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ORIGINAL ARTICLE

Diversity of cutaneous bacteria with antifungal activity isolated from female four-toed salamanders

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Among the microbiota of amphibian skin are bacteria that produce antifungal compounds. We isolated cutaneous bacteria from the skins of three populations of the nest-attending plethodontid salamander *Hemidactylium scutatum* and subsequently tested the bacterial isolates against two different fungi (related to *Mariannaea elegans* and *Rhizomucor variabilis*) that were obtained from dead salamander eggs. The culturable antifungal bacteria were phylogenetically characterized based on 16S rRNA phylogeny, and belonged to four phyla, comprising 14 bacterial families, 16 genera and 48 species. We found that about half of the antifungal bacterial genera and families were shared with a related salamander species, but there was virtually no overlap at the species level. The proportion of culturable antifungal bacterial taxa shared between two large populations of *H. scutatum* was the same as the proportion of taxa shared between *H. scutatum* and *Plethodon cinereus*, suggesting that populations within a species have unique antifungal bacterial species. Approximately 30% of individuals from both salamander species carried anti-*M. elegans* cutaneous bacteria and almost 90% of *P. cinereus* and 100% of *H. scutatum* salamanders carried anti-*R. variabilis* cutaneous bacteria. A culture independent method (PCR/DGGE) revealed a shared resident bacterial community of about 25% of the entire resident bacterial community within and among populations of *H. scutatum*. Thus, the culturable antifungal microbiota was far more variable on salamander skins than was the bacterial microbiota detected by PCR/DGGE. The resident cutaneous antifungal bacteria may play an important role in amphibians' innate defense against pathogens, including the lethal chytrid fungus *Batrachochytrium dendrobatidis*.

Introduction

Up to 90% of the cells in and on vertebrate organisms are bacteria, and animals may play host to thousands of different bacterial species. Bacteria are increasingly viewed as mutualists that are often essential for the survival of their host (McFall Ngai et al., 2005). Symbioses of microorganisms with invertebrate and vertebrate species have been studied in regard to diversity of microbial symbionts, host specificity of these symbionts, and details about beneficial effects of these symbioses have been elucidated (for example, Currie, 2001; Douglas, 2003; Nyholm and McFall-Ngai, 2004; van Oppen and Gates, 2006). Living in moist or aquatic environments, amphibians are exposed to microorganisms through contact with soil, water, plants

and each other. Indeed, amphibian skin hosts a diverse microbiota that reflects a subset of the microbiota from their habitat (Austin, 2000; Culp et al., 2007; Lauer et al., 2007; Woodhams et al., 2007). In this study, we compare culture and culture-independent assays of the cutaneous microbiota of three populations of the plethodontid salamander, *Hemidactylium scutatum*.

Salamanders, frogs and caecilians have moist skin continuously supplied with mucus, secreted through glands, to prevent desiccation and to promote efficient oxygen uptake through their skin. Cutaneous respiration is especially important for lungless amphibians such as the plethodontid salamanders (Feder and Burggren, 1992) that are the focus of this study. In addition to moisture retention, a layer of mucus plays a role in thermoregulation, reproduction and defense against microbial pathogens. Mucous glands are typically small and widely distributed over the body and produce an acidic secretion containing carbohydrates (Fontana et al., 2006). Substances in the mucus can attract bacteria and fungi, which can be a food

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source for these microorganisms (Ducklow and Michell, 1979; Duellman and Trueb, 1986; Brizzi et al., 2002).

Antimicrobial peptide secretions from amphibian granular glands, as an innate defense mechanism, can limit microbial growth on the skin and prevent pathogenic microbial infections. In general, antimicrobial peptides vary among species in diversity and activity (Simmaco et al., 1998; Rollins-Smith et al., 2005; Woodhams et al., 2006). Therefore, bacteria that live on salamander skin as residents are expected to have coevolved with the amphibian species and developed mechanisms of protection against these antimicrobial peptides. In addition, these bacteria are expected to be able to produce antimicrobial metabolites against competing microbial species. Amphibians, as their hosts, might benefit from the ability of an epibiotic bacterium to produce such antimicrobial compounds in case of an infection with a pathogenic microorganism, such as a pathogenic fungus. Therefore, the production of antifungal compounds from members of the amphibians' cutaneous microbiota can become an additional pathway for the amphibians' innate protection against pathogenic microorganisms.

Among the known potential fungal pathogens that amphibians encounter in natural environments are the embryo pathogens Cunninghamella echinulata (Austin, 2000) and Saprolegnia ferax (Kiesecker et al., 2001) as well as the lethal fungal pathogens of adults such as Basdiobolus ranarum (Nickerson and Hutchison, 1971; Muths et al., 2003), Batrachochytrium dendrobatidis (Longcore et al., 1999) and Amphibiocystidium ranae (Pereira et al., 2005). When amphibian embryos of species that attend their nests are deserted, embryos often succumb to fungal infections (Forester, 1979; Harris et al., 1995). Fungi may develop preferentially on nonviable or spoiled eggs (Villa, 1979), but in some cases fungi are pathogenic and kill healthy eggs (Villa, 1979; Warkentin et al., 2001; JL Banning et al., unpublished data).

One function of nest attendance in species that brood their eggs is prevention of pathogenic fungal infection in embryos (Forester, 1979; Ng and Wilbur, 1995; Austin, 2000). It has been observed that female salamanders weave among their embryos (Zehnder, 2002). This behavior would allow the transmission of antibiotics produced by the cutaneous microbiota and amphibian granular gland secretions (Simmaco et al., 1998; Rollins-Smith et al., 2002, 2005) to the embryos by the brooding salamander. The presence of antifungal skin bacteria on females at the nest limits the spread of pathogenic fungi in H. scutatum (JL Banning et al., unpublished data).

In previous work, antifungal bacterial species have been isolated and identified from the skin of the salamanders Salamandra salamandra (Bettin and Greven, 1986), Plethodon ventralis (Austin, 2000; Culp et al., 2007) and Plethodon cinereus (Harris et al., 2006; Lauer et al., 2007), as well as

from the frog species Rana muscosa (Woodhams et al., 2007). Some antifungal bacteria or their metabolites are used in marine and freshwater aquaculture to reduce the susceptibility of the animals toward microbial pathogens including fungi (Gil-Turnes and Fenical, 1992; Verschuere et al., 2000; Irianto and Austin, 2002). A cutaneous microbiota that includes antifungal species may be important for the health of adult and embryonic amphibians.

The objectives of this study are to (i) characterize and compare the phylogenetic diversity of the culturable cutaneous antifungal bacteria of three populations of H. scutatum based on 16S rRNA fragments ($\sim$1400 bp), (ii) characterize the proportion of individual salamanders of H. scutatum that carry culturable cutaneous antifungal bacteria, (iii) use a culture-independent method (PCR/DGGE) to characterize the bacterial microbiota of H. scutatum and (iv) compare the culturable cutaneous antifungal microbiota of H. scutatum to a related species, P. cinereus. The potential role of the cutaneous antifungal bacteria in protecting salamander eggs from infections through pathogenic fungi is interpreted in regard to the nesting biology of the two salamander species.

Materials and methods

Study species
H. scutatum is a plethodontid salamander with nest attendance behavior by females. H. scutatum is abundant in the Eastern United States where bogs or ponds are available in or near damp wooded habitats. Females nest by themselves in solitary nests or with other females in joint nests. The forming of joint nests is rare among plethodontid salamanders. Females frequently desert their eggs in joint nests, leaving only one female to brood the eggs (Blanchard, 1934; Harris et al., 1995). Females that stay at the nest provide parental care, such as aeration, moistening and cleaning of the embryos and weave among them (Forester, 1979). The female lays her eggs in spring, in a clump of moss, leaf litter or rotting wood overhanging a pond or seepage and attends them for 30–60 days.

Sampling and sampling site
At three ponds (human constructed rainwater catches) in the George Washington Forest, Virginia, USA (Pond Ridge (PR), Cline's Hacking (CH) and White Oak Flat (WOF)), 87 female H. scutatum salamanders were captured from April to June 2005. The salamanders were either found in joint nests, in solitary nests where eggs had already been laid, or were found roaming close to the brooding habitat presumably searching for a nest site or a nest to join. Forty-eight nesting salamanders from Pond Ridge, 28 salamanders from Cline's Hacking and 12

salamanders from White Oak Flat were swabbed for the isolation of bacteria and for PCR/DGGE purposes. Taken together, we found 24 joint nesting salamanders, 43 solitary nesting salamanders and 20 'roaming' salamanders.

Rinsing and swabbing salamanders
To efficiently remove transient bacteria from the skin, all salamanders were rinsed twice with sterile dechlorinated water before swabbing their ventral and lateral sides following the protocol described in Lauer et al. (2007). It is possible that some of the resident bacteria were also lost by rinsing, but we assume that far more transient species were rinsed off than resident species.

The efficiency of removing transient bacteria from the salamander skin was confirmed by DGGE of 'PCR amplified' 16S rRNA gene fragments from rinsing solution and salamander skin swabs after the rinsing process for three randomly chosen individual salamanders (data not shown) and is consistent with the removal of transient bacteria demonstrated in Lauer et al. (2007) for the cutaneous bacteria of the related salamander P. cinereus.

Isolation of bacteria and fungi
Bacteria and fungi were isolated on a low nutrient medium (R2A) following the protocol described in Lauer et al. (2007). Pure cultures were frozen at $-80\,^\circ$C in TSYE-medium (2% trypticase soy broth, and 1% yeast extract (Difco, Detroit, MI, USA)) with 20% glycerol. Fungi were isolated in pure culture from dead salamander eggs of H. scutatum on R2A medium, incubated at room temperature for 1 week. Because of our limited isolation procedure with only one type of culture medium, we are aware that we cannot represent here the entire resident bacterial community with antifungal properties. Therefore, our results are giving a not entirely complete picture of the cutaneous antifungal bacterial community.

Challenge assays
The two fungi used in this study were identified by 18S rRNA gene sequencing with primer pair SSU 817 and SSU 1536 (Borneman and Hartin, 2000). One fungus was closely related (99%) to GenBank database entries of an uncultured aposymbiotic ascomycete fungus found in pea aphids (AB074659), to Mariannaea elegans var. punicea (AB111493), and to M. camptospora (AB112029). The second fungus was 99% related to Rhizomucor variabilis (AF113425). In a laboratory test, where healthy H. scutatum embryos were exposed to both fungi, M. elegans was able to kill the embryos, whereas R. variabilis was not (JL Banning et al., unpublished data). All bacterial isolates were examined for their activity against M. elegans and

R. variabilis. In the challenge assays, two bacterial isolates were streaked in a thin line on an R2A-plate with a piece of fungus ($\sim$1 cm^2) in the middle of each plate. The plates were incubated for 2 weeks at room temperature, then scored and photographed. Challenge assays of all isolates were performed as described in Lauer et al. (2007).

The challenge assays were scored as follows:

(1) Strongly antifungal: an inhibition zone appeared between fungi and bacteria. The fungus was unable to overgrow the bacteria.
(2) Weakly antifungal: the fungal density of the hyphae was lower toward the bacterial area, whereas all bacteria free space on the plate was covered with a thick fungal carpet. The fungal growth was also slowed down considerably.
(3) Not antifungal: the whole plate was evenly covered with fungal hyphae.

All challenge assays were unambiguously placed into one of these categories. Isolates that did not show any antifungal activity against at least one of the fungi were not identified.

DNA-extraction of salamander swabs
DNA from cotton swabs was freeze-thawed twice (70 $^\circ$C and $-80\,^\circ$C; 10 min each) and extracted with the Qiagen QIAamp DNA Micro Kit (Germantown, MD, USA) according to the manufacturer's protocol. DNA from bacterial and fungal pure cultures was extracted using the protocol of the MoBio Microbial Ultra Clean DNA KIT (Carlsbad, CA, USA).

Amplification of 16S rRNA genes
DNA obtained from salamander swabs was amplified with bacterial specific 16S rRNA primers 357F (5′-GCclamp-CCTACGGGAGGCAGCAG-3′) and 907R (5′-CCGTCAATTCMTTTGACTTT-3′) (Muyzer and Smalla, 1998) designed to amplify the V4 and V5 region. The 50 μl PCR contained 0.2 μM of each primer, 0.2 mM dNTPs, 2.5 U Taq polymerase with 1 × buffer (1.5 mM MgCl$_2$, 50 mM KCl, 10 mM Tris; Fisher Scientific, Pittsburg, PA, USA) and were amplified according to Toffin et al. (2004). DNA from bacterial isolates was amplified with the bacteria specific primer 8F (5′-AGAGTTTGA TCCTGGCTCAG-3′) and the universal primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Lane, 1991). The 25 μl PCR contained 0.2 μM of each primer, 0.5 μg ml^{-1} BSA, 3.0 mM MgCl$_2$, 0.2 mM dNTPs, 2.5 U of Taq polymerase with 1 × buffer. The thermocycling parameters were as follows: 94 $^\circ$C for 4 min followed by 35 cycles of 94 $^\circ$C for 1 min, 48 $^\circ$C for 1 min, 72 $^\circ$C for 1.5 min and a final elongation for 10 min at 72 $^\circ$C. All amplification products were checked by electrophoresis in 2% agarose and ethidium bromide (0.0001%) staining. DNA from fungal isolates was amplified using the protocol described in Borneman and Hartin (2000).

Table 1 Antifungal activity of bacterial species from *H. scutatum*.

No. of H. scutatum on which the bacterial species was found	Phylogeny/closest matches in GenBank, accession no.	Mariannaea sp.			Rhizomucor sp.		
		N	WP	P	N	WP	P
	Bacteroidetes						
	Flexibacteriaceae						
1	Uncultured bacterium clone related to *Kaistomonas* sp., AF502211, AB245370	1	0	0	0	0	1
	Sphingobacteriaceae						
2	*Flavobacterium* sp., DQ339585	2	0	0	0	0	2
2	*Flavobacterium* sp., AY337599	2	0	0	0	0	2
7	***Pedobacter caeni, AJ786798***	1	0	6	0	1	6
1	*Pedobacter* sp., AM116730	1	0	0	0	0	1
1	*Pedobacter* sp., AF408950	0	0	1	0	0	1
4	***Pedobacter* sp., AK583425**	0	1	3	0	0	4
1	*Pedobacter koreensis*, DQ092871	1	0	0	0	0	1
	Flavobacteriaceae						
1	*Chryseobacterium* sp., AY345551	1	0	0	0	0	1
1	*Chryseobacterium* sp., AY468446	1	0	0	0	0	1
2	*Chryseobacterium* sp., AY599654	2	0	0	0	0	2
1	*Flavobacterium* sp., AF336355	1	0	0	0	0	1
1	*Flavobacterium* sp., AM177633	1	0	0	0	0	1
1	*Flavobacterium hydatis*, AM230487	1	0	0	0	0	1
1	*Flavobacterium* sp., AM177621	1	0	0	0	0	1
1	*Flavobacterium* sp., AM177627	1	0	0	0	0	1
2	*Flavobacterium* sp., DQ177492	2	0	0	0	0	2
4	*Flavobacterium* sp., AF493666	4	0	0	0	0	4
1	*Flavobacterium* sp., AF493639	1	0	0	0	0	1
1	*Flavobacterium* sp., AM177619	1	0	0	0	0	1
	Firmicutes						
	Paenibacillaceae						
1	***Paenibacillus* sp., AB213458**	0	0	1	0	0	1
1	Bacillaceae						
4	*Bacillus cereus*, DQ289987	2	2	0	0	2	2
	Actinobacteria						
	Streptomycetaceae						
1	***Streptomyces* sp., AJ536294**	0	0	1	0	0	1
1	*Streptomyces griseochromogenes* subsp. *suitaensis*, AB184473	0	1	0	0	0	1
1	*Streptomyces laceyi*, AY094367	1	0	0	0	1	0
1	*Streptomyces purpeofuscus*, AJ781264	0	1	0	0	1	0
2	*Agromyces* sp., DQ232617	1	1	0	0	1	1
1	*Microbacterium* sp., AF408999	0	0	0	0	0	1
	Intrasporangiaceae						
1	*Nostocoida aromativora*, AY682381	0	1	0	0	1	0
	Micrococcaceae						
1	*Arthrobacter* sp., AY452081	2	1	0	0	2	1
1	*Arthrobacter* sp., AF409000	1	0	0	0	0	1
1	*Arthobacter* sp., AJ785760	0	1	0	0	1	0
	Proteobacteria						
	Oxalobacteraceae						
13	*Janthinobacterium lividum*, Y08846	13	1	0	2	8	4
3	β-proteobacterium, AY345557	3	0	0	0	1	2
2	β-proteobacterium, AY345557	2	0	0	0	0	2
2	β-proteobacterium, AF529111	2	0	0	0	1	1
2	β-proteobacterium, AB294320	2	0	0	0	0	2
3	*Janthinobacterium agaricidamnosum*, AY167838	2	1	0	0	0	3
	Comamonadaceae						
1	*Variovorax* sp., AB098595	1	0	0	0	1	0
	Sphingomonadaceae						
1	*Sphingomonas* sp., DQ469213	1	0	0	0	0	1

Table 1 Continued

No. of H. scutatum on which the bacterial species was found	Phylogeny/closest matches in GenBank, accession no.	Mariannaea sp.			Rhizomucor sp.		
		N	WP	P	N	WP	P
1	Sphingomonas sp., DQ339616	1	0	0	0	1	0
	Xanthomonadaceae						
1	Stenotrophomonas sp., AY689084	1	0	0	0	0	1
	Pseudomonadaceae						
1	Pseudomonas aeruginosa, DQ095913	1	0	0	0	0	1
1	**Pseudomonas sp., DQ279323**	0	0	1	0	1	0
1	Pseudomonas sp., DQ279324	1	0	0	0	1	0
2	**Pseudomonas sp., DQ11923**	1	1	1	0	1	2
3	**Pseudomonas tolaasii, AF320990**	2	0	1	0	0	3
5	Pseudomonas sp., AY599719	5	0	0	0	2	3

Abbreviations: *H. scutatum*, *Hemidactylium scutatum*; *n*, no. of isolates; N, negative; NT, not tested; P, positive; WP, weak positive.
Strong positive antifungal bacteria against *M. elegans* are in bold.
Strong positive antifungal bacteria which were found on ⩾4 individual salamanders are highlighted in gray.

DNA sequencing of PCR products from bacterial pure cultures and comparative sequence analysis
DNA sequencing of antifungal bacterial isolates was performed at the DNA Sequencing Centre of the Brigham Young University, Provo, UT, USA. A consensus sequence of approximately 1400 bp was obtained by combining the forward and reverse sequences amplicons. All sequences were compared with those of reference organisms by BLAST search (http://www.ncbi.nlm.nih.gov/blast) using the Gen-Bank database (Benson *et al.*, 1999). Phylogenetic trees were constructed to put our antifungal isolates in the evolutionary context of already described bacterial species and to assess the taxonomic diversity of antifungal bacteria from a salamander species. Sequence alignments and phylogenetic trees were performed with Seqpup32 (Gilbert, 1996), and ClustalX on the basis of the neighbor-joining method (Jeanmougin *et al.*, 1998). On the basis of these data, a phylogenetic tree was viewed using TreeView1.6.6 (Page, 1996). The taxonomy browser of the NCBI server was used for determination of family affiliation. A bootstrap analysis (1000 replicates) was used to validate the reproducibility of the branching pattern of the phylogenetic trees.

Analysis of resident cutaneous bacteria by DGGE
DGGE, including the identification of excised DGGE bands, was performed as described previously (Lauer *et al.*, 2007). The statistical analysis of DGGE patterns, in the form of an unweighted pair group matching analysis (UPGMA), was performed with the QuantityOne Software of the Biodoc System (BioRad, Hercules, CA, USA). The statistics are based on Dice similarity coefficients (Carrero-Colón *et al.*, 2006), which were calculated for all pairwise combinations of DGGE lanes as two times the number of shared bands divided by the total number of bands found in the pair of samples. DGGE fingerprints are useful tools to characterize and compare microbial communities. However, it is important to recognize their limitations. Overestimates of diversity and/or dominant populations may come from the formation of heteroduplex or chimera molecules during PCR, or because of sequence heterogeneities in multiple copies of the 16S rRNA gene (Qiu *et al.*, 2001). Furthermore, bands on DGGE gels may be comprised of several amplicons, underestimating bacterial diversity. Despite these limitations, DGGE profiles have been shown to be a powerful tool for assessment of bacterial diversity in environmental samples (Muyzer and Smalla, 1998).

Nucleotide sequence accession number
All 16S rRNA gene sequences of the antifungal isolates and DGGE bands analyzed in this study were deposited in the DDBJ/EMBL/GenBank databases under accession numbers DQ156142-DQ156146, EU057127-EU057130, and EU057829-EU057893.

Results

Diversity of antifungal bacterial isolates and its frequency on H. scutatum *salamanders*
Incubation on R2A agar from streaked swabs revealed between 1 and 21 different colony morphology types from each individual salamander. Many of the bacterial colony morphologies were

Table 2 Percentage of *H. scutatum* and *P. cinereus* salamanders with at least one antifungal cutaneous bacterial species

	H. scutatum/P. cinereus with at least 1 P or WP anti-M.e. sp.	*H. scutatum/P. cinereus* with at least 1 P or WP anti-R.v. sp.
H. scutatum		
CH	35.71	100.00
(n = 28 H.s.)		
PR	27.08	89.58
(n = 48 H.s.)		
WOF	16.67	100.00
(n = 12 H.s.)		
Average	26.49	96.53
P. cinereus		
HA	33.33	88.88
(n = 27 P.c.)		

Abbreviations: CH, Cline's Hacking; H.s., *Hemidactylium scutatum*; M.e., *Mariannaea elegans*; P, positive; P.c., *Plethodon cinereus*; PR, Pond Ridge; R.v., *Rhizomucor variabilis*; WOF, White Oak Flat; WP, weak positive.

similar among individuals of the same salamander species. Fourty-eight different bacterial species with antifungal activities were identified from *H. scutatum* salamanders comprising 16 genera, 14 families and 4 phyla (Table 1). A bacterial species was defined on the basis of greater than 97% 16S rRNA gene similarity. Sixteen bacterial species (33%) showed antifungal activity (weak or strong) against *M. elegans* and 48 species (100%) were antifungal (weak or strong) against *R. variabilis.* The most frequently occurring antifungal bacterial species (weak or strong) on salamanders (found on more than three individual salamanders) were related to the Bacillaceae (*Bacillus cereus* group), the Oxalobacteraceae (related to *Janthinobacterium lividum* and *Duganella zoogloeides*), the Pseudo-monadaceae (*Pseudomonas fluorescens* group), the Flavobacteriaceae (*Flavobacterium* sp.), and the Sphingobacteriaceae (related to *Pedobacter caeni* and *Pedobacter cryoconitis*). On average, ∼27%
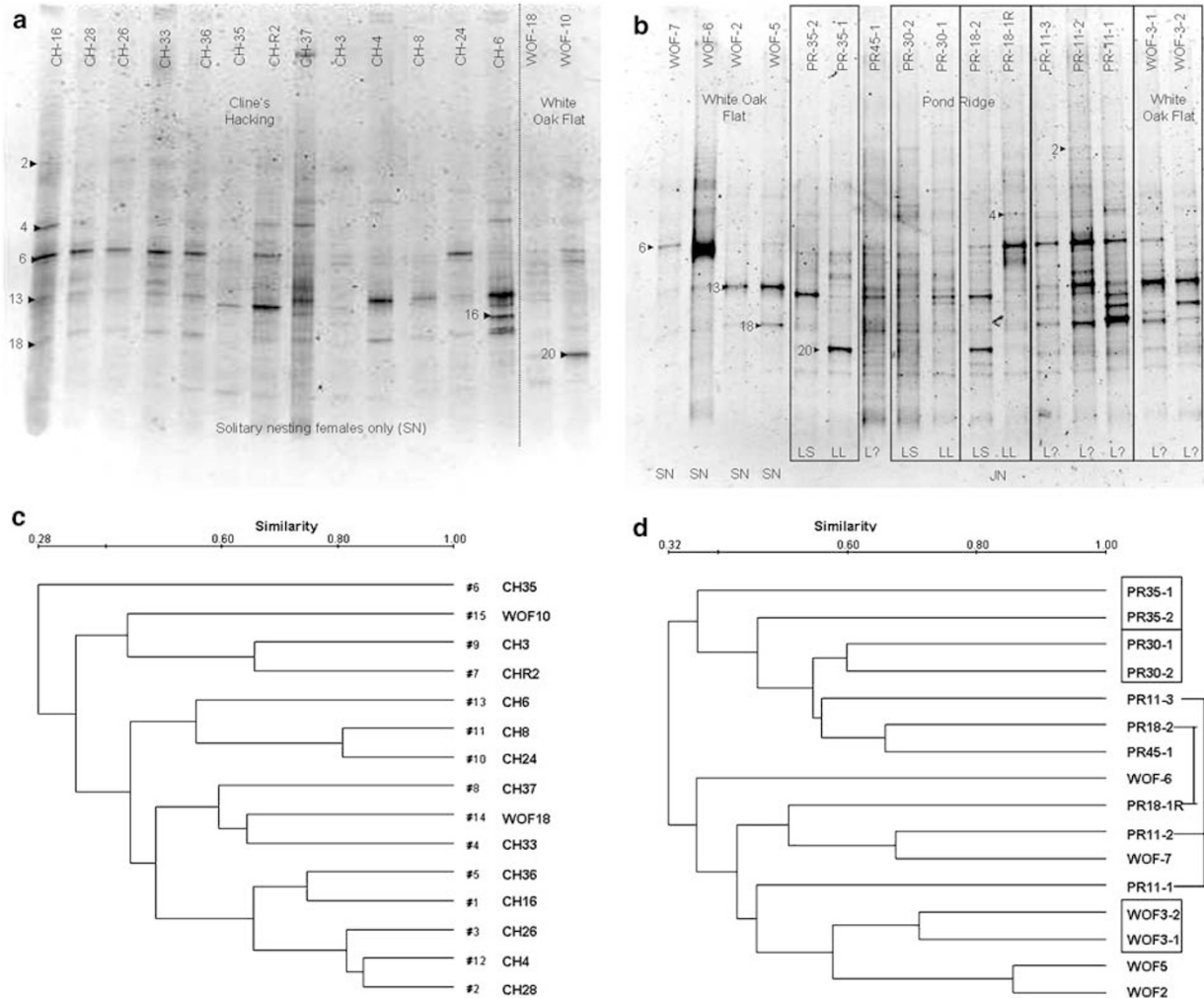

Figure 1 (**a** and **b**) DGGE of nesting female *Hemidactylium scutatum* individual salamanders (S, solitary nesting; JN, joint nesting; R, roamers; PR, Pond Ridge; CH, Cline's Hacking; WOF, White Oak Flat). Dominant bands that were identified are indicated with an arrow. Bands 2 and 20 could not be identified. Joint nests are indicated by boxes (LS, lay and stay; LL, lay and leave; L? = lay and status unknown). (**c** and **d**) UPGMA dendrograms constructed using Dice's similarity coefficient generated from the above DGGE profiles. Joint nests are indicated by boxes or connecting lines.

of *H. scutatum* salamanders were carrying anti-*M. elegans* bacteria and ~97% of *H. scutatum* salamanders carried anti-*R. variabilis* bacteria on their skin (Table 2).

Epibiotic bacterial community of H. scutatum *individual salamanders examined by DGGE*
DGGE profiles were obtained from 27 solitary nesting females (~63% of all solitary nesting females), 22 joint nesting females comprising 8 nests (~76% of all joint nesting females from 11 nests altogether) and 13 'roaming' females (65% of all 'roaming females). Each female had between 5 and 20 different bands in their DGGE profiles. At least 25% of all sequence types were shared by all females in all populations. The DGGE profiles of females at joint nests were compared among each other and with profiles of solitary nesting and roaming females. Females in a joint nest shared 50–60% of the sequence types at three of eight joint nests. Females in the other five joint nests shared

25–40% of sequence types. The sharing of sequence types among females at the three joint nests that shared a majority of sequence types suggests that these joint nesters may have transferred resident bacterial species among each other. Overall, six sequence types (bands 2, 4, 6, 13, 18 and 20) were observed in the profiles of the majority of the nesting females. Four of these bands (6, 13, 16 and 18) were identified and indicated in the Figures 1a and b. Band 6 was identified as a γ-proteobacterium 99% related to the species *Acinetobacter junii* (AF417863) and was found on almost 75% of all females. Band 13 was identified as a β-proteobacterium 99% related to the species *Acidovorax temperans* (DQ111711) and was detected on ~77% of all females. Band 16 which was found on ~48% of the females was identified as an α-proteobacterium, 98% related to *Methylobacterium* sp. (AB242722), and band 18 which was detected on ~97% of all females was identified as 100% related to the β-proteobacterium *Imtechium assamiensis* strain BPTSA16 (AY544767).
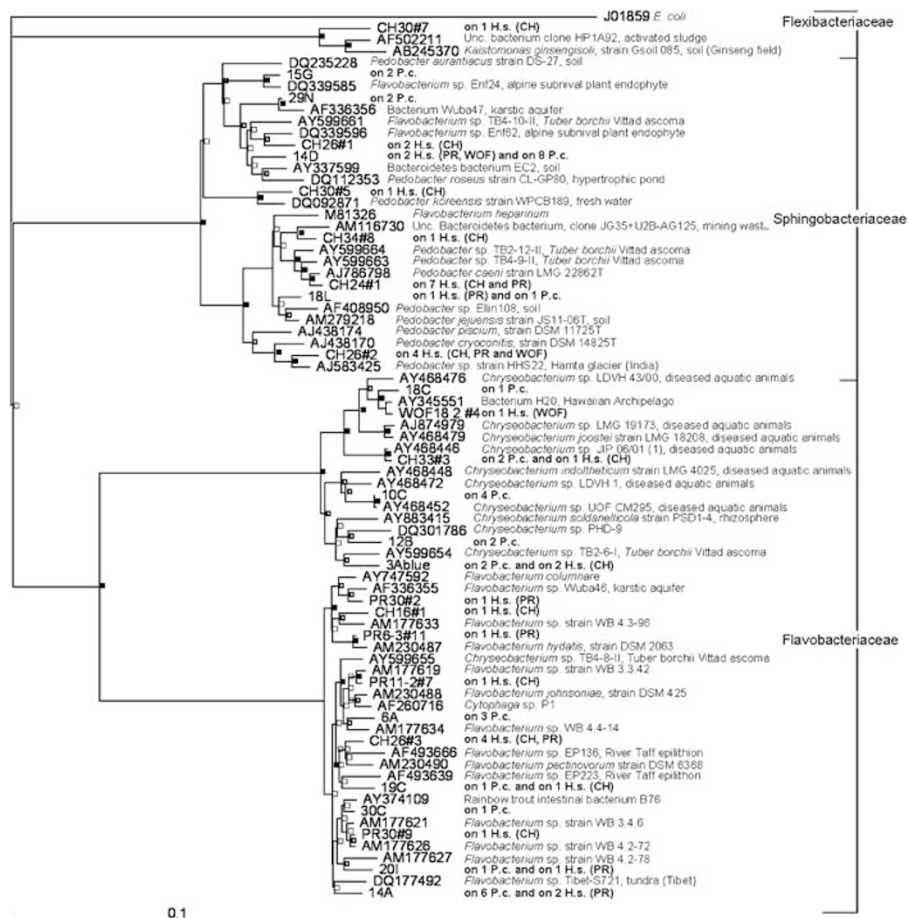

Figure 2 Phylogenetic tree based on 16S rRNA gene sequences of antifungal isolates belonging to the *Bacteroidetes* together with closest matches and representative sequences of the GenBank database. The tree was generated using the neighbor–joining method with 1000 bootstrap replicates using approximately 1400 nucleotides. The frequency of individual salamanders carrying a particular isolate is indicated (P.c., *Plethodon cinereus*; H.s., *Hemidactylium scutatum*). Branch points supported by bootstrap resampling are indicated by symbols (■, bootstrap proportion >90%; ◨, bootstrap proportion 90–50%; □, bootstrap proportion <50%).

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Discussion

In this study, we have shown that bacterial species isolated from the skin of the plethodontid salamander *H. scutatum* have antifungal activities against a saprophytic zygomycete and an ascomycete fungus that can be pathogenic to salamander embryos. Below, we compare these results to those found on *P. cinereus*, which is also a member of the family Plethodontidae (Lauer *et al.*, 2007). We did not find a closely related cutaneous bacterial community at the bacterial species level when we compared the isolated cutaneous antifungal bacterial diversity of both salamander species (Figures 2–5). Furthermore, the proportion of culturable antifungal bacterial taxa shared between two large populations of *H. scutatum* was the same as the proportion of taxa shared between *H. scutatum* and *P. cinereus* (Figure 6).

Altogether 68 different bacterial species that showed antifungal activity against at least one of the fungal species *M. elegans* and *R. variabilis* were identified from both salamander species together. Twenty-eight antifungal bacterial species from

P. cinereus (Lauer *et al.*, 2007) were compared to 48 bacterial species from the skin of *H. scutatum* (this paper) in phylogenetic trees on the basis of 16S rRNA gene similarities (Figures 2–5). Although only 27 salamanders were investigated in the study by Lauer *et al.* (2007), compared to 87 salamanders in this study, we found similar patterns of antifungal cutaneous diversity among the bacterial isolates on the bacterial family level, but significant differences were found on the bacterial species level. By comparing the antifungal bacterial diversity on the taxonomic levels, we found that the proportion of shared bacterial diversity was declining with the taxonomic hierarchy (phyla, families, genera and species). This tendency was found when the shared bacterial diversity of individual *H. scutatum* salamanders from the two largest populations (CH and PR) was compared and also when the bacterial diversity of the two salamander species was compared. *P. cinereus* and *H. scutatum* salamanders shared antifungal bacterial taxa in four phyla (100%), seven bacterial families (~53%), nine genera (~47%), and eight species (~10%) (Figure 6). Furthermore, we found that the indivi-
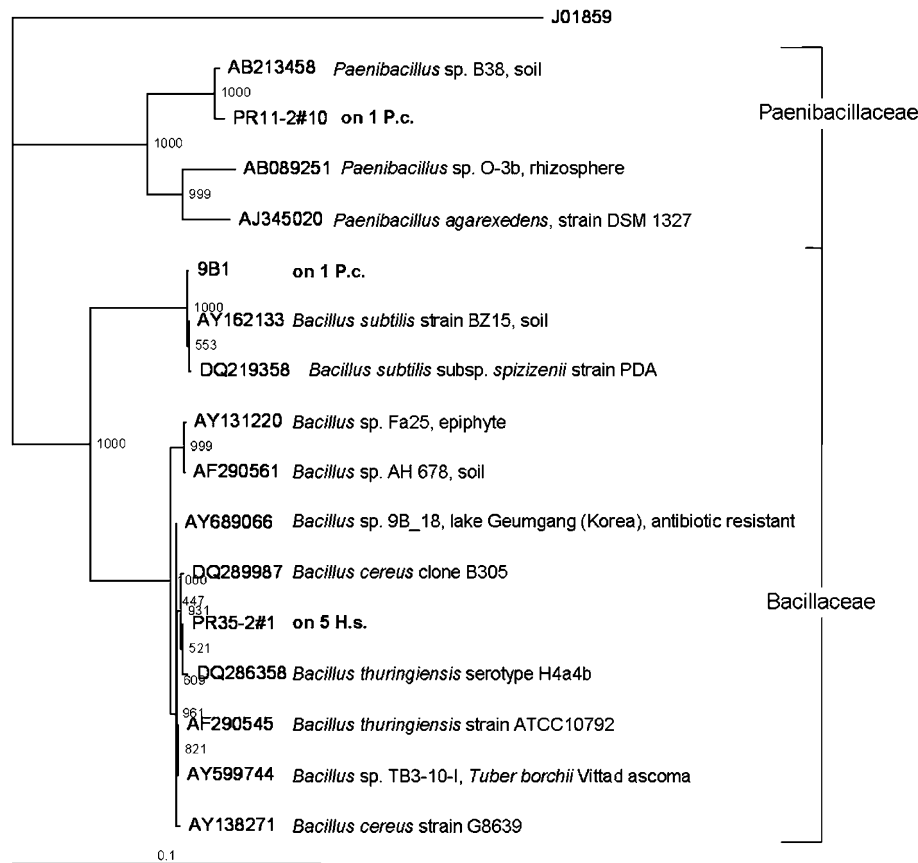

Figure 3 Phylogenetic tree based on 16S rRNA gene sequences of antifungal isolates belonging to the *Firmicutes* together with closest matches and representative sequences of the GenBank database. The tree was generated using the neighbor–joining method with 1000 bootstrap replicates using approximately 1400 nucleotides. The frequency of individual salamanders carrying a particular isolate is indicated (P.c., *Plethodon cinereus*, H.s., *Hemidactylium scutatum*).
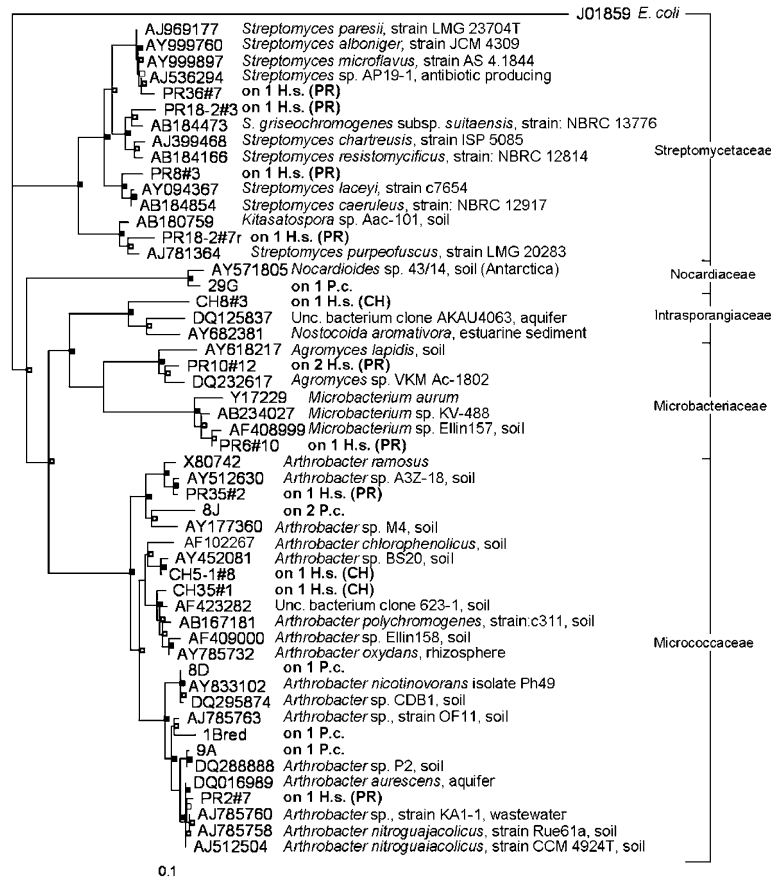
Figure 4 Phylogenetic tree based on 16S rRNA gene sequences of antifungal isolates belonging to the *Actinobacteria* together with closest matches and representative sequences of the GenBank database. The tree was generated using the neighbor–joining method with 1000 bootstrap replicates using approximately 1400 nucleotides. The frequency of individual salamanders carrying a particular isolate is indicated (P.c., *Plethodon cinereus*; H.s., *Hemidactylium scutatum*). Branch points supported by bootstrap resampling are indicated by symbols (■, bootstrap proportion >90%; ◪, bootstrap proportion 90–50%; □, bootstrap proportion <50%).

duals of both salamander species carried a similar percentage of anti-*M. elegans* and anti-*R. variabilis* bacteria on their skin (Table 2).

Members of the *Bacteroidetes* were the most common antifungal bacteria on both salamander species. Almost 40% of all antifungal bacterial species on both salamander species belonged to this phylum, comprising two families: the Sphingo-bacteriaceae (~13%) and the Flavobacteriaceae (~25%). Strong antifungal bacterial isolates obtained from *H. scutatum* were related to *Pedobacter cryoconitis* and *P. caeni*, whereas isolates related to *P. roseus* were obtained from *P. cinereus* only (Figure 2). Antifungal bacterial isolates belonging to the *Firmicutes* comprised ~2% of the antifungal cutaneous bacterial diversity of *H. scutatum* and ~7% of the antifungal bacterial diversity of *P. cinereus*, and were different for the two salamander species (Figure 3). Members of the *Actinobacteria* comprised ~23% of the entire isolated antifungal bacterial species on *H. scutatum*, but they comprised only ~10% of the cutaneous bacterial community on *P. cinereus*. There was no overlap in the *Actinobacterial* diversity on the two salaman-

der species on the bacterial species level. Each salamander species seemed to have their own cutaneous diversity of *Actinobacteria* of which most were weakly antifungal against *R. variabilis* and not active against *M. elegans*. The highest diversity of antifungal species in this group was found among the Streptomycetaceae and Micrococcaceae. Bacterial species related to the Streptomycetaceae were only found on *H. scutatum* salamanders, but different species of the Micrococcaceae (*Arthrobacter* sp.) were found on both salamander species (Figure 4). Antifungal isolates in all five families of the *Proteobacteria* were found on both salamander species, with the exception of the Sphingomonadaceae that were only found on *H. scutatum*. Only two bacterial species related to *Stenotrophomonas maltophilia* and *Pseudomonas tolaasii* in the Gamma *Proteobacteria* were shared by the two salamander species. Two of the most numerically dominant bacterial species found on both salamanders were closely related to *J. lividum* and were found on ~37% of all individual *P. cinereus* salamanders and on ~16% of all *H. scutatum* salamanders (Figure 5).

Antifungal cutaneous bacteria from salamanders
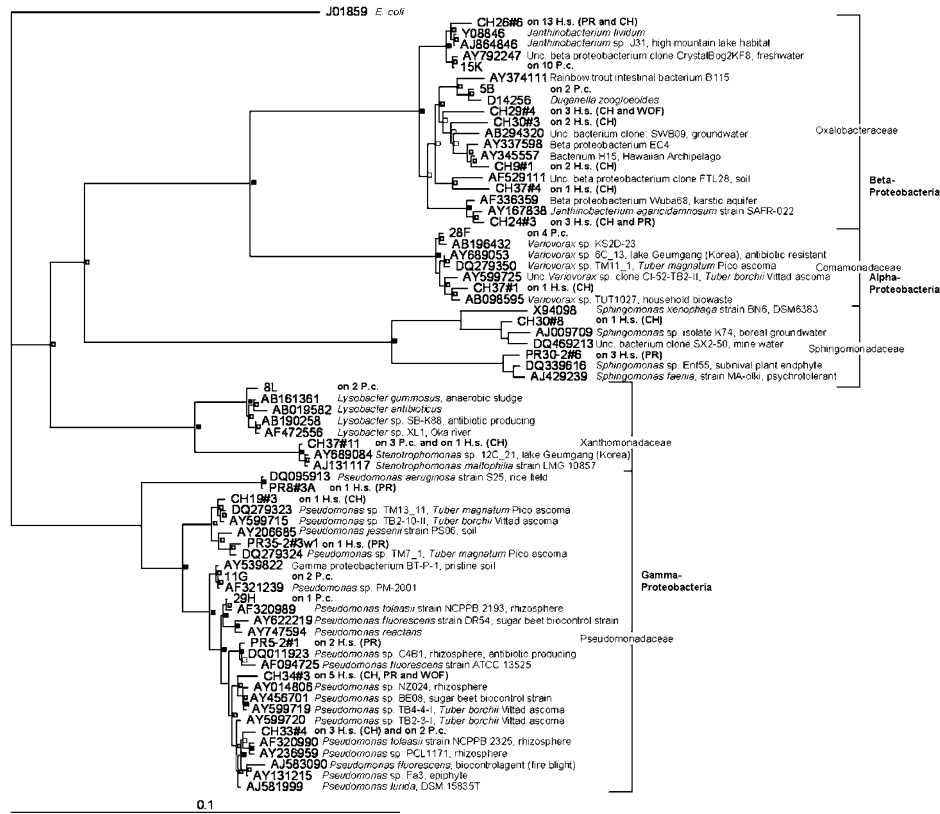A Lauer et al
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Figure 5 Phylogenetic tree based on 16S rRNA gene sequences of antifungal isolates belonging to the *Proteobacteria* together with closest matches and representative sequences of the GenBank database. The tree was generated using the neighbor–joining method with 1000 bootstrap replicates using approximately 1400 nucleotides. The frequency of individual salamanders carrying a particular isolate is indicated (P.c., *Plethodon cinereus*; H.s., *Hemidactylium scutatum*). Branch points supported by bootstrap resampling are indicated by symbols (■, bootstrap proportion >90%; ◧, bootstrap proportion 90–50%; □, bootstrap proportion <50%).

Our culture independent assay revealed that *H. scutatum* did share species of bacteria within and between populations. We identified four dominant members of the cutaneous bacterial community. The evaluation of the DGGE banding patterns from *H. scutatum* individual salamanders suggests a shared cutaneous bacterial microbiota of between 25 and 90%, with several sequence types found on most individuals. The pattern of higher similarity for uncultured species than for culturable antifungal species may be due to cultured species being relatively rare members of the cutaneous community or that selection has favored a common antifungal function to the community rather than favoring specific species. This latter situation is similar to that found among bacterial communities in the human gut, where each individual tends to have a unique community (Dethlefsen *et al.*, 2006). A statistical analysis (UPGMA) of 16S rRNA DGGE banding patterns from six male and five female individuals of *P. cinereus* salamanders showed random clustering of the genders in a dendrogram, indicating no gender specific cutaneous bacterial diversity (Lauer *et al.*, 2007). This finding is probably also true for the related *H. scutatum*, although it was not investigated in this study. By interpreting results obtained by PCR/DGGE, we have
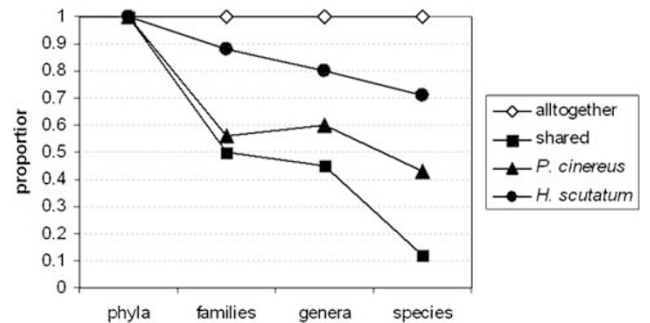
Figure 6 Shared isolated antifungal cutaneous bacterial diversity of *H. scutatum* compared to *P. cinereus* on different taxonomic levels. The proportion of shared antifungal bacterial diversity is declining with the hierarchy of the taxonomic level (phyla, families, genera and species) when the shared bacterial diversity of individual *H. scutatum* salamanders from two locations (CH and PR) was compared, and also, when the bacterial diversity of *H. scutatum* salamanders (from CH and PR together) was compared to the bacterial diversity on *P. cinereus*. The data from White Oak Flat (*H. scutatum*) is not included in this graph, because significantly less individual salamanders (12 individuals) from this location were studied, compared to the amount of individual salamanders investigated from the other two locations (Pond Creek and Cline's Hacking; 48 and 28 individuals, respectively).

to be aware that only members of the bacterial community that comprise at least 1%, can be detected, and that preferential amplification, hetero-

geneity in ribosomal RNA copy number and other PCR bias can also distort the true bacterial diversity of a sample (reviewed by Farrelly *et al.*, 1995; Suzuki and Giovannoni, 1996; van Wintzingerode *et al.*, 1997; Øvreås and Torsvik, 1998; Sekiguchi *et al.*, 2001; Coenye and Vandamme, 2003). In our study, we found that bands from different salamanders that stopped in the same melting area of the DGGE gel revealed the same bacterial sequence type.

We found that a similar percentage of individuals from both salamander species carried culturable antifungal bacteria ($\sim 30\%$ with anti-*M. elegans* bacteria and $>90\%$ anti-*R. variabilis*). This result indicates that almost all individual salamanders of *H. scutatum* and *P. cinereus* have an antifungal cutaneous bacterial microbiota that may aid in the inhibition of the opportunistic saprophyte *R. variabilis*, and nearly a third may be protected against infections with the proven embryo pathogen *M. elegans*. A frequently isolated cutaneous bacterial species (on $\sim 15\%$ of *H. scutatum* salamanders) producing a purple colony type was identified as closely related to *J. lividum* (Y08846) of the family Oxalobacteraceae. A closely related *Janthinobacterium* sp. isolate that clustered separately in the phylogenetic tree and differed in pigment production (beige colony type) compared to the isolates found on *H. scutatum* was also found on $\sim 33\%$ of the *P. cinereus* individuals (Lauer *et al.*, 2007). This finding, suggests that these isolates of the family Oxalobacteraceae are important members of the cutaneous bacterial diversity of the plethodontid salamanders investigated in this study. However, the β-proteobacterium *J. lividum* could not be detected by PCR/DGGE among the dominant bands. This result might be due to methodological reasons, such as PCR bias. It is known that *J. lividum* produces the peptide lactone antibiotics Janthinocin A, B and C (O'Sullivan *et al.*, 1990), and in our challenge assays it was found to be weakly antifungal against both fungi.

Bacteria coevolve with their host and may differentiate into subspecies or different strains with time (Steinert *et al.*, 2000). This finding may explain the related but not identical isolated cutaneous antifungal bacterial diversity on the two closely related plethodontid salamanders. Also, the fact that the larvae of *H. scutatum* complete their development in the water in contrast to *P. cinereus* salamanders that are completely terrestrial likely influences the diversity of resident cutaneous bacteria. The presence of cutaneous bacteria that produce antifungal metabolites was found in three plethodontid salamanders (Austin, 2000; Lauer *et al.*, 2007; the present study) and in two other amphibian species (*Centrolene prosoblepon* and *Rana muscosa*) (unpublished data; Woodhams *et al.*, 2007).

The ability of resident microorganisms to produce secondary metabolites that inhibit or kill transient microbiota will certainly aid in the successful reproduction of the resident microbiota and at the same time function as a barrier against potential pathogens such as *M. elegans*. Indeed, we have isolated an antifungal compound from *P. cinereus* produced by a member of its skin bacterial community (Brucker *et al.*, in press). Resource competition for nutrients and space are also mechanisms of bacterial inhibition. In other taxa, bacteria related to species found on amphibian skins have been successfully used as probiotics. In aquaculture of fish (*Salmon salar*, *Oncorhynchus mykiss* and *Arripis trutta*) and crustaceans (*Penaeus monodon*), *Bacillus* sp. including *B. subtilis* strains and strains of *Pseudomonas fluorescens* have been used as probiotics against pathogenic bacteria and fungi (for example, *Vibrio* sp. and *Saprolegnia* sp.). *Flavobacterium* strains have been used as probiotics and growth promoters in algae (*Chaetoceros gracilis*) (Verschuere *et al.*, 2000; Irianto and Austin, 2002). It seems reasonable that bacteria related to these biocontrol agents will work as powerful disease preventing agents against amphibian pathogens on their skin and embryos.

We have provided evidence of a resident antifungal cutaneous bacterial microbiota on *H. scutatum*. The transmission of cutaneous bacteria from the brooding female to her embryos may be necessary to enhance embryo survival. The evidence of an antifungal cutaneous bacterial community on amphibians that is closely related to bacteria used successfully as biocontrol agents, gives hope of finding a probiotic cure for adult amphibians threatened by other pathogenic fungi, such as *Batrachochytrium dendrobatidis* (*Bd*) which is responsible for recent amphibian population declines worldwide (Harris *et al.*, 2006; Woodhams *et al.*, 2007). Recent results showed that a subset of the bacteria that were antifungal against *M. elegans* and *R. variabilis* are also antifungal against *Bd* (unpublished data). Current work focuses on the detection and characterization of strong antifungal metabolites from different cutaneous bacteria from different salamander and frog species that are able to protect salamander embryos (Brucker *et al.*, in press). Future work will also include probiotic experiments where adult amphibians are exposed to *Bd* zoospores, monitored and compared to a group that has been inoculated with an antifungal bacterium prior to *Bd* exposure. Such studies will provide a better understanding of cutaneous bacteria on amphibians and their role as a barrier against disease.

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