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Bacterial symbionts that inhabit apothecia of the cup fungus *Scutellinia scutellata*

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With 9 figures and 1 table

Abstract: New symbiotic relationships between bacteria and members of all other eukaryotic Kingdoms – Plant, Animal, Fungal and Protist – are continually being discovered. Higher resolution imaging of eukaryotic tissues is revealing that coexistence of live bacteria among cells of eukaryotic tissues is more ubiquitous than has been conventionally reported. Apothecia of the discomycete fungus *Scutellinia scutellata* are colonized by extracellular bacteria throughout their primary tissue types as viewed in thin sections of both tissues and in scanning electron microscope images of the hymenial surface. Within the hymenium, at the termination of ascosporogenesis, these bacteria invade each ascus and associate with surfaces of mature ascospores. DNA was extracted from intact *S. scutellata*, and approximately 1500 bp of the 16S ribosomal RNA gene was amplified using general eubacterial primers. DNA sequences of the predominant bacteria found in association with *S. scutellata* do not correspond to sequences of bacteria reported from endophytic, mycorrhizal, or other fungi but surprisingly closely match sequences of members of the genera *Acidovorax* and *Verminephrobacter*, the latter being beneficial extracellular symbionts of earthworm nephridia. From a total of 74 non-chimeric clones sequenced, 52% had a high similarity to free-living *Acidovorax* bacteria (97 to 99% identity) and to members of the genus *Verminephrobacter* (95% identity), until recently also placed in the genus *Acidovorax*. All attempts to culture hyphae in the absence of bacteria failed. To our knowledge, this represents the first report of these versatile *Acidovorax*-like bacteria having established symbiotic relationships with members of both Fungal and Animal Kingdoms.

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Key words: Apothecium, symbionts, extracellular bacteria.

Introduction

New examples of the coexistence of bacterial cells among living cells of eukaryotic organisms of all Kingdoms are continually emerging (Hoffman & Arnold 2010; Scott et al. 2008; Currie 2001). The symbiotic associations of members of the Bacteria Kingdom and the Fungus Kingdom have been categorized as predatory, parasitic and mutualistic (Leveau & Preston 2008).

Although fungi secrete antibacterial compounds to repel bacteria, they also secrete nutrients such as amino acids, organic acids such as oxalic acid, sugars, and sugar alcohols that are the sole or major source(s) of nutrient substrates for some fungus-associated bacteria (de Boer et al. 2005). Certain bacterial symbionts may be entirely dependent on their fungal hosts for their survival. The bacterial symbionts involved in mutualistic relationships with fungi apparently obtain nutrients from their fungal hosts without having deleterious effects on the fungi and by often benefiting the fungus and enhancing its fitness.

The bacterial partners can influence the physiology and development of their fungal hosts in myriad ways. Bacteria exert an indirect effect on fungal cells by modulating the expression of fungal genes (Schrey et al. 2005) or through expression of bacterial genes whose products act directly by (1) enhancing the germination of spores or facilitating the opening of asci (Roesti et al. 2005), by (2) providing their fungal hosts with nutritional benefits as in the case of the cyanobacterial-fungal associations of lichens (deBoer et al. 2005), by (3) promoting fruiting body induction (Cho et al. 2003), by (4) encouraging hyphal growth of certain mycorrhizal fungi while simultaneously inhibiting growth of certain phytopathogenic fungi (Schrey et al. 2005; Maier 2004), by (5) conferring antimicrobial properties that offer protection from predatory or parasitic bacteria or by (6) even conferring resistance to antifungal chemicals (Chamilos et al. 2007) or modulating the virulence of fungi that are plant pathogens (Minerdi et al. 2008; Partida-Martinez and Hertweck 2005).

In the case of bacteria associated with mycorrhizal fungi, endophytic fungi, or pathogenic fungi, the bacterial influence extends to the plant partner in a tripartite relationship of Plant, Fungal and Bacterial Kingdoms (Hoffman & Arnold 2010; Minerdi et al. 2008; Sharma et al. 2008; Partida-Martinez & Hertweck 2005). Such multitrophic and multi-kingdom interactions of plants, fungi and bacteria are apparently ubiquitous and vastly expand the ability of hosts and their associated microbes to adapt to their environments. Bacteria and mycorrhizal fungi interact to stimulate growth of their host plants by enhancing nutrient uptake and suppressing the action of fungal pathogens (Sharma et al. 2008; Artursson et al. 2006). These so-called mycorrhizal helper bacteria (Garbaye 1994) would include bacteria with the ability to solubilize and/or transport inorganic phosphates (Hoffman & Arnold 2010; Artursson et al. 2006), to produce plant hormones (Sharma et al., 2008), to fix atmospheric nitrogen (Leveau & Preston 2007), or to enhance access to lignocellulose-rich substrates through production of pectinases and cellulases (deBoer et al. 2005).

As a decomposer of plant detritus, the Eyelash Cup Fungus, *Scutellinia scutellata* (L.) Lambotte, is a member of a common genus of discomycetes found most frequently on decaying wood (Hanson & Pfister 2006; Schumacher 1990). Thin sections of its colorful and conspicuous apothecia revealed the unexpected presence of bacteria associated with living fungal cells throughout all regions of the apothecium. A search of literature on the genus *Scutellinia* revealed that the presence of extracellular bacteria among hyphae of another member of this genus had been described earlier (Schrantz 1973, 1980, 1986). In this manuscript, the spatial relationship between fungal and bacterial cells in apothecia of *S. scutellata* as well as the identity of the symbiotic bacteria have been explored.

Materials and methods

COLLECTION OF APOTHECIA FROM THE FIELD: Over a two-year period, between April and November of 2010 and 2011, a total of 27 apothecia were collected from exposed oak wood at two different localities separated by 150 kilometers: Allerton Park in Piatt County, Illinois (40°02'02"N, 88°34'23"W) and a privately owned forest in Parke County, Indiana (39°39'42"N, 87°22'08"W). At the Parke County locality, apothecia were collected on moist, exposed oak logs at four sites, all separated by distances of at least 500 m. The presence of bacteria within apothecia was scored by microscopic examination of tissue sectioned either with a single-edge razor blade or by resin-embedded tissue sectioned with a diamond knife.

Two dry specimens collected from the Parke County, Indiana site on 12 June 2011 were deposited as voucher specimens along with a microscope slide having one micron sections of an apothecium of *Scutellinia scutellata.* Sections obtained with a diamond knife and a Reichert Ultracut E microtome were deposited in the ILLS Herbarium of the Illinois Natural History Survey.

PREPARATION OF TISSUES FOR SECTIONING AND SCANNING ELECTRON MICROSCOPY: Sections of eight apothecia for transmission electron microscopy and light microscopy and four whole mounts for scanning electron microscopy were fixed at 4°C in a primary fixative of 2.5% glutaraldehyde and 0.5% paraformaldehyde dissolved in a rinse buffer of 0.1 M cacodylate (pH 7.4) containing 0.18 mM $CaCl_2$ and 0.58 mM sucrose. After three hours in this fixative, tissues were washed three times with rinse buffer before being transferred to the secondary fixative (2% osmium tetroxide in rinse buffer). Tissues remained in this solution for 4 hours in the cold and were then washed three more times with rinse buffer. To enhance membrane contrast, rinsed tissues were placed in filtered, saturated uranyl acetate for 10 min immediately before being gradually dehydrated in a graded ethanol series (10%–100%). At this stage in the preparation, tissues for scanning electron microscopy were processed separately from the tissues for sectioning. From absolute ethanol, tissues for sectioning were transferred to propylene oxide and infiltrated with mixtures of propylene oxide and resin before being embedded in pure LX112 resin. Resin was polymerized at 60°C for three days followed by 15 repetitions of ten-second microwave treatments. Embedded tissues were sectioned with a diamond knife either at 0.35 µm for light microscopy or at ~0.09 µm for electron microscopy. Sections for light microscopy were mounted on glass slides and stained with a solution of 0.5% toluidine blue and 0.25% basic fuchsin in 1% borax. Thin sections of regions chosen for ultrastructural examination were mounted on copper grids and stained briefly with saturated aqueous uranyl acetate and Luft's lead citrate to enhance contrast. Images were taken with a Hitachi H600 transmission electron microscope operating at 75 kV.

Specimens for scanning electron microscopy were processed in parallel with specimens for resin embedding until they had been dehydrated in 100% ethanol. From absolute ethanol, specimens were critical-point dried and coated with gold-palladium. Images were taken with a Philips XL30 ESEM-FEG microscope operated by the Imaging Technology Group at the Beckman Institute, University of Illinois.

CULTURING THE FUNGUS: Repeated attempts were made to isolate *S. scutellata* in pure culture. Efforts were made to culture from single ascospores or asci of *S. scutellata.* Using sterile needles, apothecia

were removed from the substrata and carefully spread on the surface of antibiotic water agar plates (AWA: agar 20 g, streptomycin sulfate 250 mg/L, penicillin G 250 mg/L, distilled water 1 L; antibiotics were added to the molten agar immediately after autoclaving). Using sterile, fine dissecting needles, the centrum (asci, ascospores, and paraphyses) from an apothecium was spread on the agar surface. Alternatively, the apothecium was stuck to the lower surface of the petri plate with a drop of sterile distilled water, so that the ascospores were forcefully shot onto the water agar plate. Subsequently, about 5–10 ml of autoclaved distilled water was added to the AWA plates, so to spread the ascospores and asci uniformly over the agar surface. Excess water was decanted gently from the plates and the plates were then incubated for 24–72 h at a slight angle so that any remaining water could drain from the agar surface. Despite several attempts to isolate the fungus in axenic culture, we were unable to obtain germinating ascospores on AWA.

DNA ANALYSIS: DNA of *S. scutellata* was extracted from apothecia using the QIAamp DNA Micro Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The 16S ribosomal RNA gene was amplified using Illustra PuReTaq™ Ready-To-Go™ PCR beads (GE, Fairfield, CT), and general eubacterial primers 21F (5'-AYTTTGAGAGTTTGATCCTG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Courtesy of Carl Woese, University of Illinois) using the following protocol [95°C 3 min (95°C 30 sec; 53°C 30 sec; 72°C 90 sec) 40×] and a DNA Engine Peltier thermal cycler (Bio-Rad, Hercules, CA). Amplified PCR products were visualized on 1% agarose gels run at 90 V for 30 min. PCR products were cloned using the TOPO TA Cloning kit (Invitrogen, Carlsbad, CA). Positive bacterial clones were screened using modified M13 primers: M13F Long (5'-GTAAAACGACGGCCAGTGAATTG-3') and M13R Long (5'-CAGGAAACAGCTATGACCATGATTACG-3') and the following protocol [95°C 3 min (95°C 30 sec; 58°C 30 sec; 72°C 120 sec) 40×]. PCR products from positive clones were cleaned using the QiAquick PCR purification kit (Qiagen, Valencia, CA). Concentrations of PCR products were measured using a Nanodrop™ spectrophotometer (Thermo Scientific, Wilmington, DE). Sequencing reactions were performed with a DNA concentration of 5 ng/100 bp, in a volume of 20 µl, with a 2:1:1 mixture of BigDye Terminator v3.1, dGTP BigDye Terminator v3.0 and 5× sequencing buffer (Life Technologies, Carlsbad, CA), using the following protocol: [(95°C 5 min (98°C 10 sec; 50°C 5 sec; 60°C 4 min) 30×]. Clones from Sets1, 2 and 3 were sequenced with the M13F and R Long listed above as well as the following respective internal primers: Set1 (Set1ScutF 5'-GCTTAACTAGAGGGGTGCCATTGA-3' and Set1ScutR 5'-TGGCCGCGTACTGTATATCGCAC-3'); Set2 (Set2ScutF 5'-TCGGAATTAYTG GGCGTAAAGC-3' and Set2ScutR 5'-GGGTATCTAATCCTGTTTGCT-3'); Set3 (Set3ScutF 5'-AGTGTAGAGGTGGAATTCG-3' and Set3ScutR 5'-TGCGACCGTACTCCCCAGGCGG-3'). Sequencing reactions were cleaned using Performa® Ultra 96-Well Plates (EdgeBio, Gaithersburg, MD) and run on ABI3730XL capillary systems at the Keck Center of the University of Illinois. Sequences were edited using Sequencer 4.10.1 (Gene Codes Corp., Ann Arbor, MI) and aligned manually using PAUP* 4.0 (Swofford 2003).

The presence of possible chimeric sequences was tested using DECIPHER (Wright et al. 2012) (http://DECIPHER.cee.wisc.edu). From a total of 91 clones sequenced, 17 were found to be chimeric and removed from the data set. Identity of bacterial clones was determined using NCBI Blast (Zhang et al. 2000), the Ribosomal Data Base (RDP) (Cole et al. 2008) (http://rdp.cme.msu.edu/), and DECIPHER. Sequences were grouped in 4 sets. Set 1 comprised of 8 bacterial clones classified within the Class Sphingobacteria by all three databases used. Set 2 consisted of 39 bacterial clones classified by all three databases within the genera *Acidovorax.* Set 3 bacterial clones were classified by all three databases within the order Rhizobiales. Set 4 consists of a mixture of bacterial clones mostly found as singletons. For results specific to each database, for all sets of clones, see Table 1.

A phylogenetic analysis of sequences from Set 2 bacterial clones was conducted together with representatives of free living *Acidovorax, Variovorax, Rhodoferax, and Diaphorobacter* species as well as *Verminephrobacter* species, the latter genus known to be symbiotic with Lumbricidae. Phylogenetic trees were estimated using Bayesian analyses as implemented in MrBayes 3.2 (Ronquist & Huelsenbeck 2011). The Bayesian analysis was performed twice, each time for 5 million generations and four chains, sampling one tree every 100 generations. Posterior probabilities were estimated based on all trees sampled after likelihood values stabilized; in both searches likelihoods stabilized at approximately 34,000 trees. Sequences were submitted to Genbank and can be found under accession numbers JQ684104 to JQ684177.

Table 1. Bacterial Clones from the Apothecia of *Scutellinia scutellata.*

Clone	Accession number	Database	Class	Order	Family	Genus
		GenBank	Sphingobacteria	-	-	-
1-1	JQ684104	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-2	JQ684105	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-3	JQ684106	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-4	JQ684107	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-5	JQ684108	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-6	JQ684109	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-7	JQ684110	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
1-8	JQ684111	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
2-1	JQ684112	GenBank	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
to	to	DECIPHER	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
2-39	JQ684150	RDP	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
		GenBank	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Shinella*
3-1	JQ684151	DECIPHER	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Shinella*
		RDP	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Shinella*
		GenBank	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*
3-2	JQ684152	DECIPHER	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Protheco-microbium*

Clone	Accession number	Database	Class	Order	Family	Genus
		RDP	α-Proteobacteria	Rhizobiales	Hyphomicro-biaceae	*Devosia*
		GenBank	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*
3-3	JQ684153	DECIPHER	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*
		RDP	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Rhizobium*
3-4	JQ684154	GenBank	α-Proteobacteria	Rhizobiales	Hyphomicro-biaceae	*Devosia*
to	to	DECIPHER	α-Proteobacteria	Rhizobiales	Hyphomicro-biaceae	*Devosia*
3-6	JQ684156	RDP	α-Proteobacteria	Rhizobiales	Hyphomicro-biaceae	*Devosia*
		GenBank	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Rhodopseud-omonas*
4-1	JQ684157	DECIPHER	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Afipia*
		RDP	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Afipia*
		GenBank	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Rhodopseud-omonas*
4-2	JQ684158	DECIPHER	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Afipia*
		RDP	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Afipia*
		GenBank	α-Proteobacteria	Caulobacteriales	Caulobacte-raceae	*Brevundimonas*
4-3	JQ684159	DECIPHER	α-Proteobacteria	Caulobacteriales	Caulobacte-raceae	*Brevundimonas*
		RDP	α-Proteobacteria	Caulobacteriales	Caulobacte-raceae	*Brevundimonas*
		GenBank	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	*Flexibacter*
4-4	JQ684160	DECIPHER	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	-
		RDP	Sphingobacteria	Sphingobacteriales	-	-
		GenBank	-	-	-	-
4-5	JQ684161	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
4-6	JQ684162	DECIPHER	β-Proteobacteria	Burkholderiales	Comamona-daceae	-
		RDP	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
		GenBank	α-Proteobacteria	Caulobacteriales	Caulobacte-raceae	*Brevundimonas*
4-7	JQ684163	DECIPHER	α-Proteobacteria	Rhizobiales	Brucellaceae	*Mycoplana*
		RDP	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
		GenBank	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	*Flexibacter*

4-8	JQ684164	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	-	-	-
4-9	JQ684165	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	*Flexibacter*
4-10	JQ684166	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-
		GenBank	Sphingobacteria	-	-	-
4-11	JQ684167	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	α-Proteobacteria	Rhizobiales	Rhizobiaceae	*Sinorhizobium*
4-12	JQ684168	DECIPHER	α-Proteobacteria	Rhizobiales	-	-
		RDP	α-Proteobacteria	Rhizobiales	Hyphomicro-biaceae	*Devosia*
		GenBank	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Methylibium*
4-13	JQ684169	DECIPHER	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Methylibium*
		RDP	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Methylibium*
		GenBank	Cyanobacteria	Nostocales	Oscillatoriaceae	*Phormidium*
4-14	JQ684170	DECIPHER	Cyanobacteria	-	-	-
		RDP	Cyanobacteria	-	-	-
		GenBank	Sphingobacteria	-	-	-
4-15	JQ684171	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Limnohabitans*
4-16	JQ684172	DECIPHER	Sphingobacteria	Sphingobacteriales	Chitinopha-gaceae	-
		RDP	β-Proteobacteria	Burkholderiales	Comamona-daceae	*Acidovorax*
		GenBank	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	*Flexibacter*
4-17	JQ684173	DECIPHER	Sphingobacteria	Sphingobacteriales	Cyclobacteri-aceae	-
		RDP	Sphingobacteria	Sphingobacteriales	Cyclobacteri-aceae	*Algoriphagus*
		GenBank	Sphingobacteria	Sphingobacteriales	Saprospiraceae	*Haliscomeno-bacter*
4-18	JQ684174	DECIPHER	Sphingobacteria	Sphingobacteriales	Saprospiraceae	*Haliscomeno-bacter*
		RDP	Sphingobacteria	Sphingobacteriales	Saprospiraceae	*Haliscomeno-bacter*
		GenBank	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Afipia*

Clone	Accession number	Database	Class	Order	Family	Genus
4-19	JQ684175	DECIPHER	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Bosea*
		RDP	α-Proteobacteria	Rhizobiales	Bradyrhizo-biaceae	*Bosea*
		GenBank	Sphingobacteria	Sphingobacteriales	Flexibacteraceae	*Flexibacter*
4-20	JQ684176	DECIPHER	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Sporocyto-phaga*
		RDP	Sphingobacteria	Sphingobacteriales	Cytophagaceae	*Ohtaekwangia*
		GenBank	α-Proteobacteria	Rhizobiales	Hyphomicro-biaceae	*Pedomicrobium*
4-21	JQ684177	DECIPHER	α-Proteobacteria	-	-	-
		RDP	α-Proteobacteria	Rhizobiales	-	-

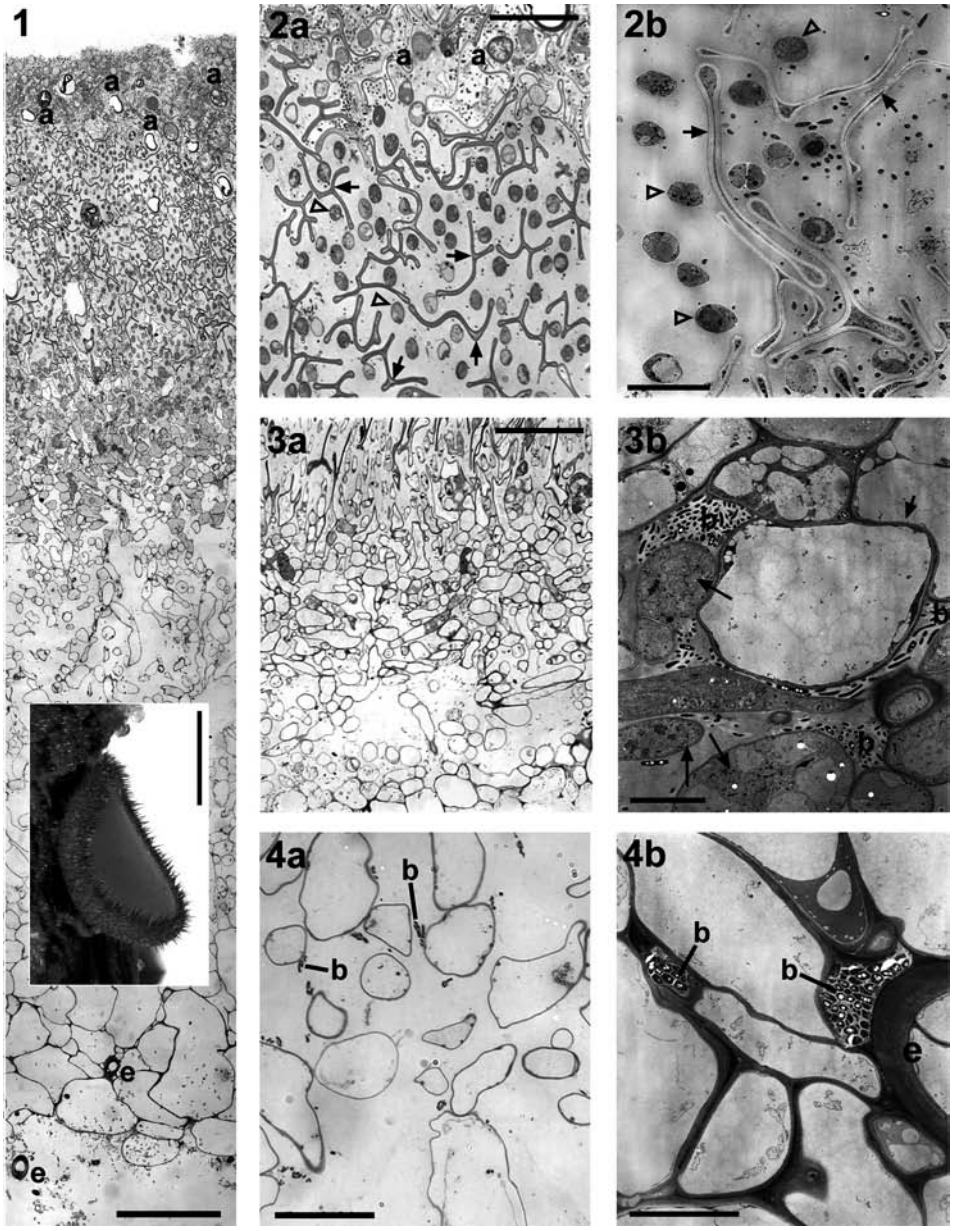
Results

Architecture of *Scutellinia* apothecia: The apothecia of *Scutellinia* are organized into distinct layers from a fleshy basal surface covered by eyelash hairs to the apical hymenial layer containing spores and paraphyses. The basal fleshy layer of the apothecium known as the excipulum has an outer (more basal) layer referred to as the ectal excipulum and an inner (more apical) layer named the medullary excipulum (Figs 1–4). The development of these layers has been described in other discomycetes: *Pyrenopeziza* by Gilles et al. (2001) and *Byssonectria* by Pfister (1993).

Within these well-defined layers, differentiation of tissue types has been observed. These tissue types have been assigned names that describe the arrangement, sizes and shapes of the fungal cells as viewed in tissue sections (Alexopoulos et al. 1996; Korf 1973). In a series of high-resolution images, what has been described as hyphal filaments of *textura intricata* (medullary excipulum) are actually noncontiguous collapsed cell walls that share an extracellular matrix with spherical fungal cells and numerous bacteria (Fig. 2). Ultrastructure of the *textura angularis* (ectal excipulum) of *S. scutellata* reveals that cells in this tissue type are almost completely devoid of cytoplasm but are associated with bacteria – within the confines of the cell walls as shown in Figs 3 and 4 as well as between walls of different cells.

This same series of ultrastructural images shows the progressive loss of cytoplasm and organelles from relatively thick-walled fungal cells (Fig. 2b, arrows). The origin

Fig. 1a. This montage shows a vertical slice through the apothecium from dorsal (top) hymenium with remnants of asci (a) to ventral (bottom) ectal excipulum. Sections of the thick-walled "eyelash" cells are indicated with (e). Scale = 100 μm. Fig. 1b. The entire apothecium of *Scutellinia* grows on a decaying log. The dorsal (hymenial) surface faces to the right. Scale = 2 mm. Fig. 2 a, b. Closer examination of the cells at the interface between the hymenium and medullary excipulum of the apothecium reveal ubiquitous bacteria interspersed among the remnants of asci (a) and paraphyses. Spherical fungal cells with thin cell walls (arrowheads) lie adjacent to the remnants of thick-walled



fungal cells (arrows). Scale for a = 20 μm; Scale for b = 10 μm. Fig. 3 a, b. Closer examination of cells at the interface of the medullary excipulum and ectal excipulum reveal ubiquitous bacteria (b) interspersed among fungal cells, some that have retained their cytoplasm (long arrow) and some that have lost cytoplasm (short arrow). Scale for a = 50 μm; Scale for b = 10 μm. Fig. 4 a, b. Images of cells within the ectal excipulum reveal that bacteria (b) are not located within fungal cells with intact walls and cytoplasm. Sections of thick-walled "eyelash" cells are marked with (e) in Fig.1a and Fig. 4b. Scale for a = 20 μm; Scale for b = 10 μm.

and fate of the separate spherical cells (Fig. 2b, arrowheads) of the *textura intricata* is not clear. These cells have walls that are conspicuously thinner than those of the cells that have collapsed. These thin-walled cells have normal cytoplasm and organelles.

Location of symbiotic bacteria within the apothecium: Without exception, all living specimens contained bacteria among their hyphae. Bacteria are always observed throughout the extracellular matrix of the apothecium (Figs 1–8). Bacteria also reside inside *S. scutellata* cells, but none of these cells can definitively be classified as viable. The intracellular bacteria are observed within the walls of fungal cells, but each of these cells is devoid of most eukaryotic organelles and cytoplasm. Within the confines of some fungal cell walls, high densities of bacterial cells (b) are often observed (Figs 3b, 4b).

Bacteria and ascosporogenesis Ascosporogenesis has been observed in some *S. scutellata* apothecia. Progressive stages in ascospore development are depicted in Figs 5–7. The cytoplasm of a given ascus is first incorporated into ascospore initials, leaving an epiplasm between the wall of the ascus and the walls of the developing spores. While the inner layers of each developing ascospore are laid down by the spore itself, the epiplasm presumably deposits the ornamented, outermost layers of each ascospore. Eventually bacteria invade each ascus and associate with the surface of liberated spores. A view of the hymenial surface of the apothecium reveals the intimate association of the rod-shaped bacteria and *S. scutellata* spores (Fig. 8).

Identity of symbiotic bacteria: During the summer of 2010, a preliminary survey of 16S ribosomal RNA genes amplified from apothecia indicated that the majority of sequences matched a bacterial sequence belonging to the genus *Acidovorax*.

The clone library constructed in 2011 using the 16S ribosomal RNA gene amplified from *S. scutellata* apothecial DNA was composed of 91 clones of which 17 were found to be chimeric; these chimeric clones were removed from the data set (Right et al. 2012). The identity of the remaining 74 clones was determined by comparing the sequences to the nucleotide databases in Genbank, the Ribosomal Database Project (RDP) and DECIPHER (See Table 1 for details). Based on these results, sequences were organized in 4 sets. Set 1 consisted of eight clones that had mean character differences between 0 and 0.28% and were identified as belonging to the genera *Ohtaekwangia* and *Sporocytophaga* from the RDP and DECIPHER databases respectively. Both of these genera are members of the family Cytophagaceae. All three databases identified Set 1 clones as belonging to the Class Sphingobacteria. Set 2 consisted of 39 clones that were identified by all three databases as belonging to the genus *Acidovorax*. The mean character differences between clones in Set 2 were found to be between 0 and 6.3%. Set 3 clones were identified by all 3 databases as belonging to the order Rhizobiales and family Rhizobiaceae with the exception of one clone that was determined by the RDP to belong to Hyphomicrobiaceae in the order Rhizobiales. Three of the clones in this set were identified as belonging to the genus *Devosia* by all three databases. The mean character differences between clones in Set 3 were found to be between 3.5 and 12.2%. The clones in Set 4 comprise a more diverse group from those in the previous sets; and they include members in the α- and β-Proteobacteria as well as Sphingobacteria and

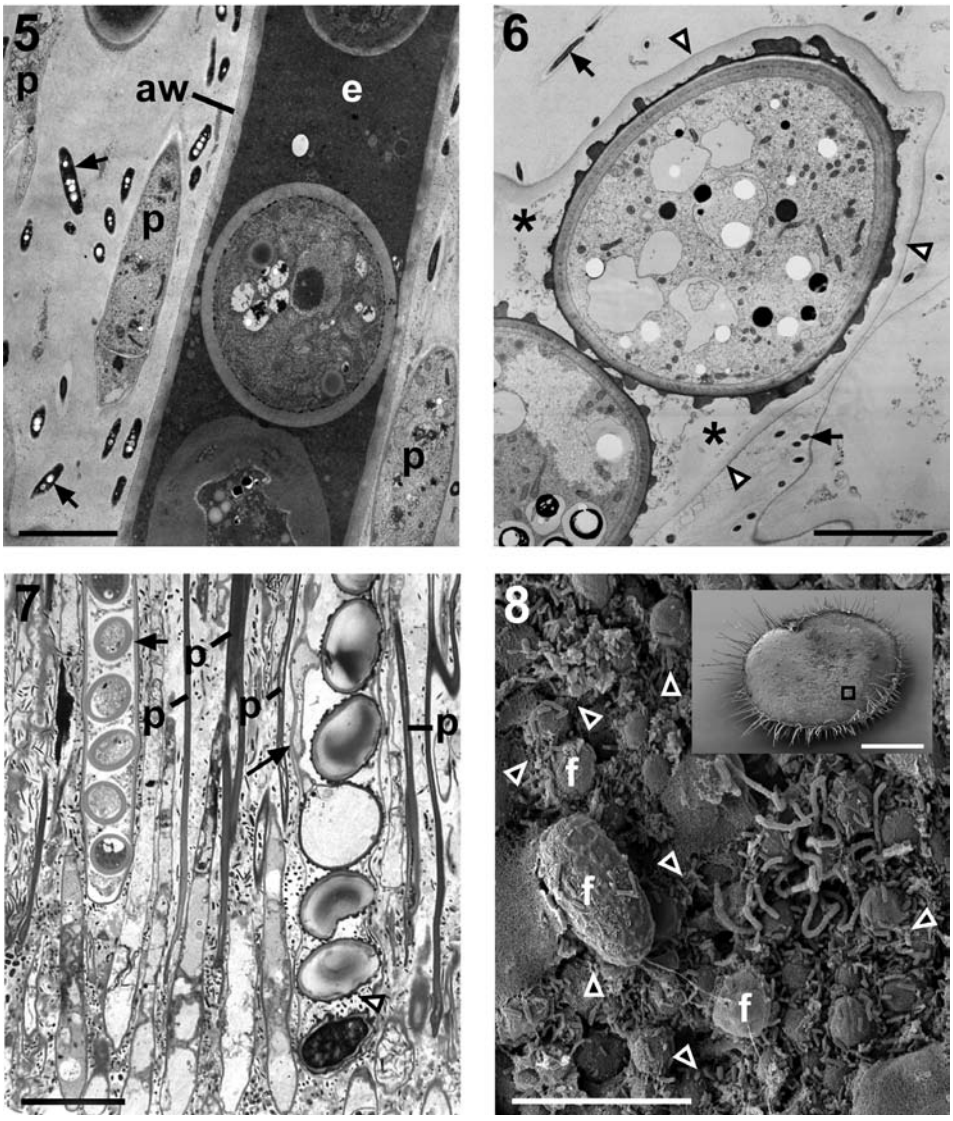


Fig. 5. A developing ascus is shown on the right side of the figure with portions of three ascospores enshrouded by the epiplasm (e) and ascus wall (aw). The ascus is surrounded by paraphyses (p) and bacteria (small arrows). Scale = 5 µm. Fig. 6. These mature ascopores with surface ornamentation lie within an ascus that has lost its epiplasm. The space once occupied by epiplasm is marked with asterisks. Bacteria (small arrows) surround the outer wall of the ascus (arrowheads) but do not lie within the ascus. Scale = 5 µm. Fig. 7. Two asci at different stages of development are surrounded by paraphyses (p) and numerous bacteria. Both asci have lost their epiplasm. The more mature ascus on the right (large arrow) whose spores have surface ornamentation has been invaded by bacteria (arrowhead); the interior of the less mature ascus on the left (small arrow), however, shows no evident bacteria. Scale = 20 µm. Fig. 8. In this surface view of the hymenial surface of the apothecium, numerous bacteria are interspersed with the larger fungal cells and spores (f). Representative, ubiquitous bacteria are indicated with several arrowheads. The location of this image is marked on the global view of the *Scutellinia* hymenium (inset, bar = 1 mm). Scale = 20 µm.

a single Cyanobacteria (Table 1). Sets 1, 2, 3 and 4 respectively represented 11, 52, 8 and 28% of the total number of clones sequenced. The members of Set 2 identified as belonging to the genus *Acidovorax* represented the largest proportion of clones sequenced from apothecia of *S. scutellata*.

A Bayesian phylogenetic analysis using the *Acidovorax* sp. clones from Set 2, free living *Acidovorax*, other closely related species, and representatives of the genus *Verminephrobacter* indicates that the *Acidovorax* sp. from *S. scutellata* and the *Verminephrobacter* sp. from the earthworm family Lumbricidae are derived from different lineages of free living *Acidovorax* species and that they are sister groups (Fig. 9).

Culturing *Scutellinia* hyphae in the absence of bacteria: Growth of *S. scutellata* hyphae without bacterial symbionts at present has not been realized *in vitro*, implying an important role of bacteria in the life cycle of this fungus.

Discussion

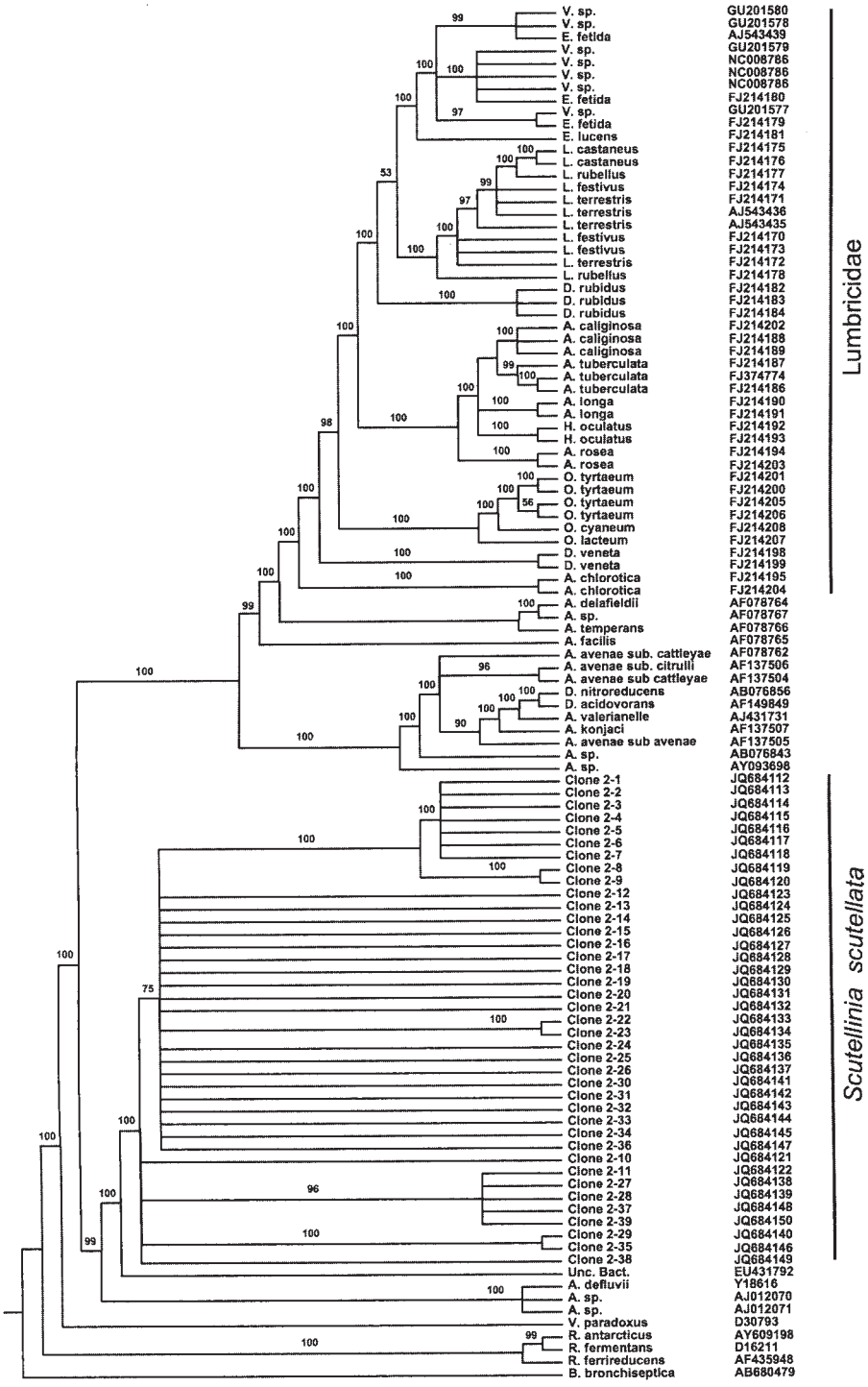
A community of bacteria inhabits each apothecium of *S. scutellinia*. The host environment of the apothecium may provide a stable environment for a well-defined set of bacterial species. Interactions among these microbes – both fungal and bacterial – may also be well defined and represented by an assemblage of mutually interdependent organisms. Each apothecium may represent a super-organism made up of these interdependent microbes. Such foci of microbes can facilitate exchange of genetic material among bacterial species as well as between bacteria and host fungus, increasing the genetic diversity of bacteria in the fungal host and the adjacent soil environment, as well as increasing genetic diversity of the fungus itself.

Intracellular symbionts or extracellular symbionts? The development and physiology of a fungus that serves as a host for symbiotic bacteria can be affected in a variety of ways whether the bacteria are entirely intracellular, extracellular, or both. Successful dispersal and vertical transmission of bacteria to new fungal hosts can also be achieved whether these bacteria are intracellular or extracellular.

Although published figures show bacteria present inside hyphal cells (Partida-Martinez & Hertweck 2005; Nurmiaho-Lassila et al. 1997; Garbaye 1994), these images do not convincingly demonstrate that these intracellular bacteria actually inhabit living fungal cells.

The ultrastructural images of arbuscular mycorrhizal cells provided by Bianciotto et al. (1996) as well as the mycorrhizal fungi in the order Sebacinales (Sharma et al.

Fig. 9. Bayesian tree based on partial (1448 bp) 16S sequences. Values above branches are posterior probabilities based on 34,000 trees. GenBank sequences of bacterial symbionts of earthworms are designated under Lumbricidae. Bacterial clones from this study are designated under *S. scutellata*. The bacterium *Bordetella bronchiseptica* was used as the outgroup.


V. sp. GU201580
V. sp. GU201578
E. fetida AJ543439
V. sp. GU201579
V. sp. NC008786
V. sp. NC008786
V. sp. NC008786
E. fetida FJ214180
V. sp. GU201577
E. fetida FJ214179
E. lucens FJ214181
L. castaneus FJ214175
L. castaneus FJ214176
L. rubellus FJ214177
L. festivus FJ214174
L. terrestris FJ214171
L. terrestris AJ543436
L. terrestris AJ543435
L. festivus FJ214170
L. festivus FJ214173
L. terrestris FJ214172
L. rubellus FJ214178
D. rubidus FJ214182
D. rubidus FJ214183
D. rubidus FJ214184
A. caliginosa FJ214202
A. caliginosa FJ214188
A. caliginosa FJ214189
A. tuberculata FJ214187
A. tuberculata FJ374774
A. tuberculata FJ214186
A. longa FJ214190
A. longa FJ214191
H. oculatus FJ214192
H. oculatus FJ214193
A. rosea FJ214194
A. rosea FJ214203
O. tyrtaeum FJ214201
O. tyrtaeum FJ214200
O. tyrtaeum FJ214205
O. tyrtaeum FJ214206
O. cyaneum FJ214208
O. lacteum FJ214207
D. veneta FJ214198
D. veneta FJ214199
A. chlorotica FJ214195
A. chlorotica FJ214204
A. delafieldii AF078764
A. sp. AF078767
A. temperans AF078766
A. facilis AF078765
A. avenae sub. cattleyae AF078762
A. avenae sub. citrulli AF137506
A. avenae sub cattleyae AF137504
D. nitroreducens AB076856
D. acidovorans AF149849
A. valerianelle AJ431731
A. konjaci AF137507
A. avenae sub avenae AF137505
A. sp. AB076843
A. sp. AY093698
Clone 2-1 JQ684112
Clone 2-2 JQ684113
Clone 2-3 JQ684114
Clone 2-4 JQ684115
Clone 2-5 JQ684116
Clone 2-6 JQ684117
Clone 2-7 JQ684118
Clone 2-8 JQ684119
Clone 2-9 JQ684120
Clone 2-12 JQ684123
Clone 2-13 JQ684124
Clone 2-14 JQ684125
Clone 2-15 JQ684126
Clone 2-16 JQ684127
Clone 2-17 JQ684128
Clone 2-18 JQ684129
Clone 2-19 JQ684130
Clone 2-20 JQ684131
Clone 2-21 JQ684132
Clone 2-22 JQ684133
Clone 2-23 JQ684134
Clone 2-24 JQ684135
Clone 2-25 JQ684136
Clone 2-26 JQ684137
Clone 2-30 JQ684141
Clone 2-31 JQ684142
Clone 2-32 JQ684143
Clone 2-33 JQ684144
Clone 2-34 JQ684145
Clone 2-36 JQ684147
Clone 2-10 JQ684121
Clone 2-11 JQ684122
Clone 2-27 JQ684138
Clone 2-28 JQ684139
Clone 2-37 JQ684148
Clone 2-39 JQ684150
Clone 2-29 JQ684140
Clone 2-35 JQ684146
Clone 2-38 JQ684149
Unc. Bact. EU431792
A. defluvii Y18616
A. sp. AJ012070
A. sp. AJ012071
V. paradoxus D30793
R. antarcticus AY609198
R. fermentans D16211
R. ferrireducens AF435948
B. bronchiseptica AB680479
Lumbricidae
Scutellinia scutellata

2008), however, show bacteria encased in membranes within living fungal cytoplasm. Arbuscular mycorrhizae are known to harbor obligate endosymbionts in their spores, vegetative mycelium and germ tubes (Lumini et al. 2006; MacDonald & Chandler 1981); however, convincing evidence has been presented that hyphae of only certain ectomycorrhizal fungi also harbor intracellular bacteria (Bertaux et al. 2005).

Although the intracellular bacteria of fungal endophytes have not been examined ultrastructurally, these bacteria also reside in living fungal cells as evidenced by using a combination of not only in situ hybridization to label bacterial cells but also Live/Dead staining to confirm the viability of the bacterial cells and their fungal host cells. Endophytic members of four classes of Ascomycota have been shown to harbor diverse bacteria within their hyphal cells (Hoffman & Arnold 2010).

Ultrastructural examination of fungal and bacterial cells in several apothecia of *S. scutellinia* revealed no instances of bacteria occurring within living fungal cells. However, throughout each apothecium, bacterial symbionts occupied all extracellular spaces and the remains of once living fungal cells. The high densities of bacterial cells (b) within some of the walls of non-living fungal cells (Figs 3b, 4b) suggests that the degenerating fungal cells offer nutrients that are being recycled for proliferation of bacteria. During maturation of asci of *S. scutellinia*, bacteria adhere to surfaces of enclosed spores (Figs 7, 8).

Relationship of extracellular symbionts of *Scutellinia* to other symbionts: Over half of the clones sequenced (52%) represent bacteria associated with fungi inhabiting forest detritus that are related to a group of bacteria associated with animals (earthworms) inhabiting the same detritus. How widespread this symbiotic association of bacteria and saprophytic fungi is remains unclear; but among earthworms, symbiotic bacteria seem to be universally present. Dense populations of bacteria reside as symbionts in the nephridia of earthworms. The sequences of 16S rRNA genes of these *Acidovorax*-like symbionts form a monophyletic group within the genus *Acidovorax* (Fig. 9). While other members of the genus *Acidovorax* are free living or plant pathogenic, this group of earthworm symbionts has been assigned membership in the newly named genus *Verminephrobacter* (Schramm et al. 2003).

Members of the family Cytophagaceae (order Sphingobacteriales) represent 11% of the clones, and members of the order Rhizobiales represent an additional 8% of clones sequenced. The former are widely distributed in soils, and the latter are well-known nitrogen-fixing symbionts (Staley et al. 2007).

Effects of bacterial symbionts on fungal hosts: Repeated attempts to culture mycelium of *S. scutellata* in the presence of antibiotics failed. However, ascospores of several species of *Scutellinia*, including *S. scutellata*, germinate and hyphae have been reported to grow on potato dextrose agar in the absence of antibiotics (Schumacher 1990). These culturing results imply an influence of bacteria on the growth of *Scutellinia*, although the exact effect(s) of the bacterial relationship on the fungus can only be inferred from the diversity of effects reported for bacterial associates of other fungi. Based on knowledge of the importance of bacterial symbionts to other fungi as enumerated in

the following two paragraphs, several influences of bacteria on the development and physiology of *S. scutellinia* are possible.

Indirect effects of bacterial symbionts could silence or induce fungal gene expression: A non-pathogenic strain of *Fusarium* is associated with ectosymbiotic bacteria that effectively silence certain fungal genes involved in the pathogenesis of *Fusarium*. Removal of these ectosymbiotic bacteria resulted in restoration of the pathogenic properties of *Fusarium* (Minerdi et al. 2008). Schrey et al. (2005) and Poole et al. (2001) using dual cultures of bacteria and hyphae showed that bacteria promote fungal growth of ectomycorrhizae. The same bacterial isolates that stimulate growth of ectomycorrhizal hyphae have been shown to inhibit growth of pathogenic fungi such as *Heterobasidion* and *Armillaria* (Maier et al. 2004).

Bacterial symbionts could exert direct effects on fungal fitness: Bacteria that colonize the mycelial surface have also been implicated in the induction of fruit body formation (Noble et al. 2009; Cho et al. 2003; Rainey et al. 1990). Bacteria might stimulate spore germination (Roesti et al. 2005; Citterio et al. 2001; Filipi et al. 1998). The bacterial symbionts also have the potentiality to confer nutritional benefits to their hosts, including fixing nitrogen and producing enzymes that increase access to lignocellulose-rich substrates (Leveau & Preston 2008; de Boer et al. 2005).

Changing views of symbioses: The pervasiveness of symbioses that span kingdoms of organisms may be greater than has been conventionally believed. New examples of cross-Kingdom mutualisms are continually emerging as cellular features of organisms are viewed at higher resolution. Many mutualisms initially thought to extend across two Kingdoms are now known to extend across a third Kingdom. The presence of intracellular bacteria in cells of both mycorrhizal fungi and endophytic fungi represents a tripartite symbiosis among the Plant, Fungal and Bacterial Kingdoms (Hoffman & Arnold 2010; Sharma et al. 2008). Mutualistic associations of fungi and insects also encompass a third association with actinobacteria that selectively maintain and promote the fungal food source of the insects by suppressing the growth of competing fungi in the same environment (Scott et al. 2008; Currie 2001).

Based on sequencing of 74 bacterial clones, over half (52%) of the extracellular bacteria that associate with apothecia of *S. scutellata* have sequences that resemble those of another group of extracellular symbiotic bacteria associated with cells of earthworm nephridia (Schramm et al. 2003). Another set of sequences (8%) resembles those of nodule-forming, nitrogen-fixing Rhizobiales that associate with roots of plants. Most bacteria associated with *S. scutellata* therefore represent a versatile group of microbes that have also established symbiotic relationships with soil organisms in both the Animal and Plant Kingdoms.

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