesis.

Laboratory culture of bacterial isolates

Chapter Three addressed potential biases in descriptions of the microbial assemblage determined using laboratory culture. My study indicated that a large fraction of the bacterial species inhabiting amphibian skin are culturable, and that bacterial colony morphology was not a good proxy indicator of species richness. Selection of all bacterial colony morphotypes from mixed assemblage agar plates under-represented the culturable bacterial genetic diversity present. When screening for potentially important bacterial symbionts, it is important to maximise detection of all culturable isolates. A simple way to accomplish this is to over-select morphologically similar colonies from the agar plate. Duplicate isolates can subsequently be identified using molecular analysis and removed from analyses. In addition, my study showed that 75% of ribotypes present were culturable on the R2A agar medium. This figure is substantially better than the 1% expected for environmental bacteria (Staley and Konopka 1985) and suggests that frog skin is much more complex than a simple environmental substrate. Evolutionary pressure, as a result of host exposure to pathogens, has likely facilitated selection for the particular cutaneous bacteria present (Cook et al. 1995). This study demonstrates potential to further investigate the functional role of culturable bacteria in pathogen control.

New assay development

To effectively test large numbers of cutaneous bacterial isolates for the ability to inhibit the pathogen *Bd*, it was necessary to develop a new *in vitro* challenge assay technique. In Chapter Four, I created a broth-based 96-well plate protocol to resolve many of the issues that arise when using agar plate challenge assays. This technique involves the production of a bacterial cell-free supernatant containing bacterial metabolites, and the subsequent culture of *Bd* in the presence of these metabolites. It is an effective *in vitro* method for screening bacterial isolates for strong *Bd*-inhibitory activity and allows visual differentiation between fungicidal and fungistatic effects. Bacteria that produce metabolites with fungicidal effects, which completely destroy *Bd* cells, may make more desirable probiotics than those that have fungistatic activity. This technique can now be used to facilitate future research with greater confidence. It is now possible to investigate the effect of a range of environmentally-relevant incubation temperatures on metabolite production, or the effect of multiple bacterial metabolites on *Bd* growth. Of particular interest are the possible hormetic effects observed (Stebbing 1982).

Further research should investigate this growth phenomenon, as it may be important in natural systems; the use of a probiotic whose antibiotic compounds enhance the growth of *Bd* may increase infection intensity and accelerate host mortality. The potential for this mishap has been demonstrated in humans. Low concentrations of some pharmaceuticals have enhanced the *in vitro* growth of cancer cells, while higher concentrations destroyed them (see Calabrese 2005). The range of inhibitory effects observed using this assay technique also provides an opportunity to study gene expression in *Bd* to identify genes involved in growth and inhibition of the pathogen.

Bacterial inhibition of *Batrachochytrium dendrobatidis* in wild frog populations

Chapter Five presents results from screening Wet Tropics frogs for *Bd*-inhibitory bacteria, using the technique described in Chapter Four, while Chapter Six identifies the totally inhibitory isolates and investigates the effect of bacterial taxonomic richness on *Bd* infection intensity. Of the totally inhibitory isolates, 82% were from the Gammaproteobacteria group and 45% of these isolates were from the genus *Pseudomonas*. Although Pseudomonads are ubiquitous in many environments (Paulsen et al. 2005), it is possible that selection pressures exerted by *Bd* have led to the preferential colonization of these antibiotic-producers (Walke et al. 2011, Online Supplementary Information). Traditionally, studies have focused on identifying bacterial isolates that exhibit total inhibition against *Bd* (Harris et al. 2006; Woodhams et al. 2007c; Lam et al. 2010; Walke et al. 2011) or on the whole bacterial community without knowledge of inhibitory taxa (McKenzie et al. 2012). However, it would be useful to know whether the taxonomy of non-inhibitory and inhibitory isolates is similar. If different taxa are involved, this could lead to the development of specific molecular probes that could be used to screen the cutaneous microbiota in frog populations of interest, for *Bd*-inhibitory potential. Conversely, if the same taxa are involved, this indicates that there may be strain-level differences in the ability to produce antibiotic compounds.

There was a negative relationship between the number of bacterial genera present per frog and *Bd* infection, suggesting that, in accordance with some community ecology theory (Robinson and Valentine 1979), increased richness of *Bd*-inhibitory taxa on frogs may play a role in controlling *Bd* infection intensity, and in facilitating coexistence with enzootic infection. Unfortunately, it is not possible to say whether frogs with naturally lower bacterial taxonomic richness could simply be more susceptible to *Bd,* or whether *Bd* infection caused a reduction in taxonomic richness. Future research should attempt to resolve this conundrum. However, the two possibilities are not necessarily mutually exclusive. Assuming bacteria have played a role

in recolonisation of upland sites with enzootic *Bd* infection, it is possible that upland frogs have evolved a more diverse *Bd*-inhibitory microbiota that helps prevent or reduce infection by *Bd*. However, despite this, if *Bd* infection does occur, it might still disrupt the established interactions among members of the bacterial assemblage. This could occur because the pathogen produces compounds that inactivate antibiotics, as has been demonstrated in plant-symbiont-pathogen systems (Duffy et al. 2003; Schouten et al. 2004). When selecting possible bacteria for bioaugmentation trials, cocktails of several bacteria from different genera may be more effective against *Bd* than a single bacterial isolate, as functional redundancy could reduce the probability of antibiotic deactivation. Consideration should be given to the inclusion of *Pseudomonas* spp. in this cocktail, due their widespread occurrence on anuran skin, and the diverse range of inhibitory compounds they produce.

One upland site had a significantly higher proportion of frogs with *Bd*-inhibitory bacteria, suggesting that herd immunity may be operating in this population. Frogs at this site also had more inhibitory bacterial genera, supporting the idea that the increased diversity of *Bd*-inhibitory taxa offers protection against *Bd* infection. This protection could arise because more inhibitory compounds are produced by the higher diversity of taxa present on frogs. However, frogs sampled at the other upland site did not have increased bacterial taxon richness, and yet recolonisation had also occurred there. It is, therefore, quite possible that recolonisation and coexistence of frogs with enzootic *Bd* infection is facilitated by different mechanisms at each upland site.

Uninfected frogs, from both *Bd*-exposed and unexposed populations, did not have significantly different numbers of cultured bacteria, or proportions of inhibitory bacteria. This lack of difference suggests that, on average, frogs in populations subject to *Bd* infection have not acquired large numbers of novel *Bd*-inhibitory bacteria. Future research should concentrate on gathering further evidence to demonstrate that natural selection for frogs with better or greater antifungal capacity can occur.

Stability of the bacterial assemblage

Chapter Seven addresses natural changes in the (uncultured) cutaneous bacterial microbiota in individual wild frogs tracked over a year. Significant changes in assemblage composition occurred throughout the year, with the majority of changes detected immediately after Cyclone Yasi. Severe alterations to frog habitat could cause changes to the host bacterial assemblage. Flood surge can increase bacterial cell numbers in stream water (Muirhead et al. 2004) and could have led to host exposure to different environmental bacteria, as *L. rheocola*

spends a considerable amount of time submerged in flowing water. Alternatively, increased UV exposure through a more open canopy may have caused observed changes in the bacterial assemblage (Kadivar and Stapleton 2003). My bacterial assemblages had quite consistent dominant species, with more variable subdominants, as many relatively stable assemblages of macroscopic species do. The five "core" bacterial species were present in a high proportion of samples collected throughout the year. All core bacterial species were present in a higher proportion of uninfected frogs than frogs that were infected, or had recently recovered from *Bd* infection, indicating that these taxa may have specific roles within the microbial assemblage, such as the ability to produce *Bd*-inhibitory metabolites.

The greatest differences in bacterial assemblages were related to differences in host *Bd* infection. Frogs had fairly consistent bacterial assemblages if they were not infected immediately prior to, or at the time of sampling, whereas infected frogs, and frogs that had recently recovered from *Bd* infection had more divergent microbiotas. Unfortunately, the results did not suggest a mechanism for these observed differences. Frogs with a more consistent bacterial assemblage may have been more susceptible to *Bd* infection, or *Bd* infection may make the bacterial assemblage more divergent; both scenarios are equally plausible. Future laboratory studies should attempt to resolve the nature of this host-pathogen-bacterial symbiont interaction.

Future directions for probiotic development and use

There is a pressing need for effective frog probiotics to control *Bd*. Much remains unknown. For example, many interactions in microbial assemblages are highly context-dependent (Daskin and Alford 2012). We do not yet know whether a bacterial strain that is *Bd*-inhibitory *in vitro*, will always produce inhibitory compounds *in vivo*. One trial, involving two *Bacillus* species that produced antifungal compounds *in vitro,* which were active against a plant pathogen, found that only one of them was effective *in vivo* (Leifert et al. 1995). Changing from a laboratory to a field environment may have changed nutrient availability, which can affect antibiotic production (Martin and Demain 1980). Therefore, developing a laboratory medium that more closely resembles frog skin mucosa may provide more realistic assay results and lead to a reduction in the subsequent failure rate of time-consuming *in vivo* trials.

A number of factors deserve consideration when developing an effective probiotic. These include, but are not limited to, interactions between the probiotic and its host, environment, and other cutaneous microbiota, including the pathogen. Some of these criteria have been discussed above and within the thesis chapters; others deserve a brief mention here. An ideal

bacterium or bacterial cocktail should readily become part of the resident microbiota without major imbalance of other community members (Tagg and Dierksen 2003) or ill-effects to the host or environment. Furthermore, a probiotic should work synergistically with other resident bacteria and with host adaptive and innate immune defences (see Myers et al. 2012). Those that can establish and grow rapidly *in situ,* producing effective antibiotic defence at low cell densities, are model candidiates.

Ideally, the host-probiotic partnership should become long-lasting. However, amphibians that shed skin very frequently, or those that produce a waxy layer to prevent water loss may pose unique problems for probiotic therapy. In addition, cutaneous bacteria that are already present on multiple amphibian species in the target population may make more effective probiotics than a novel bacterium. For example, the bacterium *Janthinobacterium lividum*, provided *in vivo* protection against *Bd* for *Plethodon cinereus* and *Rana muscosa* in laboratory trials (Becker et al. 2009; Harris et al. 2009a), but failed to provide protection when used as a probiotic in captive Panamanian golden frogs, *Atelopus zeteki* , possibly because it was not native to that species (Becker et al. 2012).

The most effective way to apply probiotic bacteria is likely to vary among species and habitats. In some laboratory experiments, amphibians had their existing microbiota depleted by treatment with 3% hydrogen peroxide, antibiotics, or both, to create a niche for the applied probiotic to occupy (Becker & Harris, 2010; Harris et al. 2009). This pre-treatment increases the probability that the target bacterium will be incorporated. Bacteria are then applied by bathing amphibians in washed bacterial cells suspended in a sterile buffered medium such as Provosoli (Wyngaard & Chinnappa, 1982; Becker & Harris, 2010; Harris et al., 2009) or Holtfreter's solution (Schadich & Cole, 2010). This is likely to remain an effective means of inoculation for captive animals prior to reintroduction from assurance colonies. However, in a large-scale field trial, this process is only likely to work effectively in an isolated pond environment where there is a high probability that the majority of animals could be caught. On flowing streams, only a small proportion of the frog population is captured in any given survey (Richards and Alford 2005). Therefore, to ensure that sufficiently high numbers of animals are exposed, probiotic bacteria would ideally be applied to frogs by hand-spray without capture or depletion of existing microbiota. This could be repeated on multiple occasions, as necessary, to ensure that a sufficiently high proportion of frogs are exposed to the probiotic. Further consideration of alternative means of inoculation such as aerial spraying over large or remote areas may be necessary. This technique is used successfully to apply probiotics for the control

of parasitic insects (Roversi 2008; Sanchis and Bourguet 2009). Recent laboratory trials have demonstrated that environmental transmission of bacteria to amphibians is possible. A probiotic bacterium inoculated to soil was readily transferred to salamanders providing disease resistance against *Bd* (Muletz et al. 2012). This possibility offers another mode of application, and may be ideal for some host species. Further investigation of possible ecosystem effects should precede any application of bacterial probiotics *via* environmental routes.

To assess bioaugmentation trial success, it will be essential to develop effective molecular and biochemical tools. For example, specific molecular probes and primers for quantitative PCR (Nadkarni et al. 2002) will enable monitoring of changes in bacterial cell numbers over time. Another useful tool would be a non-invasive, rapid screening technique to test frog skin directly for the presence and concentration of specific antibiotic compounds from common bacterial genera.

To develop effective probiotics to control the effects of *Bd* in wild and captive populations, a multidisciplinary approach involving ecologists, biochemists, microbiologists and chemists will be required. There may be environmental, and potentially, legislative matters to attend to, and the issue of probiotic production scale-up will need to be addressed before inoculants can be used in large-scale field applications. Probiotic research to date has demonstrated that the protection offered against pathogens is often only effective when the probiotic is regularly applied (Verschuere et al. 2000; Fric 2007). Amphibian probiotic research should therefore aim to maximize the duration of protection offered while host innate and adaptive immune systems adjust to provide longer-lasting means of defence against *Bd*.

LITERATURE CITED

---------- (2006) Threat abatement plan: Infection of amphibians with chytrid fungus resulting in chytridiomycosis. Australian Department of the Environment and Heritage. http://www.environment.gov.au/biodiversity/threatened/publications/tap/chytrid.html, Canberra

Aguirre, A.A., Tabor, G.M. (2008) Global factors driving emerging infectious diseases. Annals of the New York Academy of Sciences 1149:1-3

Alford, R.A. (2010) Declines and the Global Status of Amphibians. In: Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K. (eds) Ecotoxicology of Amphibians and Reptiles. CRC Press, Boca Raton

Alford, R.A., Richards, S.J. (1999) Global amphibian declines: A problem in applied ecology. Annual Review of Ecology and Systematics 30:133-165

Andersen, R.J., Wolfe, M.S., Faulkner, D.J. (1974) Autotoxic antibiotic production by a marine Chromobacterium. Marine Biology 27:281-285

Anderson, R.M., May, R.M. (1985) Vaccination and herd immunity to infectious diseases. Nature 318:323-329

Anderson, S.A., Northcote, P.T., Page, M.J. (2010) Spatial and temporal variability of the bacterial community in different chemotypes of the New Zealand marine sponge *Mycale hentscheli*. Fems Microbiology Ecology 72:328-342

Austin, R.M.J. (2000) Cutaneous microbial flora and antibiosis in *Plethodon ventralis*. In: Bruce, R.C., Jaeger, R.G., Houck, L.D. (eds) The Biology of Plethodontid Salamanders. Kluwer, Plenum, New York

Backhed, F., Ley, R.E., Sonnenburg, J.L., Peterson, D.A., Gordon, J.I. (2005) Host-bacterial mutualism in the human intestine. Science 307:1915-1920

Baker, G.C., Smith, J.J., Cowan, D.A. (2003) Review and re-analysis of domain-specific 16S primers. Journal of Microbiological Methods 55:541-555

Bally, M., Garrabou, J. (2007) Thermodependent bacterial pathogens and mass mortalities in temperate benthic communities: a new case of emerging disease linked to climate change. Global Change Biology 13:2078-2088

Banning, J.L., Weddle, A.L., Wahl, G.W., Simon, M.A., Lauer, A., Walters, R.L., Harris, R.N. (2008) Antifungal skin bacteria, embryonic survival, and communal nesting in four-toed salamanders, *Hemidactylium scutatum*. Oecologia 156:423-429

Barra, D., Simmaco, M., Boman, H.G. (1998) Gene-encoded peptide antibiotics and innate immunity.: Do "animalcules" have defence budgets? FEBS Letters 430:130-134

Barry, A.L. (1980) Procedure for testing antibiotics in agar media: Theoretical considerations. In: Lorian, V. (ed) Antibiotics in Laboratory Medicine. Williams and Wilkins, Baltimore

Becker, M.H., Brucker, R.M., Schwantes, C.R., Harris, R.N., Minbiole, K.P.C. (2009) The bacterially produced metabolite violacein is associated with survival of amphibians infected with a lethal fungus. Applied and Environmental Microbiology 75:6635-6638

Becker, M.H., Harris, R.N. (2010) Cutaneous bacteria of the redback salamander prevent morbidity associated with a lethal disease. PLoS ONE 5:e10957

Becker, M.H., Harris, R.N., Minbiole, K.P.C., Schwantes, C.R., Rollins-Smith, L.A., Reinert, L.K., Brucker, R.M., Domangue, R.J., Gratwicke, B. (2012) Towards a better understanding of the use of probiotics for preventing chytridiomycosis in Panamanian golden frogs. Ecohealth 8:501-506

Belden, L.K., Harris, R.N. (2007) Infectious diseases in wildlife: The community ecology context. Frontiers in Ecology and the Environment 5:533-539

Bell, B.D., Carver, S., Mitchell, N.J., Pledger, S. (2004) The recent decline of a New Zealand endemic: How and why did populations of Archey's frog *Leiopelma archeyi* crash over 1996-2001? Biological Conservation 120:189-199

Bell, R.C., MacKenzie, J.B., Hickerson, M.J., Chavarria, K.L., Cunningham, M., Williams, S., Moritz, C. (2012) Comparative multi-locus phylogeography confirms multiple vicariance events in co-distributed rainforest frogs. Proceedings of the Royal Society B: Biological Sciences 279:991-999

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., Sayers, E.W. (2011) GenBank. Nucleic Acids Research 39:D32-D37

Bentley, R., Meganathan, R. (1982) Biosynthesis of vitamin K (menaquinone) in bacteria. Microbiological Reviews 46:241-280

Berger, L., Marantelli, G., Skerratt, L.L., Speare, R. (2005) Virulence of the amphibian chytrid fungus *Batrachochytrium dendrobatidis* varies with the strain. Diseases of Aquatic Organisms 68:47-50

Berger, L., Speare, R., Daszak, P., Green, D.E., Cunningham, A.A., Goggin, C.L., Slocombe, R., Ragan, M.A., Hyatt, A.D., McDonald, K.R., Hines, H.B., Lips, K.R., Marantelli, G., Parkes, H. (1998) Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. Proceedings of the National Academy of Sciences of the United States of America 95:9031-9036

Berger, L., Speare, R., Hines, H.B., Marantelli, G., Hyatt, A.D., McDonald, K.R., Skerratt, L.F., Olsen, V., Clarke, J.M., Gillespie, G., Mahony, M., Sheppard, N., Williams, C., Tyler, M.J. (2004) Effect of season and temperature on mortality in amphibians due to chytridiomycosis. Australian Veterinary Journal 82:434-439

Berger, L., Speare, R., Hyatt, A. (1999) Chytrid fungi and amphibian declines: Overview, implications and future directions. In: Campbell, A. (ed) Declines and Disappearances of Australian Frogs. Environment Australia

Berger, L., Speare, R., Pessier, A., Voyles, J., Skerratt, L.F. (2010) Treatment of chytridiomycosis requires urgent clinical trials. Diseases of Aquatic Organisms 92:165-174

Bettin, C., Greven, H. (1986) Bacteria on the skin of *Salamandra salamandra* (L) (Amphibia, Urodela) with notes on their possible significance. Zoologischer Anzeiger 216:267-270

Blaustein, A.R., Romansic, J.M., Scheessele, E.A., Han, B.A., Pessier, A.P., Longcore, J.E. (2005) Interspecific variation in susceptibility of frog tadpoles to the pathogenic fungus *Batracbochytrium dendrobatidis*. Conservation Biology 19:1460-1468

Blomberg, L., Henriksson, A., Conway, P.L. (1993) Inhibition of adhesion of *Escherichia coli* K88 to piglet ileal mucus by *Lactobacillus* spp. Applied and Environmental Microbiology 59:34-39

Boman, H.G. (2000) Innate immunity and the normal microflora. Immunological Reviews 173:5-16

Bosch, J., Martinez-Solano, I., Garcia-Paris, M. (2001) Evidence of a chytrid fungus infection involved in the decline of the common midwife toad (*Alytes obstetricans*) in protected areas of central Spain. Biological Conservation 97:331-337

Boskey, E.R., Telsch, K.M., Whaley, K.J., Moench, T.R., Cone, R.A. (1999) Acid production by vaginal flora *in vitro* is consistent with the rate and extent of vaginal acidification. Infection and Immunity 67:5170-5175

Boyle, D.G., Boyle, D.B., Olsen, V., Morgan, J.A.T., Hyatt, A.D. (2004) Rapid quantitative detection of chytridiomycosis (*Batrachochytrium dendrobatidis*) in amphibian samples using real-time Taqman PCR assay. Diseases of Aquatic Organisms 60:141-148

Bray, J.R., Curtis, J.T. (1957) An ordination of the upland forest communities of southern Wisconsin. Ecological Monographs 27:325-349

Briggs, C.J., Knapp, R.A., Vredenburg, V.T. (2010) Enzootic and epizootic dynamics of the chytrid fungal pathogen of amphibians. Proceedings of the National Academy of Sciences of the United States of America 107:9695-9700

Brucker, R.M., Baylor, C.M., Walters, R.L., Lauer, A., Harris, R.N., Minbiole, K.P.C. (2008a) The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:39-43

Brucker, R.M., Harris, R.N., Schwantes, C.R., Gallaher, T.N., Flaherty, D.C., Lam, B.A., Minbiole, K.P.C. (2008b) Amphibian chemical defense: Antifungal metabolites of the microsymbiont *Janthinobacterium lividum* on the salamander *Plethodon cinereus*. Journal of Chemical Ecology 34:1422-1429

Brunham, R.C., Plummer, F.A., Stephens, R.S. (1993) Bacterial antigenic variation, host immune response, and pathogen-host coevolution. Infection and Immunity 61:2273

Bruno, M.E.C., Montville, T.J. (1993) Common mechanisitic action of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiology 59:3003-3010

Brutyn, M., D'Herde, K., Dhaenens, M., Rooij, P.V., Verbrugghe, E., Hyatt, A.D., Croubels, S., Deforce, D., Ducatelle, R., Haesebrouck, F., Martel, A., Pasmans, F. (2012) *Batrachochytrium dendrobatidis* zoospore secretions rapidly disturb intercellular junctions in frog skin. Fungal Genetics and Biology 49:830-837

Buck, J.C., Lisa, T., Blaustein, A.R. (2011) Predation by zooplankton on *Batrachochytrium dendrobatidis*: Biological control of the deadly amphibian chytrid fungus? Biodiversity and Conservation 20:3549-3553

Burkhead, K.D., Schisler, D.A., Slininger, P.J. (1994) Pyrrolnitrin production by biological control agent *Pseudomonas cepacia* B37w in culture and in colonized wounds of potatoes. Applied and Environmental Microbiology 60:2031-2039

Cade, B.S., Richards, J.D. (2005) User Manual for Blossom Statistical Software. Open-File Report 2005-1353 U. S. Department of the Interior, U. S. Geological Survey. www.fort.usgs.gov/products/software/blossom/

Cain, C.C., Lee, D., Waldo III, R.H., Henry, A.T., Casida Jr, E.J., Wani, M.C., Wall, M.E., Oberlies, N.H., Falkinham III, J.O. (2003) Synergistic antimicrobial activity of metabolites produced by a nonobligate bacterial predator. Antimicrobial agents and chemotherapy 47:2113-2117

Calabrese, E.J. (2005) Cancer biology and hormesis: Human tumor cell lines commonly display hormetic (biphasic) dose responses. Critical Reviews in Toxicology 35:463-582

Cartwright, D.K., Chilton, W.S., Benson, D.M. (1995) Pyrrolnitrin and phenazine production by *Pseudomonas cepacia*, strain 5.5 B, a biocontrol agent of *Rhizoctonia solani*. Applied microbiology and biotechnology 43:211-216

Chabrillon, M., Ouwehand, A.C., Diaz-Rosales, P., Arijo, S., Martinez-Manzanares, E., Balebona, M.C. (2006) Adhesion of lactic acid bacteria to mucus of farmed gilthead seabream, and interactions with fish pathogenic microorganisms. Bulletin of the European Association of Fish Pathologists 26:202-210

Chandler, D.P., Li, S.-M., Spadoni, C.M., Drake, G.R., Balkwill, D.L., Fredrickson, J.K., Brockman, F.J. (1997) A molecular comparison of culturable aerobic heterotrophic bacteria and 16S rDNA clones derived from a deep subsurface sediment. Fems Microbiology Ecology 23:131-144

Chatfield, M.W.H., Richards-Zawacki, C.L. (2011) Elevated temperature as a treatment for *Batrachochytrium dendrobatidis* infection in captive frogs. Diseases of Aquatic Organisms 94:235-238

Chernin, L., Brandis, A., Ismailov, Z., Chet, I. (1996) Pyrrolnitrin production by an *Enterobacter agglomerans* strain with a broad spectrum of antagonistic activity towards fungal and bacterial phytopathogens. Current Microbiology 32:208-212

Collins, J.P. (2010) Amphibian decline and extinction: What we know and what we need to learn. Diseases of Aquatic Organisms 92:93-99

Collins, J.P., Storfer, A. (2003) Global amphibian declines: Sorting the hypotheses. Diversity and Distributions 9:89-98

Conlon, J.M. (2011) The contribution of skin antimicrobial peptides to the system of innate immunity in anurans. Cell and Tissue Research 343:201-212

Cook, R.J., Thomashow, L.S., Weller, D.M., Fujimoto, D., Mazzola, M., Bangera, G., Kim, D.S. (1995) Molecular mechanisms of defense by rhizobacteria against root disease. Proceedings of the National Academy of Sciences 92:4197-4201

Corthesy, B., Gaskins, H.R., Mercenier, A. (2007) Cross-talk between probiotic host immune system. Journal of Nutrition 137:781S-790S

Coyne, M.J., Reinap, B., Lee, M.M., Comstock, L.E. (2005) Human symbionts use a host-like pathway for surface fucosylation. Science 307:1778-1781

Crosby, L.D., Criddle, C.S. (2003) Understanding bias in microbial community analysis techniques due to rrn operon copy number heterogeneity. Biotechniques 34:790-802

Cruz-Perez, P., Buttner, M.P., Stetzenbach, L.D. (2001) Detection and quantitation of *Aspergillus fumigatus* in pure culture using polymerase chain reaction. Molecular and Cellular Probes 15:81-88

Culp, C.E., Falkinham, J.O., Belden, L.K. (2007) Identification of the natural bacterial microflora on the skin of eastern newts, bullfrog tadpoles and redback salamanders. Herpetologica 63:66-71

Cunningham, A.A., Dobson, A.P., Hudson, P.J. (2012) Disease invasion: Impacts on biodiversity and human health. Philosophical Transactions of the Royal Society B: Biological Sciences 367:2804-2806

Cunningham, M. (2001) Vicariance, speciation and diversity in Australopapuan rainforest frogs. Ph.D., University of Queensland, Brisbane

Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J., Billen, J. (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311:81-83

Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. Nature 398:701-704

D'Haeze, W., Holsters, M. (2004) Surface polysaccharides enable bacteria to evade plant immunity. Trends in Microbiology 12:555-561

Daskin, J.H., Alford, R.A. (2012) Context-dependent symbioses and their potential roles in wildlife diseases. Proceedings of the Royal Society B: Biological Sciences 279:1457-1465

Daskin, J.H., Alford, R.A., Puschendorf, R. (2011) Short-term exposure to warm microhabitats could explain amphibian persistence with *Batrachochytrium dendrobatidis*. PLoS ONE 6:e26215

Daszak, P., Berger, L., Cunningham, A.A., Hyatt, A.D., Green, D.E., Speare, R. (1999) Emerging infectious diseases and amphibian population declines. Emerging Infectious Diseases 5:735-748

Daszak, P., Cunningham, A.A., Hyatt, A.D. (2000) Emerging infectious diseases of wildlife - Threats to biodiversity and human health. Science 287:443-449

Daszak, P., Cunningham, A.A., Hyatt, A.D. (2003) Infectious disease and amphibian population declines. Diversity and Distributions 9:141-150

De Boer, W., Wagenaar, A.-M., Klein Gunnewiek, P.J.A., Van Veen, J.A. (2007) *In vitro* suppression of fungi caused by combinations of apparently non-antagonistic soil bacteria. Fems Microbiology Ecology 59:177-185

de Castro, F., Bolker, B. (2005) Mechanisms of disease-induced extinction. Ecology Letters 8:117-126

Dethlefsen, L., Eckburg, P.B., Bik, E.M., Relman, D.A. (2006) Assembly of the human intestinal microbiota. Trends in Ecology & Evolution 21:517-523

Dethlefsen, L., McFall-Ngai, M., Relman, D.A. (2007) An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449:811-818

Dice, L.R. (1945) Measures of the amount of ecologic association between species. Ecology 26:297-302

Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K. (2005) Diversity of locust gut bacteria protects against pathogen invasion. Ecology Letters 8:1291-1298

Drummond, A.J., Ashton, B., Buxton, S., Cheung, M., Cooper, A., Heled, J., Kearse, M., Moir, R., Stones-Havas, S., Sturrock, S., Thierer, T., Wilson, A. (2010) Geneious v5.3 Biomatters Ltd. http://www.geneious.com

du Toit, E.A., Rautenbach, M. (2000) A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. Journal of Microbiological Methods 42:159-165

Duffy, B., Schouten, A., Raaijmakers, J.M. (2003) Pathogen self-defense: Mechanisms to counteract microbial antagonism. Annual Review of Phytopathology 41:501-538

Duffy, B.K., Defago, G. (1999) Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Applied and Environmental Microbiology 65:2429-2438

Farrelly, V., Rainey, F.A., Stackebrandt, E. (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. Applied and Environmental Microbiology 61:2798-2801

Farrer, R.A., Weinert, L.A., Bielby, J., Garner, T.W.J., Balloux, F., Clare, F., Bosch, J., Cunningham, A.A., Weldon, C., du Preez, L.H., Anderson, L., Pond, S.L.K., Shahar-Golan, R., Henk, D.A., Fisher, M.C. (2011) Multiple emergences of genetically diverse amphibian-infecting chytrids include a globalized hypervirulent recombinant lineage. Proceedings of the National Academy of Sciences of the United States of America 108:18732-18736

Fernando, W.G.D., Nakkeeran, S., Zhang, Y., Savchuk, S. (2007) Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Protection 26:100-107

Ferris, M.J., Muyzer, G., Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Applied and Environmental Microbiology 62:340-346

Fierer, N., Jackson, R.B. (2006) The diversity and biogeography of soil bacterial communities. Proceedings of the National Academy of Sciences of the United States of America 103:626-631

Fisher, M.C., Bosch, J., Yin, Z., Stead, D.A., Walker, J., Selway, L., Brown, A.J.P., Walker, L.A., Gow, N.A.R., Stajich, J.E., Garner, T.W.J. (2009) Proteomic and phenotypic profiling of the amphibian pathogen *Batrachochytrium dendrobatidis* shows that genotype is linked to virulence. Molecular Ecology 18:415-429

Fisher, M.C., Garner, T.W.J., Walker, S.F. (2009) Global emergence of *Batrachochytrium dendrobatidis* and amphibian chytridiomycosis in space, time, and host. Annual Review of Microbiology 63:291-310

Fisher, M.C., Henk, D.A., Briggs, C.J., Brownstein, J.S., Madoff, L.C., McCraw, S.L., Gurr, S.J. (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186-194

Fox, G.E., Wisotzkey, J.D., Jurtshuk, P. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. International Journal of Systematic Bacteriology 42:166-170

Fraune, S., Bosch, T.C.G. (2007) Long-term maintenance of species-specific bacterial microbiota in the basal metazoan *Hydra*. Proceedings of the National Academy of Sciences of the United States of America 104:13146-13151

Fravel, D.R. (1988) Role of antibiosis in the biocontrol of plant diseases. Annual Review of Phytopathology 26:75-91

Fric, P. (2007) Probiotics and prebiotics — renaissance of a therapeutic principle. Central European Journal of Medicine 2:237-270

Frith, C.B., Frith, D.W. (1985) Seasonality of insect abundance in an Australian upland tropical rainforest. Australian Journal of Ecology 10:237-248

Fuller, R. (1989) Probiotics in man and animals: A review. Journal of Applied Bacteriology 66:365-378

Gaboriau-Routhiau, V., Rakotobe, S., Lécuyer, E., Mulder, I., Lan, A., Bridonneau, C., Rochet, V., Pisi, A., De Paepe, M., Brandi, G., Eberl, G., Snel, J., Kelly, D., Cerf-Bensussan, N. (2009) The key role of segmented filamentous bacteria in the coordinated maturation of gut helper T cell responses. Immunity 31:677-689

Gagliardo, R., Crump, P., Griffith, E., Mendelson, J., Ross, H., Zippel, K. (2008) The principles of rapid response for amphibian conservation, using the programmes in Panama as an example. International Zoo Yearbook 42:125-135

Gajjar, Y., Mehta, C., Patel, N.G., Senchaudhuri, P. (1998) StatXact 4. Cytel Software. http://www.cytel.com

Gantz, J.D., Sheafor, B.A. (2012) Behavioral thermoregulation and its role in decreasing morbidity and mortality associated with chytridiomycosis. Integrative and Comparative Biology 52:E249-E249

Garmyn, A., Van Rooij, P., Pasmans, F., Hellebuyck, T., Van den Broeck, W., Haesebrouck, F., Martel, A. (2012) Waterfowl: Potential environmental reservoirs of the chytrid fungus *Batrachochytrium dendrobatidis*. PLoS ONE 7:e35038

Garner, T.W.J., Garcia, G., Carroll, B., Fisher, M.C. (2009) Using itraconazole to clear *Batrachochytrium dendrobatidis* infection, and subsequent depigmentation of *Alytes muletensis* tadpoles. Diseases of Aquatic Organisms 83:257-260

Geiger, C.C., Kupfer, E., Schar, S., Wolf, S., Schmidt, B.R. (2011) Elevated temperature clears chytrid fungus infections from tadpoles of the midwife toad, *Alytes obstetricans*. Amphibia-Reptilia 32:276-280

Gerber, N.N. (1975) Prodigiosin-like pigments. Critical Reviews in Microbiology 3:469-485

Gil-Turnes, M.S., Fenical, W. (1992) Embryos of *Homarus americanus* are protected by epibiotic bacteria. Biological Bulletin 182:105-108

Gil-Turnes, M.S., Hay, M.E., Fenical, W. (1989) Symbiotic marine-bacteria chemically defend crustacean embryos from a pathogenic fungus. Science 246:116-118

Graf, J., Ruby, E.G. (1998) Host-derived amino acids support the proliferation of symbiotic bacteria. Proceedings of the National Academy of Sciences of the United States of America 95:1818-1822

Gram, L., Lovold, T., Nielsen, J., Melchiorsen, J., Spanggaard, B. (2001) *In vitro* antagonism of the probiont *Pseudomonas fluorescens* strain AH2 against *Aeromonas salmonicida* does not confer protection of salmon against furunculosis. Aquaculture 199:1-11

Gram, L., Melchiorsen, J., Spanggaard, B., Huber, I., Nielsen, T.F. (1999) Inhibition of *Vibrio anguillarum* by *Pseudomonas fluorescens* AH2, a possible probiotic treatment of fish. Applied and Environmental Microbiology 65:969-973

Green, S.J., Leigh, M.B., Neufeld, J.D. (2009) Denaturing gradient gel electrophoresis (DGGE) for microbial community analysis. In: Timmis, K.N. (ed) Microbiology of Hydrocarbons, Oils, Lipids, and Derived Compounds. Springer, Heidelberg, Germany

Grice, E.A., Kong, H.H., Renaud, G., Young, A.C., Bouffard, G.G., Blakesley, R.W., Wolfsberg, T.G., Turner, M.L., Segre, J.A. (2008) A diversity profile of the human skin microbiota. Genome Research 18:1043-1050

Haas, D., Défago, G. (2005) Biological control of soil-borne pathogens by fluorescent pseudomonads. Nature Reviews Microbiology 3:307-319

Hacker, J., Carniel, E. (2001) Ecological fitness, genomic islands and bacterial pathogenicity. Embo Reports 2:376-376

Hamdache, A., Lamarti, A., Aleu, J., Collado, I.G. (2011) Non-peptide metabolites from the genus *Bacillus*. Journal of natural products 74:893-899

Hamilton, P.T., Richardson, J.M.L., Anholt, B.R. (2012) *Daphnia* in tadpole mesocosms: trophic links and interactions with *Batrachochytrium dendrobatidis*. Freshwater Biology 57:676-683

Hammer, Ø., Harper, D.A.T., Ryan, P.D. (2001) PAST: Paleontological Statistics Software Package for Education and Data Analysis. In: Palaeontologia Electronica http://folkuiono/ohammer/past/

Harris, R.N., Brucker, R.M., Walke, J.B., Becker, M.H., Schwantes, C.R., Flaherty, D.C., Lam, B.A., Woodhams, D.C., Briggs, C.J., Vredenburg, V.T., Minbiole, K.P.C. (2009a) Skin microbes on frogs prevent morbidity and mortality caused by a lethal skin fungus. The ISME Journal 3:818-824

Harris, R.N., James, T.Y., Lauer, A., Simon, M.A., Patel, A. (2006) Amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of Amphibian species. Ecohealth 3:53-56

Harris, R.N., Lauer, A., Simon, M.A., Banning, J.L., Alford, R.A. (2009b) Addition of antifungal skin bacteria to salamanders ameliorates the effects of chytridiomycosis. Diseases of Aquatic Organisms 83:11-16

Hashidoko, Y., Nakayama, T., Homma, Y., Tahara, S. (1999) Structure elucidation of xanthobaccin A, a new antibiotic produced from Stenotrophomonas sp. strain SB-K88. Tetrahedron letters 40:2957-2960

Hastings, J.W., Greenberg, E.P. (1999) Quorum sensing: the explanation of a curious phenomenon reveals a common characteristic of bacteria. Journal of Bacteriology 181:2667-2668

Hentschel, U., Dobrindt, U., Steinert, M. (2003) Commensal bacteria make a difference. Trends in Microbiology 11:148-150

Hooper, L.V. (2009) Do symbiotic bacteria subvert host immunity? Nature Reviews Microbiology 7:367-374

Hoskin, C.J., Higgie, M., McDonald, K.R., Moritz, C. (2005) Reinforcement drives rapid allopatric speciation. Nature 437:1353-1356

Imai, H., Suzuki, K., Miyazaki, S., Tanaka, K., Watanabe, S., Iwanami, M. (1983) A new antibiotic Y-T0678H produced by a *Chromobacterium* species. Journal of Antibiotics 36:911-912

Irianto, A., Austin, B. (2002) Probiotics in aquaculture. Journal of Fish Diseases 25:633-642

Ivanov, I.I., Atarashi, K., Manel, N., Brodie, E.L., Shima, T., Karaoz, U., Wei, D., Goldfarb, K.C., Santee, C.A., Lynch, S.V., Tanoue, T., Imaoka, A., Itoh, K., Takeda, K., Umesaki, Y., Honda, K., Littman, D.R. (2009) Induction of iIntestinal Th17 cells by segmented filamentous bacteria. Cell 139:485-498

Jackson, J.A., Tinsley, R.C. (2005) Geographic and within-population structure in variable resistance to parasite species and strains in a vertebrate host. International Journal for Parasitology 35:29-37

Jacques, M.-A., Morris, C.E. (1995) A review of issues related to the quantification of bacteria from the phyllosphere. Fems Microbiology Ecology 18:1-14

Jakobi, M., Winkelmann, G., Kaiser, D., Kempler, C., Jung, G., Berg, G., Bahl, H. (1996) Maltophilin: a new antifungal compound produced by Stenotrophomonas maltophilia R3089. The Journal of antibiotics 49:1101-1104

James, T.Y., Litvintseva, A.P., Vilgalys, R., Morgan, J.A.T., Taylor, J.W., Fisher, M.C., Berger, L., Weldon, C., du Preez, L., Longcore, J.E. (2009) Rapid global expansion of the fungal disease chytridiomycosis into declining and healthy amphibian populations. PLoS Pathogens 5:e1000458

Janda, J.M., Abbott, S.L. (2007) 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. Journal of Clinical Microbiology 45:2761-2764

Janse, I., Bok, J., Zwart, G. (2004) A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. Journal of Microbiological Methods 57:279-281

Jarchum, I., Pamer, E.G. (2011) Regulation of innate and adaptive immunity by the commensal microbiota. Current Opinion in Immunology 23:353-360

Jenkins, K.M., Jensen, P.R., Fenical, W. (1998) Bioassays with Marine Microorganisms. In: Haynes, K.F., Millar, J.G. (eds) Methods in Chemical Ecology: Bioassay Methods, Book 2. Kluwer Academic Publishers, Norwell

Jiao, Y., Yoshihara, T., Ishikuri, S., Uchino, H., Ichihara, A. (1996) Structural identification of cepaciamide A, a novel fungitoxic compound from *Pseudomonas cepacia* D-202. Tetrahedron letters 37:1039-1042

Johnson, M.L., Berger, L., Philips, L., Speare, R. (2003) Fungicidal effects of chemical disinfectants, UV light, desiccation and heat on the amphibian chytrid *Batrachochytrium dendrobatidis*. Diseases of Aquatic Organisms 57:255-260

Jones, K.E., Patel, N.G., Levy, M.A., Storeygard, A., Balk, D., Gittleman, J.L., Daszak, P. (2008) Global trends in emerging infectious diseases. Nature 451:990-U994

Kadivar, H., Stapleton, A. (2003) Ultraviolet radiation alters maize phyllosphere bacterial diversity. Microbial Ecology 45:353-361

Kaltenpoth, M., Göttler, W., Herzner, G., Strohm, E. (2005) Symbiotic bacteria protect wasp larvae from fungal infestation. Current Biology 15:475-479

Kang, Y., Carlson, R., Tharpe, W., Schell, M.A. (1998) Characterization of genes involved in biosynthesis of a novel antibiotic from *Burkholderia cepacia* BC11 and their role in biological control of *Rhizoctonia solani*. Applied and Environmental Microbiology 64:3939-3947

Kang, Y., Cheng, J., Mei, L., Hu, J., Piao, Z., Yin, S. (2010) Multiple copies of 16S rRNA gene affect the restriction patterns and DGGE profile revealed by analysis of genome database. Microbiology 79:655-662

Keesing, F., Holt, R.D., Ostfeld, R.S. (2006) Effects of species diversity on disease risk. Ecology Letters 9:485-498

Kennedy, P.G., Bruns, T.D. (2005) Priority effects determine the outcome of ectomycorrhizal competition between two Rhizopogon species colonizing Pinus muricata seedlings. New Phytologist 166:631-638

Kenyon, N. (2008) Variable susceptibility to an emerging infectious disease, chytridiomycosis, in anurans. Ph.D., James Cook University, Townsville

Kerr, J.R. (1996) Inhibition of growth of fungi pathogenic to man by Stenotrophomonas maltophilia. Journal of Medical Microbiology 45:380-382

Kilburn, V.L., Ibanez, R., Green, D.M. (2011) Reptiles as potential vectors and hosts of the amphibian pathogen *Batrachochytrium dendrobatidis* in Panama. Diseases of Aquatic Organisms 97:127-134

Kimbell, J.R., McFall-Ngai, M.J. (2003) The squid-*Vibrio* Symbioses: From demes to genes. Integrative and Comparative Biology 43:254-260

Kirshtein, J.D., Anderson, C.W., Wood, J.S., Longcore, J.E., Voytek, M.A. (2007) Quantitative PCR detection of *Batrachochytrium dendrobatidis* DNA from sediments and water. Diseases of Aquatic Organisms 77:11-15

Kleessen, B., Blaut, M. (2005) Modulation of gut mucosal biofilms. British Journal of Nutrition 93:S35-S40

Kleessen, B., Hartmann, L., Blaut, M. (2003) Fructans in the diet cause alterations of intestinal mucosal architecture, released mucins and mucosa-associated bifidobacteria in gnotobiotic rats. British Journal of Nutrition 89:597-606

Kloepper, J.W., Leong, J., Teintze, M., Schroth, M.N. (1980) *Pseudomonas* siderophores: A mechanism explaining disease-suppressive soils. Current Microbiology 4:317-320

Knappe, T.A., Linne, U., Zirah, S., Rebuffat, S., Xie, X., Marahiel, M.A. (2008) Isolation and structural characterization of capistruin, a lasso peptide predicted from the genome sequence of *Burkholderia thailandensis* E264. Journal of the American Chemical Society 130:11446-11454

Kong, H.H., Oh, J., Deming, C., Conlan, S., Grice, E.A., Beatson, M.A., Nomicos, E., Polley, E.C., Komarow, H.D., Murray, P.R., Turner, M.L., Segre, J.A. (2012) Temporal shifts in the skin microbiome associated with disease flares and treatment in children with atopic dermatitis. Genome Research 22:850-859

Konopka, A. (2009) What is microbial community ecology? The ISME Journal 3:1223-1230

Kriger, K.M., Hero, J.M. (2006) Survivorship in wild frogs infected with chytridiomycosis. Ecohealth 3:171-177

Kriger, K.M., Hero, J.M. (2007) Large-scale seasonal variation in the prevalence and severity of chytridiomycosis. Journal of Zoology 271:352-359

Kroiss, J., Kaltenpoth, M., Schneider, B., Schwinger, M.-G., Hertweck, C., Maddula, R.K., Strohm, E., Svatoš, A. (2010) Symbiotic streptomycetes provide antibiotic combination prophylaxis for wasp offspring. Nature Chemical Biology 6:261-263

Kunin, C.M., Edmondson, W.P. (1968) Inhibitor of Antibiotics in Bacteriologic Agar. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY) 129:118-122

Kusrini, M.D., Skerratt, L.F., Garland, S., Berger, L., Endarwin, W. (2008) Chytridiomycosis in frogs of Mount Gede Pangrango, Indonesia. Diseases of Aquatic Organisms 82:187-194

Lai, Y., Di Nardo, A., Nakatsuji, T., Leichtle, A., Yang, Y., Cogen, A.L., Wu, Z.-R., Hooper, L.V., Schmidt, R.R., von Aulock, S., Radek, K.A., Huang, C.-M., Ryan, A.F., Gallo, R.L. (2009) Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. Nature Medicine 15:1377-1382

Lam, B.A., Walke, J.B., Vredenburg, V.T., Harris, R.N. (2010) Proportion of individuals with anti-*Batrachochytrium dendrobatidis* skin bacteria is associated with population persistence in the frog *Rana muscosa*. Biological Conservation 143:529-531

Lane, D.J. (1991) 16S/23S rRNA sequencing. In: Stackebrandt, E., Goodfellow, M. (eds) Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, Chichester, United Kingdom

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23:2947-2948

Lategan, M.J., Booth, W., Shimmon, R., Gibson, L.F. (2006) An inhibitory substance produced by *Aeromonas media* A199, an aquatic probiotic. Aquaculture 254:115-124

Lauer, A., Simon, M.A., Banning, J.L., Andre, E., Duncan, K., Harris, R.N. (2007) Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. Copeia 3:630-640

Lauer, A., Simon, M.A., Banning, J.L., Lam, B.A., Harris, R.N. (2008) Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders. The ISME Journal 2:145-157

Laurance, W.F., McDonald, K.R., Speare, R. (1996) Epidemic disease and the catastrophic decline of Australian rain forest frogs. Conservation Biology 10:406-413

Lebaron, P., Ghiglione, J.-F., Fajon, C., Batailler, N., Normand, P. (1998) Phenotypic and genetic diversity within a colony morphotype. FEMS microbiology letters 160:137-143

Lee, Y.K., Puong, K.Y. (2002) Competition for adhesion between probiotics and human gastrointestinal pathogens in the presence of carbohydrate. British Journal of Nutrition 88 Suppl 1:S101-108

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S., Harbour, A. (1995) Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Microbiology 78:97-108

Li, W.J., Zhang, Y.Q., Park, D.J., Li, C.T., Xu, L.H., Kim, C.J., Jiang, C.L. (2004) *Duganella violaceinigra* sp. nov., a novel mesophilic bacterium isolated from forest soil. International journal of systematic and evolutionary microbiology 54:1811-1814

Lindgren, E., Andersson, Y., Suk, J.E., Sudre, B., Semenza, J.C. (2012) Monitoring EU emerging infectious disease risk due to climate change. Science 336:418-419

Lipson, D.A., Schadt, C.W., Schmidt, S.K. (2002) Changes in soil microbial community structure and function in an Alpine dry meadow following spring snow melt. Microbial Ecology 43:307-314

Liu, C.H., Chen, X., Liu, T.T., Lian, B., Gu, Y., Caer, V., Xue, Y.R., Wang, B.T. (2007a) Study of the antifungal activity of *Acinetobacter baumannii* LCH001 *in vitro* and identification of its antifungal components. Applied microbiology and biotechnology 76:459-466

Liu, X., Bimerew, M., Ma, Y., Müller, H., Ovadis, M., Eberl, L., Berg, G., Chernin, L. (2007b) Quorum sensing signaling is required for production of the antibiotic pyrrolnitrin in a rhizospheric biocontrol strain of *Serratia plymuthica*. FEMS microbiology letters 270:299-305

Liu, Y.S., Shyu, Y.T. (2006) Adulteration identification of citrus juice by denaturing gradient gel electrophoresis (DGGE). Journal of Food and Drug Analysis 14:44-49

Longcore, J.E., Pessier, A.P., Nichols, D.K. (1999) *Batrachochytrium dendrobatidis* gen. et sp. nov., a chytrid pathogenic to amphibians. Mycologia 91:219-227

Lubick, N. (2010) Ecology: Emergency medicine for frogs. Nature 465:680-681

Mahenthiralingam, E., Song, L., White, J., Wilmot, C., Sass, A., Marchbank, A., Challis, G.L. (2010) *Burkholderia ambifaria* produces a polyketide antibiotic that inhibits *Burkholderia multivorans* & other *Burkholderia cepacia* complex species. Pediatric Pulmonology S33:345-345

Makarasen, A., Yoza, K., Isobe, M. (2009) Higher structure of cereulide, an emetic toxin from *Bacillus cereus*, and special comparison with valinomycin, an antibiotic from *Streptomyces fulvissimus*. Chemistry–An Asian Journal 4:688-698

Margulies, M., Egholm, M., Altman, W.E., Attiya, S., Bader, J.S., Bemben, L.A., Berka, J., Braverman, M.S., Chen, Y.J., Chen, Z.T., Dewell, S.B., Du, L., Fierro, J.M., Gomes, X.V., Godwin, B.C., He, W., Helgesen, S., Ho, C.H., Irzyk, G.P., Jando, S.C., Alenquer, M.L.I., Jarvie, T.P., Jirage, K.B., Kim, J.B., Knight, J.R., Lanza, J.R., Leamon, J.H., Lefkowitz, S.M., Lei, M., Li, J., Lohman, K.L., Lu, H., Makhijani, V.B., McDade, K.E., McKenna, M.P., Myers, E.W., Nickerson, E., Nobile, J.R., Plant, R., Puc, B.P., Ronan, M.T., Roth, G.T., Sarkis, G.J., Simons, J.F., Simpson, J.W., Srinivasan, M., Tartaro, K.R., Tomasz, A., Vogt, K.A., Volkmer, G.A., Wang, S.H., Wang, Y., Weiner, M.P., Yu, P.G., Begley, R.F., Rothberg, J.M. (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437:376-380

Martel, A., Van Rooij, P., Vercauteren, G., Baert, K., Van Waeyenberghe, L., Debacker, P., Garner, T.W.J., Woeltjes, T., Ducatelle, R., Haesebrouck, F., Pasmans, F. (2011) Developing a safe antifungal treatment protocol to eliminate *Batrachochytrium dendrobatidis* from amphibians. Medical Mycology 49:143-149

Martín-Vivaldi, M., Peña, A., Peralta-Sánchez, J.M., Sánchez, L., Ananou, S., Ruiz-Rodríguez, M., Soler, J.J. (2010) Antimicrobial chemicals in hoopoe preen secretions are produced by symbiotic bacteria. Proceedings of the Royal Society B: Biological Sciences 277:123-130

Martin, J.F., Demain, A.L. (1980) Control of antibiotic biosynthesis. Microbiological Reviews 44:230-251

Matos, A., Kerkhof, L., Garland, J.L. (2005) Effects of microbial community diversity on the survival of *Pseudomonas aeruginosa* in the wheat rhizosphere. Microbial Ecology 49:257-264

May, S., Zeisset, I., Beebee, T.J.C. (2011) Larval fitness and immunogenetic diversity in chytrid-infected and uninfected natterjack toad (*Bufo calamita*) populations. Conservation Genetics 12:805-811

McDonald, K., Alford, R. (1999) A review of Declining Frogs in Northern Queensland. In: Campbell, A. (ed) Declines and disappearances of Australian frogs. Environment Australia

McFall-Ngai, M. (2006) Love the one you're with: Vertebrate guts shape their microbiota. Cell 127:247-249

McKenzie, V.J., Bowers, R.M., Fierer, N., Knight, R., Lauber, C.L. (2012) Co-habiting amphibian species harbor unique skin bacterial communities in wild populations. The ISME Journal 6:588-596

McMahon, T.A., Brannelly, L.A., Chatfield, M.W.H., Johnson, P.T.J., Joseph, M.B., McKenzie, V.J., Richards-Zawacki, C.L., Venesky, M.D., Rohr, J.R. (2013) Chytrid fungus

Batrachochytrium dendrobatidis has nonamphibian hosts and releases chemicals that cause pathology in the absence of infection. Proceedings of the National Academy of Sciences 110:210-215

Medzhitov, R. (2007) Recognition of microorganisms and activation of the immune response. Nature 449:819-826

Meyers, E., Bisacchi, G.S., Dean, L., Liu, W.C., Minassian, B., Slusarchyk, D.S., Sykes, R.B., Tanaka, S.K., Trejo, W. (1987) Xylocandin - A new complex of antifungal peptides. 1. Taxonomy, isolation and biological activity. Journal of Antibiotics 40:1515-1519

Michener, C.D., Sokal, R.R. (1957) A quantitative approach to a problem in classification. Evolution 11:130-162

Miller, M.B., Bassler, B.L. (2001) Quorum sensing in bacteria. Annual Review of Microbiology 55:165-199

Moon, S.S., Kang, P.M., Park, K.S., Kim, C.H. (1996) Plant growth promoting and fungicidal 4-quinolinones from *Pseudomonas cepacia*. Phytochemistry 42:365-368

Moran, M.D. (2003) Arguments for rejecting the sequential bonferroni in ecological studies. Oikos 100:403-405

Moran, N.A. (2006) Symbiosis. Current Biology 16:R866-R871

Moran, N.A., Telang, A. (1998) Bacteriocyte-associated symbionts of iInsects. Bioscience 48:295-304

Morehouse, E.A., James, T.Y., Ganley, A.R., Vilgalys, R., Berger, L., Murphy, P.J., Longcore, J.E. (2003) Multilocus sequence typing suggests the chytrid pathogen of amphibians is a recently emerged clone. Molecular Ecology 12:395-403

Morell, V. (1999) Are pathogens felling frogs? Science 284:728(721)

Mori, T., Yamashita, T., Furihata, K., Nagai, K., Suzuki, K., Hayakawa, Y., Shin-Ya, K. (2007) Burkholone, a new cytotoxic antibiotic against IGF-I dependent cells from *Burkholderia* sp. Journal of Antibiotics 60:713-716

Muirhead, R.W., Davies-Colley, R.J., Donnison, A.M., Nagels, J.W. (2004) Faecal bacteria yields in artificial flood events: quantifying in-stream stores. Water Research 38:1215-1224

Muletz, C.R., Myers, J.M., Domangue, R.J., Herrick, J.B., Harris, R.N. (2012) Soil bioaugmentation with amphibian cutaneous bacteria protects amphibian hosts from infection by *Batrachochytrium dendrobatidis*. Biological Conservation 152:119-126

Murphy, P.J., St-Hilaire, S., Corn, P.S. (2011) Temperature, hydric environment, and prior pathogen exposure alter the experimental severity of chytridiomycosis in boreal toads. Diseases of Aquatic Organisms 95:31-42

Murray, A.E., Hollibaugh, J.T., Orrego, C. (1996) Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Applied and Environmental Microbiology 62:2676-2680

Muyzer, G., de Waal, E.C., Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-

amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59:695-700

Muyzer, G., Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73:127-141

Myers, J.M., Ramsey, J.P., Blackman, A.L., Nichols, A.E., Minbiole, K.P.C., Harris, R.N. (2012) Synergistic inhibition of the lethal fungal pathogen *Batrachochytrium dendrobatidis*: The combined effect of symbiotic bacterial metabolites and antimicrobial peptides of the frog *Rana muscosa*. Journal of Chemical Ecology 38:958-965

Nadkarni, M.A., Martin, F.E., Jacques, N.A., Hunter, N. (2002) Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 148:257-266

Naik, S., Bouladoux, N., Wilhelm, C., Molloy, M.J., Salcedo, R., Kastenmuller, W., Deming, C., Quinones, M., Koo, L., Conlan, S., Spencer, S., Hall, J.A., Dzutsev, A., Kong, H., Campbell, D.J., Trinchieri, G., Segre, J.A., Belkaid, Y. (2012) Compartmentalized control of skin immunity by resident commensals. Science 337:1115-1119

Nakagawa, S. (2004) A farewell to Bonferroni: the problems of low statistical power and publication bias. Behavioral Ecology 15:1044-1045

Neilands, J.B. (1981) Microbial iron compounds. Annual Review of Biochemistry 50:715-731

Nissimov, J., Rosenberg, E., Munn, C.B. (2009) Antimicrobial properties of resident coral mucus bacteria of *Oculina patagonica*. FEMS microbiology letters 292:210-215

Olsen, G.J., Lane, D.J., Giovannoni, S.J., Pace, N.R., Stahl, D.A. (1986) Microbial ecology and evolution - A ribosomal-RNA approach. Annual Review of Microbiology 40:337-365

Orr, H.A. (2000) Adaptation and the cost of complexity. Evolution 54:13-20

Ownley, B.H., Weller, D.M., Thomashow, L.S. (1992) Influence of *in situ* and *in vitro* pH on suppression of *Gaeumannomyces graminis* var. *tritici* by *Psudomonas fluorescens* 2-79. Phytopathology 82:178-184

Paracer, S., Ahmadjian, V. (2000) Symbiosis: An Introduction to Biological Associations. Oxford University Press, New York

Parker, J.M., Mikaelian, I., Hahn, N., Diggs, H.E. (2002) Clinical diagnosis and treatment of epidermal chytridiomycosis in African clawed frogs (*Xenopus tropicalis*). Comparative Medicine 52:265-268

Parker, W.L., Rathnum, M.L., Johnson, J.H., Wells, J.S., Principe, P.A., Sykes, R.B. (1988) Aerocyanidin, a new antibiotic produced by *Chromobacterium violaceum*. Journal of Antibiotics 41:454-460

Parker, W.L., Rathnum, M.L., Seiner, V., Trejo, W.H., Principe, P.A., Sykes, R.B. (1984) Cepacin A and cepacin B, two new antibiotics produced by *Pseudomonas cepacia*. Journal of Antibiotics 37:431-440

Parker, W.L., Rathnum, M.L., Wells Jr, J.S., Trejo, W.H., Principe, P.A., Sykes, R.B. (1982) SQ 27, 860, a simple carbapenem produced by species of *Serratia* and *Erwinia*. Journal of Antibiotics 35:653-660

Pask, J.D., Woodhams, D.C., Rollins-Smith, L.A. (2012) The ebb and flow of antimicrobial skin peptides defends northern leopard frogs (*Rana pipiens*) against chytridiomycosis. Global Change Biology 18:1231-1238

Paulsen, I.T., Press, C.M., Ravel, J., Kobayashi, D.Y., Myers, G.S.A., Mavrodi, D.V., DeBoy, R.T., Seshadri, R., Ren, Q., Madupu, R., Dodson, R.J., Durkin, A.S., Brinkac, L.M., Daugherty, S.C., Sullivan, S.A., Rosovitz, M.J., Gwinn, M.L., Zhou, L., Schneider, D.J., Cartinhour, S.W., Nelson, W.C., Weidman, J., Watkins, K., Tran, K., Khouri, H., Pierson, E.A., Pierson, L.S., Thomashow, L.S., Loper, J.E. (2005) Complete genome sequence of the plant commensal *Pseudomonas fluorescens* Pf-5. Nature Biotechnology 23:873-878

Peay, K.G., Belisle, M., Fukami, T. (2012) Phylogenetic relatedness predicts priority effects in nectar yeast communities. Proceedings of the Royal Society B-Biological Sciences 279:749-758

Pessier, A.P. (2002) An overview of amphibian skin disease. Seminars in Avian and Exotic Pet Medicine 11:162-174

Pessier, A.P., Nichols, D.K., Longcore, J.E., Fuller, M.S. (1999) Cutaneous chytridiomycosis in poison dart frogs (*Dendrobates* spp.) and White's tree frogs (*Litoria caerulea*). Journal of Veterinary Diagnostic Investigation 11:194-199

Piotrowski, J.S., Annis, S.L., Longcore, J.E. (2004) Physiology of *Batrachochytrium dendrobatidis*, a chytrid pathogen of amphibians. Mycologia 96:9-15

Porat, R., Clark, B.D., et al. (1991) Enhancement of growth of virulent strains of *Escherichia coli* by Interleukin-1. Science 254:430-430

Potter, J.S., Norman, J.d. (2006) Veterinary care of captive Archey's frogs, *Leiopelma archeyi*, at Auckland Zoo. Kokako 13:19-26

Poulsen, M., Cafaro, M.J., Erhardt, D.P., Little, A.E.F., Gerardo, N.M., Tebbets, B., Klein, B.S., Currie, C.R. (2010) Variation in *Pseudonocardia* antibiotic defence helps govern parasite-induced morbidity in *Acromyrmex* leaf-cutting ants. Environmental Microbiology Reports 2:534-540

Pragash, M.G., Naik, P.R. (2009) Characterization of *Chryseobacterium aquaticum* strain PUPC1 producing a novel antifungal protease from rice rhizosphere soil. Journal of Microbiology and Biotechnology 19:99-107

Puschendorf, R. (2009) Environmental effects on a host-pathogen system: Frogs and *Batrachochytrium dendrobatidis* in wet and dry habitat. Ph.D., James Cook University, Townsville

Puschendorf, R., Hoskin, C.J., Cashins, S.D., McDonald, K., Skerratt, L.F., Vanderwal, J., Alford, R.A. (2011) Environmental refuge from disease-driven amphibian extinction. Conservation Biology 25:956-964

Raaijmakers, J.M., Vlami, M., de Souza, J.T. (2002) Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81:537-547

Raffel, T.R., Rohr, J.R., Kiesecker, J.M., Hudson, P.J. (2006) Negative effects of changing temperature on amphibian immunity under field conditions. Functional Ecology 20:819-828

Ramsey, J.P., Reinert, L.K., Harper, L.K., Woodhams, D.C., Rollins-Smith, L.A. (2010) Immune defenses against *Batrachochytrium dendrobatidis*, a fungus linked to global amphibian declines, in the South African clawed frog, *Xenopus laevis*. Infection and Immunity 78:3981-3992

Rawls, J.F., Mahowald, M.A., Ley, R.E., Gordon, J.I. (2006) Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell 127:423-433

Reeder, N.M.M., Pessier, A.P., Vredenburg, V.T. (2012) A reservoir species for the emerging amphibian pathogen *Batrachochytrium dendrobatidis* thrives in a landscape decimated by disease. PLoS ONE 7:e33567

Rendueles, O., Ferrières, L., Frétaud, M., Bégaud, E., Herbomel, P., Levraud, J.-P., Ghigo, J.-M. (2012) A new zebrafish model of oro-intestinal pathogen colonization reveals a key role for adhesion in protection by probiotic bacteria. Plos Pathogens 8:e1002815

Reshef, L., Koren, O., Loya, Y., Zilber-Rosenberg, I., Rosenberg, E. (2006) The Coral Probiotic Hypothesis. Environmental Microbiology 8:2068-2073

Retallick, R.W.R. (2002) Using experimental translocations to learn about declines in Queensland's frog populations. Implementation of Queensland's threatened frog recovery plans - Experimental Ecology. Project 6422, Scope 2001.06. Final Report to Environment Australia

Retallick, R.W.R., Miera, V. (2007) Strain differences in the amphibian chytrid *Batrachochytrium dendrobatidis* and non-permanent, sub-lethal effects of infection. Diseases of Aquatic Organisms 75:201-207

Ribas, L., Li, M.S., Doddington, B.J., Robert, J., Seidel, J.A., Kroll, J.S., Zimmerman, L.B., Grassly, N.C., Garner, T.W.J., Fisher, M.C. (2009) Expression profiling the temperature-dependent amphibian response to infection by *Batrachochytrium dendrobatidis*. PLoS ONE 4:e8408

Richards-Zawacki, C.L. (2010) Thermoregulatory behaviour affects prevalence of chytrid fungal infection in a wild population of Panamanian golden frogs. Proceedings of the Royal Society B-Biological Sciences 277:519-528

Richards, S.J., Alford, R.A. (2005) Structure and dynamics of a rainforest frog (*Litoria genimaculata*) population in northern Queensland. Australian Journal of Zoology 53:229-236

Richards, S.J., Hoskin, C.J., Cunningham, M.J., McDonald, K., Donnellan, S.C. (2010) Taxonomic re-assessment of the Australian and New Guinean green-eyed treefrogs *Litoria eucnemis*, *L. genimaculata* and *L. serrata* (Anura: Hylidae). Zootaxa 2391:33-46

Richards, S.J., McDonald, K.R., Alford, R.A. (1993) Declines in populations of Australia's endemic tropical rainforest frogs. Pacific Conservation Biology 1:66-77

Richmond, J.Q., Savage, A.E., Zamudio, K.R., Rosenblum, E.B. (2009) Toward immunogenetic studies of amphibian chytridiomycosis: Linking innate and acquired immunity. Bioscience 59:311-320

Ritchie, K.B. (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Marine Ecology Progress Series 322:1-14

Riveros, R., Haun, M., Campos, V., Durán, N. (1988) Bacterial Chemistry-IV. Complete characterization of violacein: An antibiotic and trypanocide pigment from *Chromobacterium violaceum*. Arquivos de Biologia e Technologia 31:475-487

Robertson, P.A.W., O'Dowd, C., Burrells, C., Williams, P., Austin, B. (2000) Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture 185:235-243

Robinson, J.V., Valentine, W.D. (1979) The concepts of elasticity, invulnerability and invadability. Journal of Theoretical Biology 81:91-104

Rohwer, F., Seguritan, V., Azam, F., Knowlton, N. (2002) Diversity and distribution of coral-associated bacteria. Marine Ecology Progress Series 243:1-10

Rollins-Smith, L.A. (2009) The role of amphibian antimicrobial peptides in protection of amphibians from pathogens linked to global amphibian declines. Biochimica et Biophysica Acta - Biomembranes 1788:1593-1599

Rollins-Smith, L.A., Carey, C., Longcore, J.E., Doersam, J.K., Reinert, L.K., Boutte, A., Bruzgal, J., Conlon, J.M. (2002a) Antimicrobial peptide defenses against *Batrachochytrium dendrobatidis*, the chytrid fungus associated with global amphibian declines. Faseb Journal 16:A291-A291

Rollins-Smith, L.A., Conlon, J.M. (2005) Antimicrobial peptide defenses against chytridiomycosis, an emerging infectious disease of amphibian populations. Developmental and Comparative Immunology 29:589-598

Rollins-Smith, L.A., Doersam, J.K., Longcore, J.E., Taylor, S.K., Shamblin, J.C., Carey, C., Zasloff, M.A. (2002b) Antimicrobial peptide defenses against pathogens associated with global amphibian declines. Developmental and Comparative Immunology 26:63-72

Rollins-Smith, L.A., Ramsey, J.P., Pask, J.D., Reinert, L.K., Woodhams, D.C. (2011) Amphibian immune defenses against chytridiomycosis: Impacts of changing environments. Integrative and Comparative Biology 51:552-562

Rollins-Smith, L.A., Ramsey, J.P., Reinert, L.K., Woodhams, D.C., Livo, L.J., Carey, C. (2009) Immune defenses of *Xenopus laevis* against *Batrachochytrium dendrobatidis*. Frontiers in Bioscience 1S:68-91

Rollins-Smith, L.A., Woodhams, D.C. (2011) Amphibian Immunity: Staying in Tune with the Environment. In: Demas, G.E., Nelson, R.J. (eds) Ecoimmunology. Oxford University Press, New York

Ron, S.R., Merino, A. (2000) Amphibian declines in Ecuador: Overview and first report of chytridiomycosis from South America. Froglog 42:2-3

Rosenberg, E., Sharon, G., Atad, I., Zilber-Rosenberg, I. (2010) The evolution of animals and plants via symbiosis with microorganisms. Environmental Microbiology Reports 2:500-506

Rosenberg, E., Zilber-Rosenberg, I. (2011) Symbiosis and development: The hologenome concept. Birth Defects Research (Part C) 93:56-66

Rosenblum, E.B., Poorten, T.J., Settles, M., Murdoch, G.K., Robert, J., Maddox, N., Eisen, M.B. (2009) Genome-wide transcriptional response of *Silurana* (*Xenopus*) *tropicalis* to infection with the deadly chytrid fungus. PLoS ONE 4:e6494

Roversi, P.F. (2008) Aerial spraying of *Bacillus thuringiensis* var. *kurstaki* for the control of *Thaumetopoea processionea* in turkey oak woods. Phytoparasitica 36:175-186

Rowley, J.J.L. (2006) Why does chytridiomycosis drive some frog populations to extinction and not others? The effects of interspecific variation in host behaviour. Ph.D., James Cook University, Townsville

Rowley, J.J.L., Alford, R.A. (2007a) Behaviour of Australian rainforest stream frogs may affect the transmission of chytridiomycosis. Diseases of Aquatic Organisms 77:1-9

Rowley, J.J.L., Alford, R.A. (2007b) Movement patterns and habitat use of rainforest stream frogs in northern Queensland, Australia: implications for extinction vulnerability. Wildlife Research 34:371-378

Rowley, J.J.L., Skerratt, L.F., Alford, R.A., Campbell, R. (2007) Retreat sites of rain forest stream frogs are not a reservoir for Batrachochytrium dendrobatidis in northern Queensland, Australia. Diseases of Aquatic Organisms 74:7-12

Roznik, E.A., Alford, R.A. (2012) Does waterproofing Thermochron iButton dataloggers influence temperature readings? Journal of Thermal Biology 37:260-264

Salle, A.J. (1961) Laboratory Manual on Fundamental Principles of Bacteriology. McGraw-Hill, London

Sánchez, O., Gasol, J.M., Massana, R., Mas, J., Pedrós-Alió, C. (2007) Comparison of different denaturing gradient gel electrophoresis primer sets for the study of marine bacterioplankton communities. Applied and Environmental Microbiology 73:5962-5967

Sánchez, S., Chavez, A., Forero, A., Garcia-Huante, Y., Romero, A., Sanchez, M., Rocha, D., Sanchez, B., Avalos, M., Guzman-Trampe, S., Rodriguez-Sanoja, R., Langley, E., Ruiz, B. (2010) Carbon source regulation of antibiotic production. Journal of Antibiotics 63:442-459

Sanchis, V., Bourguet, D. (2009) *Bacillus thuringiensis*: Applications in agriculture and insect resistance management - A review. In: Lichtfouse, E., Navarrete, M., Debaeke, P., Véronique, S., Alberola, C. (eds) Sustainable Agriculture. Springer Netherlands

Santoyo, G., Orozco-Mosqueda, M.d.C., Govindappa, M. (2012) Mechanisms of biocontrol and plant growth-promoting activity in soil bacterial species of *Bacillus* and *Pseudomonas*: a review. Biocontrol Science and Technology 22:855-872

Savage, A.E., Zamudio, K.R. (2011) MHC genotypes associate with resistance to a frog-killing fungus. Proceedings of the National Academy of Sciences 108:16705-16710

Schloegel, L.M., Ferreira, C.M., James, T.Y., Hipolito, M., Longcore, J.E., Hyatt, A.D., Yabsley, M., Martins, A., Mazzoni, R., Davies, A.J., Daszak, P. (2010) The North American bullfrog as a reservoir for the spread of *Batrachochytrium dendrobatidis* in Brazil. Animal Conservation 13:53-61

Schloegel, L.M., Toledo, L.F., Longcore, J.E., Greenspan, S.E., Vieira, C.A., Lee, M., Zhao, S., Wangen, C., Ferreira, C.M., Hipolito, M., Davies, A.J., Cuomo, C.A., Daszak, P., James, T.Y. (2012) Novel, panzootic and hybrid genotypes of amphibian chytridiomycosis associated with the bullfrog trade. Molecular Ecology 21:5162-5177

Schneider, C.J., Cunningham, M., Moritz, C. (1998) Comparative phylogeography and the history of endemic vertebrates in the Wet Tropics rainforests of Australia. Molecular Ecology 7:487-498

Schouten, A., van den Berg, G., Edel-Hermann, V., Steinberg, C., Gautheron, N., Alabouvette, C., de Vos, C.H., Lemanceau, P., Raaijmakers, J.M. (2004) Defense responses of *Fusarium oxysporum* to 2,4-diacetylphloroglucinol, a broad-spectrum antibiotic produced by *Pseudomonas fluorescens*. Molecular Plant-Microbe Interactions 17:1201-1211

Scott, J.J., Oh, D.C., Yuceer, M.C., Klepzig, K.D., Clardy, J., Currie, C.R. (2008) Bacterial protection of beetle-fungus mutualism. Science 322:63-63

Selvin, J., Shanmughapriya, S., Gandhimathi, R., Seghal Kiran, G., Rajeetha Ravji, T., Natarajaseenivasan, K., Hema, T.A. (2009) Optimization and production of novel antimicrobial agents from sponge associated marine actinomycetes *Nocardiopsis dassonvillei* MAD08. Applied microbiology and biotechnology 83:435-445

Seyedsayamdost, M.R., Chandler, J.R., Blodgett, J.A.V., Lima, P.S., Duerkop, B.A., Oinuma, K.I., Greenberg, E.P., Clardy, J. (2010) Quorum-sensing-regulated bactobolin production by *Burkholderia thailandensis* E264. Organic letters 12:716-719

Shanahan, P., Osullivan, D.J., Simpson, P., Glennon, J.D., Ogara, F. (1992) Isolation of 2,4-diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Applied and Environmental Microbiology 58:353-358

Sharma, R., Ranjan, R., Kapardar, R.J., Grover, A. (2005) 'Unculturable' bacterial diversity: An untapped resource. Current Science 89:72-77

Sharon, G., Rosenberg, E. (2008) Bacterial growth on coral mucus. Current Microbiology 56:481-488

Shen, S., Piao, F., Lee, B., Park, C. (2007) Characterization of antibiotic substance produced by *Serratia plymuthica* A21-4 and the biological control activity against pepper *Phytophthora* blight. PLANT PATHOLOGY JOURNAL 23:180-186

Shnit-Orland, M., Kushmaro, A. (2009) Coral mucus-associated bacteria: a possible first line of defense. Fems Microbiology Ecology 67:371-380

Shoji, J., Hinoo, H., Sakazaki, R., Kato, T., Hattori, T., Matsumoto, K., Tawara, K., Kikuchi, J., Terui, Y. (1989) Isolation of CB-25-I, an antifungal antibiotic, from *Serratia plymuthica*. Journal of Antibiotics 42:869-874

Singh, P.D., Liu, W.C., Gougoutas, J.Z., Malley, M.F., Porubcan, M.A., Trejo, W.H., Wells, J.S., Sykes, R.B. (1988) Aerocavin, a new antibiotic produced by *Chromobacterium violaceum*. Journal of Antibiotics 41:446-453

Skerratt, L.F., Berger, L., Speare, R., Cashins, S., McDonald, K.R., Phillott, A.D., Hines, H.B., Kenyon, N. (2007) Spread of chytridiomycosis has caused the rapid global decline and extinction of frogs. Ecohealth 4:125-134

Skerratt, L.F., McDonald, K.R., Hines, H.B., Berger, L., Mendez, D., Phillott, A.D., Cashins, S.D., Murray, K.A., Speare, R. (2010) Application of the survey protocol for chytridiomycosis to Queensland, Australia. Diseases of Aquatic Organisms 92:117-129

Smith, K.F., Acevedo-Whitehouse, K., Pedersen, A.B. (2009) The role of infectious diseases in biological conservation. Animal Conservation 12:1-12

Smith, K.F., Sax, D.F., Lafferty, K.D. (2006) Evidence for the role of infectious disease in species extinction and endangerment. Conservation Biology 20:1349-1357

Smith, P., Davey, S. (1993) Evidence for the competitive exclusion of *Aeromonas salmonicida* from fish with stress-inducible furunculosis by a fluorescent Psedomonad. Journal of Fish Diseases 16:521-524

Soler, J.J., Martín-Vivaldi, M., Ruiz-Rodríguez, M., Valdivia, E., Martín-Platero, A.M., Martínez-Bueno, M., Peralta-Sánchez, J.M., Méndez, M. (2008) Symbiotic association between hoopoes and antibiotic-producing bacteria that live in their uropygial gland. Functional Ecology 22:864-871

Southam, C.M., Ehrlich, J. (1943) Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. Phytopathology 33:517-524

Spanggaard, B., Huber, I., Nielsen, J., Sick, E.B., Pipper, C.B., Martinussen, T., Slierendrecht, W.J., Gram, L. (2001) The probiotic potential against vibriosis of the indigenous microflora of rainbow trout. Environmental Microbiology 3:755-765

Staley, J.T., Konopka, A. (1985) Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annual Review of Microbiology 39:321-346

Stebbing, A.R.D. (1982) Hormesis - The stimulation of growth by low levels of inhibitors. The Science of The Total Environment 22:213-234

Steinert, M., Hentschel, U., Hacker, J. (2000) Symbiosis and pathogenesis: Evolution of the microbe-host interaction. Naturwissenschaften 87:1-11

Stewart, E.J. (2012) Growing unculturable bacteria. Journal of Bacteriology

Stice, M.J., Briggs, C.J. (2010) Immunization is ineffective at preventing infection and mortality due to the amphibain chytrid fungus *Batrachochytrium dendrobatidis* Journal of Wildlife Diseases 46:70-77

Stockwell, M.P., Clulow, J., Mahony, M.J. (2012) Sodium chloride inhibits the growth and infective capacity of the amphibian chytrid fungus and increases host survival rates. PLoS ONE 7:e36942

Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L., Waller, R.W. (2004) Status and trends of amphibian declines and extinctions worldwide. Science 306:1783-1786

Sunagawa, S., DeSantis, T.Z., Piceno, Y.M., Brodie, E.L., DeSalvo, M.K., Voolstra, C.R., Weil, E., Andersen, G.L., Medina, M. (2009) Bacterial diversity and White Plague Disease-associated community changes in the Caribbean coral *Montastraea faveolata*. The ISME Journal 3:512-521

Suzuki, M., Rappe, M.S., Giovannoni, S.J. (1998) Kinetic bias in estimates of coastal picoplankton community structure obtained by measurements of small-subunit rRNA gene PCR amplicon length heterogeneity. Applied and Environmental Microbiology 64:4522-4529

Tagg, J.R., Dierksen, K.P. (2003) Bacterial replacement therapy: adapting "germ warfare" to infection prevention. Trends in Biotechnology 21:217-223

Thakur, N.L., Anil, A.C., Muller, W.E.G. (2004) Culturable epibacteria of the marine sponge *Ircinia fusca*: temporal variations and their possible role in the epibacterial defense of the host. Aquatic Microbial Ecology 37:295-304

Trenerry, M.P., Laurance, W.F., McDonald, K.R. (1994) Further evidence for the precipitous decline of endemic rainforest frogs in tropical Australia. Pacific Conservation Biology 1:150-153

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., Gordon, J.I. (2007) The Human Microbiome Project. Nature 449:804-810

Utagawa, T., Morisawa, H., Yamanaka, S., Yamazaki, A., Hirose, Y. (1986) Enzymatic synthesis of virazole by purine nucleoside phosphorylase of *Enterobacter aerogenes*. Agricultural and biological chemistry 50:121-126

van der Waaij, D., Vries, J.M.B.-d., Wees, J.E.C.L.-v.d. (1971) Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. The Journal of Hygiene 69:405-411

Vartoukian, S.R., Palmer, R.M., Wade, W.G. (2010) Strategies for culture of 'unculturable' bacteria. FEMS microbiology letters 309:1-7

Venesky, M.D., Wilcoxen, T.E., Rensel, M.A., Rollins-Smith, L., Kerby, J.L., Parris, M.J. (2012) Dietary protein restriction impairs growth, immunity, and disease resistance in southern leopard frog tadpoles. Oecologia 169:23-31

Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiology and Molecular Biology Reviews 64:655-671

von Wintzingerode, F., Göbel, U.B., Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. Fems Microbiology Reviews 21:213-229

Voyles, J. (2011) Phenotypic profiling of *Batrachochytrium dendrobatidis*, a lethal fungal pathogen of amphibians. Fungal Ecology 4:196-200

Voyles, J., Berger, L., Young, S., Speare, R., Webb, R., Warner, J., Rudd, D., Campbell, R., Skerratt, L.F. (2007) Electrolyte depletion and osmotic imbalance in amphibians with chytridiomycosis. Diseases of Aquatic Organisms 77:113-118

Voyles, J., Rosenblum, E.B., Berger, L. (2011) Interactions between *Batrachochytrium dendrobatidis* and its amphibian hosts: A review of pathogenesis and immunity. Microbes and Infection 13:25-32

Voyles, J., Young, S., Berger, L., Campbell, C., Voyles, W.F., Dinudom, A., Cook, D., Webb, R., Alford, R.A., Skerratt, L.F., Speare, R. (2009) Pathogenesis of chytridiomycosis, a cause of catastrophic amphibian declines. Science 326:582-585

Vredenburg, V.T., Briggs, C.J., Harris, R.N. (2011) Host pathogen dynamics of amphibian chytridiomycosis: The role of the skin microbiome in health and disease. In: Olsen, L., Choffnes, E., Relman, D., Pray, L. (eds) Fungal Diseases: An Emerging Threat to Human, Animal, and Plant Health. The National Academies Press, Washington D.C.

Wake, D.B., Vredenburg, V.T. (2008) Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences of the United States of America 105:11466-11473

Walke, J.B., Harris, R.N., Reinert, L.K., Rollins-Smith, L.A., Woodhams, D.C. (2011) Social immunity in amphibians: Evidence for vertical transmission of innate defenses. Biotropica 43:396-400

Walker, S.F., Salas, M.B., Jenkins, D., Garner, T.W.J., Cunningham, A.A., Hyatt, A.D., Bosch, J., Fisher, M.C. (2007) Environmental detection of *Batrachochytrium dendrobatidis* in a temperate climate. Diseases of Aquatic Organisms 77:105-112

Walters, R.L. (2007) Antibiotic production by cutaneous bacteria isolated from two species of salamanders. M.Sc., James Madison University, Harrisonburg

Waters, C.M., Bassler, B.L. (2005) Quorum sensing: Cell-to-cell communication in bacteria. Annual Review of Cell and Developmental Biology 21:319-346

Weller, D.M. (2007) *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. Phytopathology 97:250-256

Whitaker, R.J., Banfield, J.F. (2006) Population genomics in natural microbial communities. Trends in Ecology & Evolution 21:508-516

Wiggins, P.J., Smith, J.M., Harris, R.N., Minbiole, K.P.C. (2011) Gut of red-backed salamanders (*Plethodon cinereus*) may serve as a reservoir for an antifungal cutaneous bacterium. Journal of Herpetology 45:329-332

Williams, S.T., Vickers, J.C. (1986) The ecology of antibiotic production. Microbial Ecology 12:43-52

Wilson, B., Aeby, G.S., Work, T.M., Bourne, D.G. (2012) Bacterial communities associated with healthy and *Acropora* white syndrome-affected corals from American Samoa. FEMS Microbiology Ecology 80:509-520

Wittebolle, L., Marzorati, M., Clement, L., Balloi, A., Daffonchio, D., Heylen, K., De Vos, P., Verstraete, W., Boon, N. (2009) Initial community evenness favours functionality under selective stress. Nature 458:623-626

Wold, A.E. (2001) Immune effects of probiotics. Scandinavian Journal of Nutrition 45:76-85

Wolda, H. (1980) Seasonality of tropical insects. Journal of Animal Ecology 49:277-290

Woodhams, D.C. (2003) The ecology of chytridiomycosis, an emerging infectious disease of Australian rainforest frogs. Ph.D., James Cook University, Townsville

Woodhams, D.C., Alford, R.A. (2005) Ecology of chytridiomycosis in rainforest stream frog assemblages of tropical Queensland. Conservation Biology 19:1449-1459

Woodhams, D.C., Alford, R.A., Briggs, C.J., Johnson, M., Rollins-Smith, L.A. (2008a) Life-history trade-offs influence disease in changing climates: Strategies of an amphibian pathogen. Ecology 89:1627-1639

Woodhams, D.C., Alford, R.A., Marantelli, G. (2003) Emerging disease of amphibians cured by elevated body temperature. Diseases of Aquatic Organisms 55:65-67

Woodhams, D.C., Ardipradja, K., Alford, R.A., Marantelli, G., Reinert, L.K., Rollins-Smith, L.A. (2007a) Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. Animal Conservation 10:409-417

Woodhams, D.C., Bosch, J., Briggs, C.J., Cashins, S., Davis, L.R., Lauer, A., Muths, E., Puschendorf, R., Schmidt, B.R., Sheafor, B., Voyles, J. (2011) Mitigating amphibian disease: strategies to maintain wild populations and control chytridiomycosis. Frontiers in Zoology 8:8

Woodhams, D.C., Hyatt, A.D., Boyle, D.G., Rollins-Smith, L.A. (2008b) The northern leopard frog *Rana pipiens* is a widespread reservoir species harboring *Batrachochytrium dendrobatidis* in North America. Herpetological Review 39:66-68

Woodhams, D.C., Kenyon, N., Bell, S.C., Alford, R.A., Chen, S., Billheimer, D., Shyr, Y., Rollins-Smith, L.A. (2010) Adaptations of skin peptide defences and possible response to the amphibian chytrid fungus in populations of Australian green-eyed treefrogs, *Litoria genimaculata*. Diversity and Distributions 16:703-712

Woodhams, D.C., Rollins-Smith, L.A., Alford, R.A., Simon, M.A., Harris, R.N. (2007b) Innate immune defenses of amphibian skin: Antimicrobial peptides and more. Animal Conservation 10:425-428

Woodhams, D.C., Rollins-Smith, L.A., Carey, C., Reinert, L., Tyler, M.J., Alford, R.A. (2006a) Population trends associated with skin peptide defenses against chytridiomycosis in Australian frogs. Oecologia 146:531-540

Woodhams, D.C., Voyles, J., Lips, K.R., Carey, C., Rollins-Smith, L.A. (2006b) Predicted disease susceptibility in a panamanian amphibian assemblage based on skin peptide defenses. Journal of Wildlife Diseases 42:207-218

Woodhams, D.C., Vredenburg, V.T., Simon, M.A., Billheimer, D., Shakhtour, B., Shyr, Y., Briggs, C.J., Rollins-Smith, L.A., Harris, R.N. (2007c) Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. Biological Conservation 138:390-398

Yannarell, A.C., Kent, A.D., Lauster, G.H., Kratz, T.K., Triplett, E.W. (2003) Temporal patterns in bacterial communities in three temperate lakes of different trophic status. Microbial Ecology 46:391-405

Yim, G., Huimi Wang, H., Davies, J. (2006) The truth about antibiotics. International Journal of Medical Microbiology 296:163-170

Youssef, N., Sheik, C.S., Krumholz, L.R., Najar, F.Z., Roe, B.A., Elshahed, M.S. (2009) Comparison of species richness estimates obtained using nearly complete fragments and simulated pyrosequencing-generated fragments in 16S rRNA gene-based environmental surveys. Applied and Environmental Microbiology 75:5227-5236

Yuan, W.M., Crawford, D.L. (1995) Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. Applied and Environmental Microbiology 61:3119-3128

Zhou, X., Bent, S.J., Schneider, M.G., Davis, C.C., Islam, M.R., Forney, L.J. (2004) Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology 150:2565-2573

Zilber-Rosenberg, I., Rosenberg, E. (2008) Role of microorganisms in the evolution of animals and plants: the hologenome theory of evolution. Fems Microbiology Reviews 32:723-735

Zippel, K., Johnson, K., Gagliardo, R., Gibson, R., McFadden, M., Browne, R., Martinez, C., Townsend, E. (2011) The amphibian ark: A global community for *ex situ* conservation of amphibians. Herpetological Conservation and Biology 6:340-352

Zoetendal, E.G., Akkermans, A.D.L., De Vos, W.M. (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. Applied and Environmental Microbiology 64:3854-3859

APPENDIX A: ANTIMICROBIAL COMPOUNDS PRODUCED BY *BD*-INHIBITORY BACTERIAL GENERA IDENTIFIED IN THIS STUDY

Taxonomy	Antimicrobial compounds produced by genus	Reference
Bacteriodetes: Bacillaceae		
Bacillus	Cereulide	Makarasen et al. (2009)
	23 polyketides, 4 terpenoids, 6 isocoumarins, Zwittermycin A, Kanosamine, 3,3'-neotrehalosadiamine, Azoxybacilin, Bacisubin, Cispentacin, Bacilysocin, Lipoamides A, B and C	See review by Hamdache et al. (2011)
Firmicutes: Flavobacteriaceae		
Chryseobacterium	Unnamed antifungal protease	Pragash and Naik (2009)
Proteobacteria		
Betaproteobacteria		
Burkholderiaceae		
Burkholderia	Unnamed polyketide antibiotic	Mahenthiralingam et al. (2010)
	Xylocandin	Meyers et al. (1987)
	Bactobolin	Seyedsayamdost et al. (2010)
	Capistruin	Knappe et al. (2008)
	Phenazine	Cartwright et al. (1995)
	4-quinolinones	Moon et al. (1996)
	Banegasine, Maculosin	Cain et al. (2003)
	Cepaciamide	Jiao et al. (1996)
	Cepacins A & B	Parker et al. (1984)
	Burkholone	Mori et al. (2007)
	AFC-BC11 lipopeptide	Kang et al. (1998)
	Pyrrolnitrin	Burkhead et al. (1994)
Neisseriaceae		
Chromobacterium	Violacein	Riveros et al. (1988)
	Aerocavin	Singh et al. (1988)
	Aerocyanidin	Parker et al. (1988)
	Y-T0678H	Imai et al. (1983)
	Tetrabromopyrrole, 2-(2'-hydroxy-3',5'-dibromophenyl)-3,4,5-tribromopyrrole and 4-hydroxybenzaldehyde	Andersen et al. (1974)
Oxalobacteraceae		
Duganella	Violacein	Li et al. (2004)
Gammaproteobacteria		
Aeromonadaceae		
Aeromonas	Indole (2,3 benzopyrrole)	Lategan et al. (2006)
Enterobacteriaceae		
Enterobacter	Virazole	Utagawa et al. (1986)
	Pyrrolnitrin	Chernin et al. (1996)
Serratia	Macrocyclic lactone A21-4	Shen et al. (2007)
	Prodigiosin	Gerber (1975)
	Carbapenem	Parker et al. (1982)
	Pyrrolnitrin	Liu et al. (2007b)
	CB-25-1	Shoji et al. (1989)
Moraxellaceae		
Acinetobacter	Iturin A2, A3, and A6	Liu et al. (2007a)
Pseudomonadaceae		
Pseudomonas	Phenazines, Phloroglucinols, Pyoluteorin, Pyrrolnitrin, cyclic Lipopeptides and hydrogen cyanide	See review by Haas and Defago (2005)
	N-BBS, DDR, Butyrolactones, Oomycin A	See review by Raiijmakers et al. (2002)
Xanthomonadaceae		
Stenotrophomonas	Xanthobaccin A	Hashidoko et al. (1999)
	Maltophilin	Jakobi et al. (1996)
	Unnamed antifungal, possibly Pyrrolnitrin	Kerr (1996)