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ORIGINAL ARTICLE

A novel symbiosis between chemoautotrophic bacteria and a freshwater cave amphipod

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Symbioses involving animals and chemoautotrophic bacteria form the foundation of entire ecosystems at deep-sea hydrothermal vents and cold seeps, but have so far not been reported in terrestrial or freshwater environments. A rare example of a terrestrial ecosystem sustained by chemoautotrophy is found within the sulfide-rich Frasassi limestone cave complex of central Italy. In this study, we report the discovery of abundant filamentous bacteria on the exoskeleton of *Niphargus ictus*, a macroinvertebrate endemic to Frasassi. Using 16S rDNA sequencing and fluorescence *in situ* hybridization (FISH), we show that *N. ictus* throughout the large cave complex are colonized by a single phylotype of bacteria in the sulfur-oxidizing clade *Thiothrix*. The epibiont phylotype is distinct from *Thiothrix* phylotypes that form conspicuous biofilms in the cave streams and pools inhabited by *N. ictus*. Using a combination of ^{13}C labeling, FISH, and secondary ion mass spectrometry (SIMS), we show that the epibiotic *Thiothrix* are autotrophic, establishing the first known example of a non-marine chemoautotroph-animal symbiosis. Conditions supporting chemoautotrophy, and the *N. ictus*-*Thiothrix* association, likely commenced in the Frasassi cave complex between 350 000 and 1 million years ago. Therefore, the *N. ictus*-*Thiothrix* symbiosis is probably significantly younger than marine chemoautotrophic symbioses, many of which have been evolving for tens to hundreds of million years.

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Introduction

Symbioses play a pivotal role in the evolution of life on our planet. Novel symbiotic associations can substantially alter metabolic capabilities of the partners involved, with important evolutionary and ecological ramifications. In sulfide-rich marine habitats such as at hydrothermal vents, methane seeps and organic-rich coastal margins, invertebrates that are symbiotic with sulfur-oxidizing chemoautotrophic bacteria are ubiquitous, and often numerically and ecologically dominant (Van Dover *et al.*, 2002; Dubilier *et al.*, 2008). Such symbioses have evolved independently in seven different metazoan phyla, and vary in nature from epibiotic to intracellular associations. Sulfidic habitats are also found in terrestrial limestone caves, such as Movile cave in Romania (Sarbu *et al.*, 1996) and the Frasassi cave system in Italy (Sarbu *et al.*, 2000). Some sulfidic caves, including Frasassi, are isolated from surface photosynthetic primary productivity and contain ecosystems entirely based on microbial chemoautotrophy (Sarbu *et al.*, 2000). Initial descriptions of these cave ecosystems noted the absence of chemoautotrophic symbioses despite other ecological and geochemical similarities with marine vents and seeps (Sarbu *et al.*, 1996; Forti *et al.*, 2002). Here, we report for the first time that *Niphargus ictus*, a macroinvertebrate belonging to the Frasassi cave ecosystem, is symbiotic with filamentous sulfur-oxidizing chemoautotrophic bacteria of the clade *Thiothrix*. Although several symbioses between animals and chemoautotrophic bacteria have been discovered in marine

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environments, to the best of our knowledge, this is the first known example from a freshwater or terrestrial habitat.

The Frasassi cave complex is actively forming by sulfuric acid dissolution of the limestone host rock. The sulfuric acid is generated by microbial and abiotic oxidation of sulfide dissolved in water rising from a deep aquifer (Sarbu *et al.*, 2000). The cave complex contains over 23 km of passages, including numerous sulfidic streams and lakes accessible by technical caving routes. Sulfide and oxygen concentrations range over an order of magnitude within the cave system due to seasonal and spatial variability in the dilution of sulfidic groundwater by oxygenated meteoric water. Conspicuous mat-like white biofilms cover surfaces near the water table where sulfidic and oxygenated waters mix, and where sulfidic springs contact the oxygenated cave air (Supplementary Figure 1). Detailed studies conducted earlier by our group revealed that the biofilms are predominantly composed of sulfur-cycling bacteria within the β-, δ-, γ-, and ε-proteobacterial clades (Macalady *et al.*, 2006). Specifically, filamentous *Thiothrix*, *Beggiatoa*, and ε-proteobacteria dominate the biomass of microbial biofilms, inhabiting separate niches defined by water chemistry and stream flow characteristics (Macalady *et al.*, 2008).

Microbial chemoautotrophy within Frasassi supports a rich ecosystem that includes several species of macroinvertebrates (Sarbu *et al.*, 2000). The macroinvertebrate that dominates the biomass of Frasassi cave waters is an amphipod species called *N. ictus*, which is endemic to the Frasassi cave ecosystem (Bertolani *et al.*, 1994; Sarbu *et al.*, 2000). This amphipod is typically found in large numbers in Frasassi cave lakes and streams, including lakes located deep within the cave system, more than 500 m interior to the natural cave entrances. Our initial microscopic observations of *N. ictus* revealed abundant filamentous bacteria attached to its exoskeleton. The goal of our study was to characterize the nature of the bacteria colonizing the amphipod exoskeleton, and we used a combination of 16 s rDNA sequencing, fluorescence *in situ* hybridization (FISH), ^{13}C labeling, and secondary ion mass spectrometry (SIMS) to find that amphipods throughout the cave system are colonized by a single phylotype of chemoautotrophic bacteria belonging to the sulfur-cycling clade *Thiothrix*.

Materials and methods

Sample collection

The Grotta Grande del Vento-Grotta del Fiume (Frasassi) cave system is located in the Marches region of central Italy. For a detailed description of the geochemistry and microbial communities within the cave system, please refer to Macalady *et al.* (2008) and Macalady *et al.* (2006). *N. ictus* and biofilms were collected in August 2006, December 2006, May–June 2007, and December 2008 from seven different locations within the Frasassi cave system (see Supplementary Figure 2 for map of the Frasassi cave system and location of collection sites). Samples for FISH and clone library construction were collected into sterile tubes, stored on ice, and transferred to four parts RNAlater (Ambion/Applied Biosystems, Foster City, CA, USA) to one part sample (v/v) within 4–6 h after collection, and stored at −20 °C until further analysis. *N. ictus* individuals used for scanning electron microscopy were transferred to a 2.5% glutaraldehyde solution made in phosphate buffer saline (PBS), and stored at 4 °C until analysis. Dissolved sulfide and oxygen concentrations of cave waters at the various study sites were determined as described earlier (Macalady *et al.*, 2008).

Clone library and phylogenetic analyses

DNA was obtained from three intact *N. ictus* individuals collected from Lago Verde (see Supplementary Figure 2 for location) as described in Bond *et al.* (2000) except that bead beating was replaced by three freeze-thaw cycles (3 min at −197 °C, 5 min at 80 °C). Libraries were constructed using the bacterial domain-specific primer 27f and universal primer 1492r. The 50-μl reaction mixture contained: environmental DNA template (~50 ng), 1.25 U Ex-Taq DNA polymerase (TaKaRa Bio Inc., Shiga, Japan), 0.2 mM each dNTPs, 1 × PCR buffer, 0.2 μM 1492r primer (5′-GGT TAC CTT GTT ACG ACT T-3′) and 0.2 μM 27f primer (5′-AGA GTT TGA TCC TGG CTC AG-3′). Thermal cycling was as follows: initial denaturation 5 min at 94 °C, 42 cycles of 94 °C for 1 min, 50 °C for 25 s and 72 °C for 2 min, followed by a final elongation at 72 °C for 20 min. The PCR products were cloned into the pCR4-TOPO plasmid and used to transform chemically competent One-Shot MACH1 T1 *E. coli* cells as specified by the manufacturer (TOPO TA cloning kit, Invitrogen, Carlsbad, CA, USA). Colonies containing inserts were isolated by streak-plating onto LB agar containing 50 μg/mL kanamycin. Plasmid inserts were screened using colony PCR with M13 primers (5′-CAG GAA ACA GCT ATG AC-3′ and 5′-GTA AAA CGA CGG CCA G-3′). Colony PCR products of the correct size were purified using the QIAquick PCR purification kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. Clones were sequenced at the Penn State University Biotechnology Center using T3 and T7 plasmid-specific primers. Sequences were assembled with Phred base calling using CodonCode Aligner v.1.2.4 (CodonCode Corp., Dedham, MA, USA) and manually checked for ambiguities. The nearly full-length gene sequences were compared against sequences in public databases using BLAST (Altschul *et al.*, 1990) and submitted to the online analyses CHIMERA_CHECK v.2.7 (Cole *et al.*, 2003)

and Bellerophon 3 (Huber *et al.*, 2004). Putative chimeras were excluded from subsequent analyses.

A total of 28 non-chimeric 16S rDNA sequences were obtained (Genbank accession numbers EU884085–EU884112), aligned using the NAST aligner (DeSantis *et al.*, 2006), and added to an existing alignment containing >150,000 nearly full length bacterial sequences in ARB (Ludwig *et al.*, 2004). The alignments were manually refined using the ARB sequence editor, and minimized using the Lane mask (1286 nucleotide positions) (Lane, 1991). Phylogenetic trees were computed using maximum parsimony (1000 bootstrap replicates), maximum likelihood (general time reversible model, site-specific rates and estimated base frequencies) and neighbor joining (general time reversible model). All trees were computed using the software program PAUP* (Swofford, 2000).

Fluorescence in situ Hybridization

Paired *N. ictus* and biofilm samples were collected from multiple sample sites within the cave (see Supplementary Figure 2 and Table 1 for sample locations and sizes). Samples were fixed in 4% paraformaldehyde, transferred to a 1:1 ethanol-PBS solution, and stored at −20 °C. *N. ictus* pereopods were dissected and sonicated briefly to separate bacterial filaments, or in some cases, whole pereopods were used intact for FISH. The epibiont-specific probe NSB1185 was designed and evaluated as described in Hugenholtz *et al.* (2001), including checks against all publicly available sequences using megaBLAST searches of the non-redundant databases at NCBI. FISH experiments were carried out as described in Amann (1995) using the epibiont-specific probe NSB1185 (5′-CTT GCT TCC CTC TGT ACC-3′) and a 1:1:1 mix (EUBMIX) of the bacterial domain probes EUB338 (5′-GCT GCC TCC CGT AGG AGT-3′), EUB338-II (5′-GCA GCC ACC CGT AGG TGT-3′) and EUB338-III (5′-GCT GCC ACC CGT AGG TGT-3′). Oligonucleotide probes were synthesized and labeled at the 5′ ends with fluorescent dyes (Cy3 and FLC) at Sigma-Genosys (St Louis, MO, USA). Hybridization stringencies were determined using positive and negative controls in experiments with formamide concentrations from 0 to 50%. Optimal formamide concentration for NSB1185 (42%) was determined using a pure culture of *Thiothrix eikelboomii* (negative control, 1-bp mismatch). Cells were counterstained after hybridization with 4′,6′-diamidino-2-phenylindole, mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and viewed on a Nikon E800 epifluorescence microscope (Nikon Instruments Inc., Melville, NY, USA). Images were collected and analyzed using NIS Elements AR 2.30, Hotfix (Build 312) image analysis software (Nikon Instruments Inc.).

^{13}C labeling and FISH-SIMS

Three *N. ictus* were collected from Lago Verde and incubated for 24 h with 25 ml of filter-sterilized sulfide-rich cave water containing 6 mg of $NaH^{13}CO_3$. Three control animals were incubated in 25 ml of filter-sterilized cave water for the same time period. At the end of the incubation period, animals were fixed using RNAlater and stored at −20 °C until further analysis. *N. ictus* individuals were washed three times in 1× PBS. They were then fixed for 24 h in 4% paraformaldehyde in PBS, washed again in PBS, and finally stored in 1:1 (v:v) PBS:ethanol solution. Bacterial filaments were separated from *N. ictus* using sonication and were loaded onto a

Table 1 Summary of FISH results and sample characteristics. Dominant microbial clades, and oxygen and sulfide concentrations were determined as described earlier (Macalady *et al.*, 2008)

Site name	*Description*	*Predominant biofilm clade*	*Collection date*	*[O_2] (μM)*	*[H_2S] (μM)*	*FISH analyses (NSB1185 probe)*			
						N. ictus		*Biofilms*	
						Sample size	*Probe binding*	*Sample size*	*Probe binding*
Cave Spring	Spring exiting cave	*Thiothrix*	August 2006	6.9	154	1	+	2	–
Fissure Spring	Spring exiting cave	ε-Proteobacteria	August 2006	2.8	447	1	+	1	–[a]
Grotta Sulfurea	Cave stream	*Thiothrix*, *Beggiatoa*	May 2007	1.2	201	4	+	3	–[a]
Lago Blanco	Stratified lake[b]	No biofilms present	June 2007	ND	ND	2	+		
Lago Verde	Stratified lake[b]	No biofilms present	December 2006	ND	ND	1	+		
			May 2007	3.6	301	3	+		
			December 2008	ND	ND	6	+		
Pozzo di Cristalli	Cave stream	ε-Proteobacteria	August 2006	0.2	322	1	+	1	–
			May 2007	2.5	542	4	+	3	–
Ramo Sulfureo	Cave stream	*Thiothrix*, *Beggiatoa*	August 2006	1.0	195	1	+	1	–
			May 2007	1.6	240	2	+	2	–

Abbreviation: ND, not determined.
[a]Binding to <1% filaments in biofilm.
[b]Sulfidic bottom layer, oxygenated top layer.

silicon wafer. Filaments were identified and mapped by hybridization with the FISH probes EUBMIX and NSB1185 (as described above). Carbon isotope values of filaments were obtained using an ion microprobe/secondary ion mass spectrometer at the University of Wisconsin (Wisc-SIMS; CAMECA IMS-1280). Samples were sputtered using a focused Cs^+ micro-beam (2pA, 1 μm diameter) scanned over 5–25 μm square areas. Negative secondary ions of ^{12}C and ^{13}C were accelerated by −10 kV at the sample surface, and their mass was detected using two electron multipliers in ion counting mode. A small electron multiplier (Hamamatsu, Bridgewater, NJ, USA) on a multi-collection trolley was used for ^{12}C and a conventional electron multiplier (ETP) in the axial position was used for ^{13}C. Mass resolution was set to ~2000 for ^{12}C and ~3000 for ^{13}C, to completely remove ^{12}CH interference from ^{13}C. Carbon isotope images ($64 \times 64 = 4096$ pixels) were simultaneously obtained for ^{12}C and ^{13}C. For each area of interest, a 25-μm square area was scanned for 200 s to identify the shape of the filament. Then, 5 μm square areas were scanned for 12–20 min (4 min for one cycle, integrated for 3–5 cycles). Non-filamentous objects on silicon wafers containing labeled epibiont samples were also targeted for analysis. Raw data files were processed using ImageJ software to obtain ^{12}C and ^{13}C total counts, and $^{13}C/^{12}C$ isotope ratio maps. For each filament analyzed, two 1-μm square areas were chosen within the analyzed filament area and data combined to calculate C isotope ratios.

Maintenance of Niphargus ictus in captivity

N. ictus were collected from Lago Verde and maintained in an aquarium containing cave sediment and sulfide-rich cave water, and kept in the dark at the *in situ* temperature (13 °C). Sulfide-rich cave water was added to the aquarium every 30–45 days.

Sulfide and oxygen profiles were obtained in the aquarium at the end of 3 months. Sulfide concentrations changed exponentially from <0.15 μM (detection limit of sulfide test) at the air–water interface to 1.2 mM at the water–sediment interface. Oxygen was at saturation levels at the air–water interface, whereas it was <6 μM at the water–sediment interface. Two *N. ictus* individuals were sacrificed after the profiles were obtained, and analyzed for the presence of *Thiothrix* epibionts using FISH with the oligonucleotide probe NSB1185 as described above.

Results

Microscopy

Five *N. ictus* individuals collected from various study sites (two from Fissure Spring, one from Pozzo di Cristalli, and two from Grotta sulfurea; see map in Supplementary Figure 2 for site locations) were examined using scanning electron microscopy. The exoskeleton of all five animals contained abundant 'rosettes' of filamentous microorganisms, located primarily at the joints of appendages (Supplementary Figure 3). Bacterial filaments were attached at the base of most of the spines and hairs on the amphipod appendages, including antennae and 'gnathopods' (appendages used by the amphipod for grooming and feeding). Phase contrast microscopy revealed that the 1–2 micron diameter filaments contain copious internal sulfur globules and have morphology consistent with *Thiothrix*, a clade of sulfur-oxidizing γ-Proteobacteria (Supplementary Figure 4).

16S rDNA clone library

To identify the filamentous epibionts, we constructed a bacterial 16S rDNA library from three intact *N. ictus* individuals. The library contained 26 clones belonging to a single phylotype of *Thiothrix* (Figure 1) and two clones related to filamentous, Sulfuricurvales-group ε-proteobacteria (Supplementary Figure 5). The *Thiothrix* sequences had >0.99 nucleotide identity to each other, and formed a coherent clade in maximum likelihood, maximum parsimony (63% bootstrap support), and neighbor joining phylogenies (78% bootstrap support). Surprisingly, the *Niphargus*-associated *Thiothrix* clade did not include any of the numerous *Thiothrix* sequences retrieved from Frasassi biofilms collected in *Niphargus* habitats (Figure 1). The nucleotide identity between epibiotic *Thiothrix* and biofilm sequences ranged from 88.5–99.7%.

Fluorescence in situ hybridization

To confirm that the filamentous *Niphargus* epibionts correspond to the *Thiothrix* phylotype from the 16S rDNA library, we designed a fluorescently labeled oligonucleotide probe (NSB1185) that binds uniquely to the *Niphargus*-associated *Thiothrix* rRNA (Supplementary Figure 6). We then used this probe along with probes designed to bind to all bacteria to test the specificity of the *Niphargus-Thiothrix* association in *Niphargus* populations throughout the Frasassi cave system. The stringency of the hybridization conditions was optimized and checked for each hybridization using a pure culture of *Thiothrix eikelboomii*, whose 16S rRNA sequence has a single nucleotide mismatch to NSB1185 (Supplementary Figure 6). Environmental sequences retrieved from Frasassi biofilms have 2–3 nucleotide mismatches to the probe. We performed FISH with 26 *N. ictus* individuals collected from seven locations within Frasassi (Supplementary Figure 2; Table 1). The NSB1185 probe bound strongly to epibiotic bacteria on all amphipods we examined (Figure 2, Table 1 and Supplementary Figure 7), and did not bind to *Thiothrix eikelboomii* controls. All epibiotic filaments that bound to bacterial domain probes also bound to NSB1185, suggesting that the two ε-proteobacterial clones obtained in the 16S

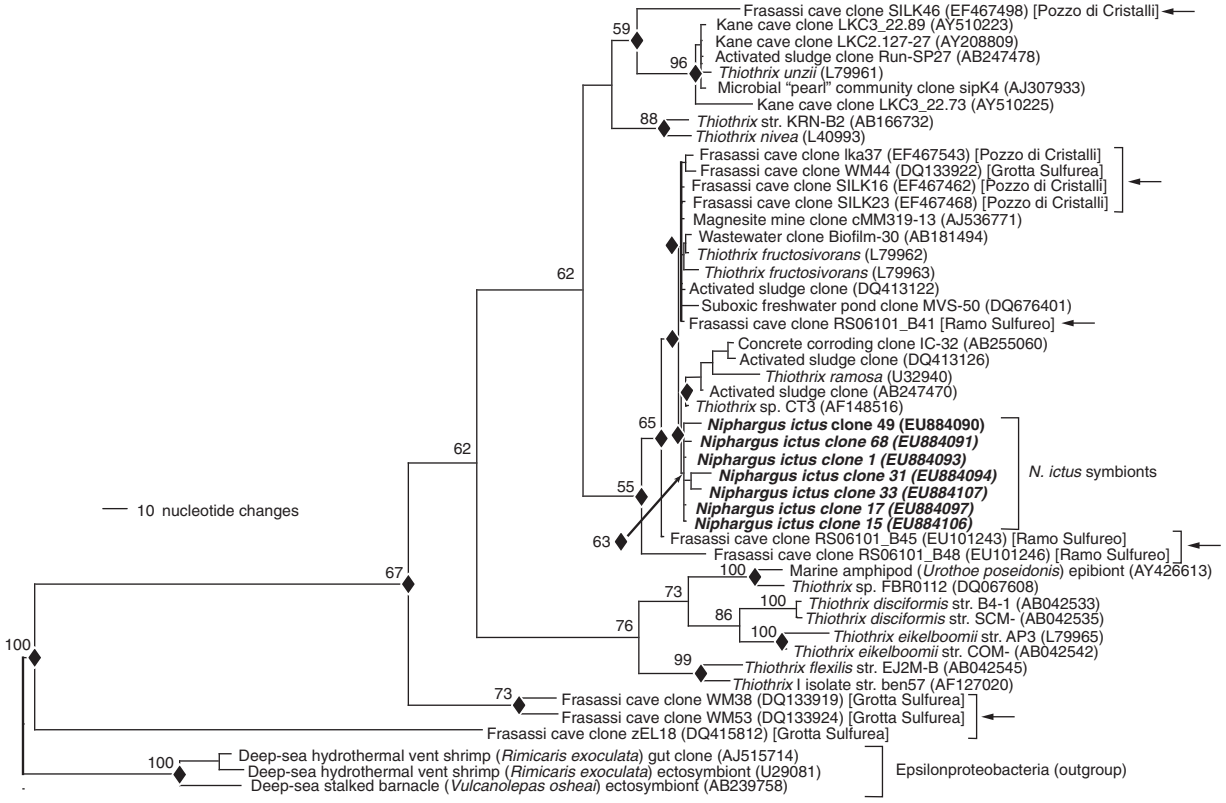


Figure 1 16S rDNA-based phylogenetic tree showing relationships among Niphargus ictus clones (shown in bold type) and cultivated and uncultivated strains in the Thiothrix clade of γ-proteobacteria. Sequences retrieved from 5 microbial biofilms in *N. ictus* habitats within the Frasassi cave system are indicated by arrows. Sample sites are indicated in brackets (see map in Supplementary Figure 2 for locations). The tree was generated using a maximum parsimony method with 1000 bootstrap replicates. Bootstrap values > 50%, showing support for the branching order, are shown. Black diamonds indicate nodes present in the maximum likelihood phylogeny. GenBank accession numbers are listed in parentheses. The scale bar indicates 10 nucleotide changes.

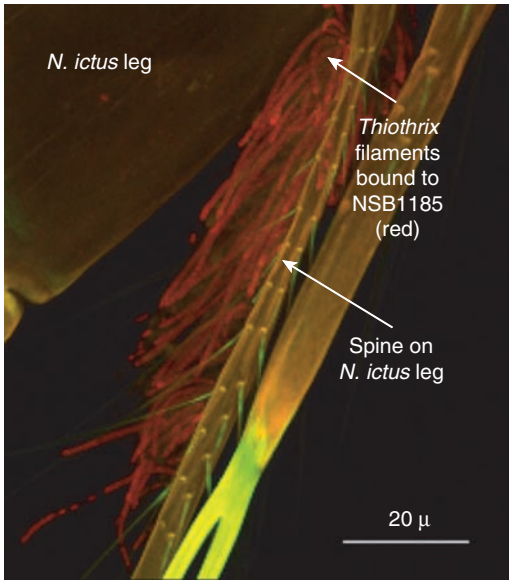


Figure 2 Confocal epifluorescence micrograph showing *Thiothrix* filaments bound to the NSB1185 probe (red) on a *N. ictus* leg spine (green and red autofluorescence).

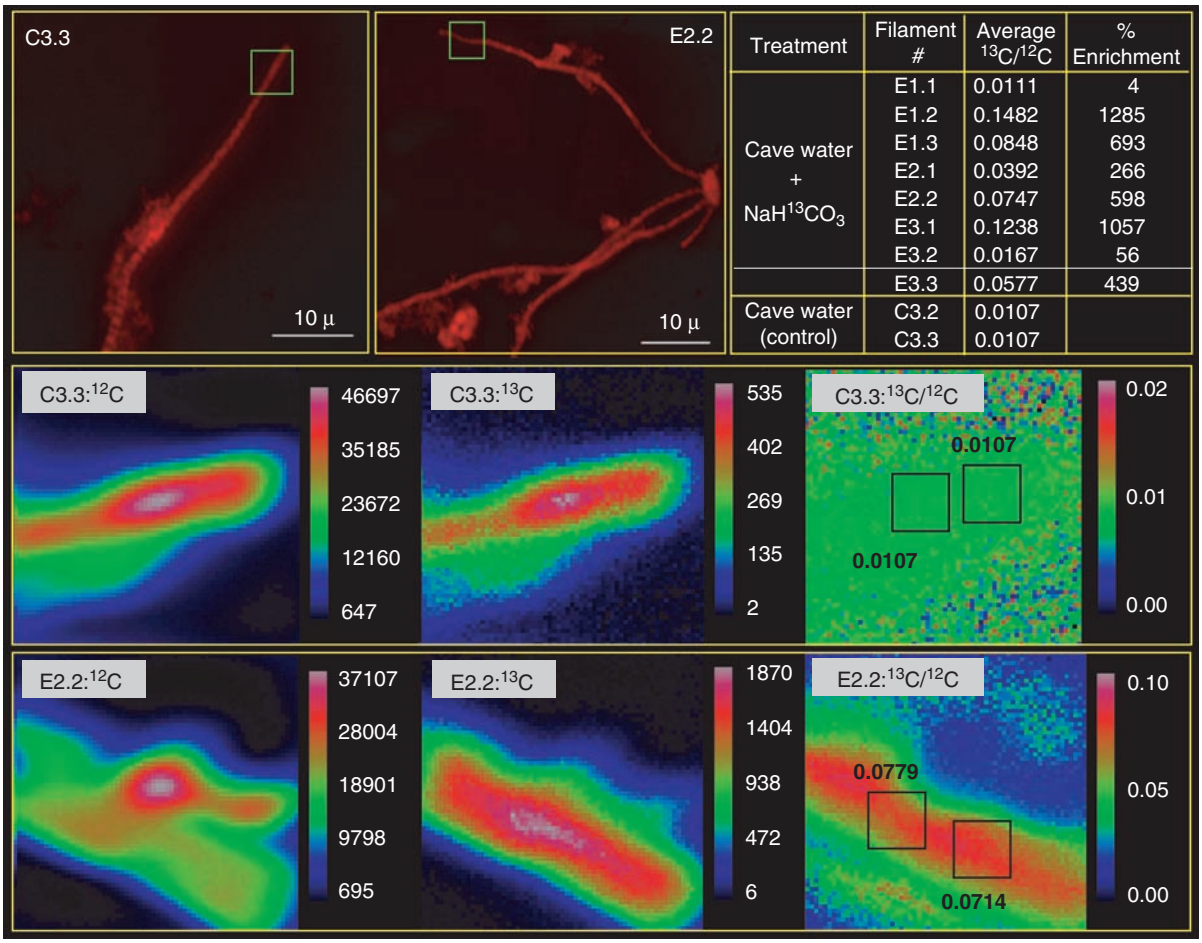
rDNA library are not ecologically significant epibiont populations. FISH analyses of microbial mats collected from *N. ictus* sample sites showed that the epibiotic *Thiothrix* phylotype is either extremely rare or completely absent (Table 1) in stream biofilms. These results are consistent with the results of 16S rDNA cloning and suggest that the *N. ictus* epibiont phylotype proliferates only on the amphipod exoskeletons.

Live animal maintenance and behavior

We maintained *N. ictus* alive and active over a period exceeding 1 year in an aquarium supplied with sulfidic cave water. All *N. ictus* individuals spent a majority of their time in the oxygen-rich zone of the aquarium close to the air–water interface. They occasionally dove down to the sulfide-rich sediment–water interface, and crawled on the sediment surface for 1–2 min before returning to the oxygen-rich zone. We examined *N. ictus* maintained in the aquarium for a 3-month period using FISH, and found that they retained a dense cover of the epibiotic *Thiothrix* phylotype.

Secondary ion mass spectrometry

We used a combination of ^{13}C-labeling, FISH, and SIMS to determine the trophic nature of the epibiotic filaments. The epibiotic *Thiothrix* rapidly



Treatment	Filament #	Average $^{13}C/^{12}C$	% Enrichment
Cave water + $NaH^{13}CO_3$	E1.1	0.0111	4
	E1.2	0.1482	1285
	E1.3	0.0848	693
	E2.1	0.0392	266
	E2.2	0.0747	598
	E3.1	0.1238	1057
	E3.2	0.0167	56
	E3.3	0.0577	439
Cave water (control)	C3.2	0.0107	
	C3.3	0.0107	

Figure 3 Results of secondary ion mass spectrometry (SIMS) of *Thiothrix* filaments after identification and mapping using fluorescence *in situ* hybridization (FISH). Three *Niphargus ictus* individuals (E1 through E3) were exposed to cave water enriched in $NaH^{13}CO_3$. Two to three *Thiothrix* filaments from each animal (for example, E1.1, E1.2) were analyzed using SIMS. Filaments from a control animal (C1) incubated without added ^{13}C were also analyzed. The upper panels show epifluoresence micrographs of filaments C1.1 and E2.2 bound to FISH probe NSB1185 (red). The green boxes indicate 5-μm square areas that were rastered using a one-micron diameter cesium ion beam to obtain the ^{12}C, ^{13}C, and $^{13}C/^{12}C$ maps shown in lower panels. Values corresponding to colors are shown on the scale to the right of each map. *Thiothrix* filaments, and other organic particles when present, are apparent as carbon-rich regions. Boxes in the lower panels show two 1-μm square areas that were averaged to obtain $^{13}C/^{12}C$ values shown in the table in the top right panel. Percent enrichment values in the table were calculated with respect to the control filaments.

incorporated ^{13}C into their cells during the experiment, demonstrating their chemoautotrophic metabolism. Two to three filaments from three different *N. ictus* exposed to ^{13}C bicarbonate for a period of 24 h were analyzed, and all filaments were enriched in ^{13}C in relation to control filaments (Figure 3). Enrichment in ^{13}C varied between 4 and 1285% for the various filaments. A non-filamentous carbon-rich object 15 microns away from filament E2.2 had a $^{13}C/^{12}C$ ratio of 0.0105, similar to filaments from the control incubation. As this non-filamentous organic matter likely derives from *N. ictus* tissue, this result implies that only epibiont carbon was labeled during the 24-h incubation.

Discussion

In this study, we used a combination of microscopy, 16S rDNA cloning and FISH to demonstrate that *N. ictus*, an amphipod that dominates the biomass of the Frasassi cave macroinvertebrate community, is symbiotic with a specific phylotype of filamentous bacteria belonging to the sulfur-cycling clade *Thiothrix*. Amphipods throughout the large cave system are colonized by a single phylotype of *Thiothrix* that is extremely rare or absent in stream biofilms (Table 1). Several types of filamentous sulfur-oxidizing bacteria abundant in the Frasassi ecosystem are adapted to life in flowing water and attach themselves to surfaces using structures called 'holdfasts' (Campbell *et al.*, 2006; Macalady *et al.*, 2008). These clades include *Thiothrix* and filamentous ε-proteobacteria, both of which form thick mats in *N. ictus* habitats. *Thiothrix* epibionts have been reported earlier on a marine amphipod species (Gillian and Dubilier, 2004), and filamentous ε-proteobacteria are the most common epibionts on deep-sea hydrothermal vent invertebrates (Polz *et al.*, 1998; Goffredi *et al.*, 2004; Campbell *et al.*,

2006). We have observed that *N. ictus* individuals regularly swim through dense mats composed of *Thiothrix* and filamentous ε-proteobacteria in Frasassi cave waters. Thus, it is not surprising to find filamentous bacteria attached to *N. ictus* exoskeletons. However, the presence of a unique phylotype of *Thiothrix*, to the exclusion of filamentous ε-proteobacteria and closely related *Thiothrix* phylotypes many times more numerous in the environment, is indeed remarkable. The phylogenetic specificity demonstrated by the *N. ictus-Thiothrix* association is extremely unusual for epibiotic associations (Wahl and Mark, 1999) and is an important hallmark of symbioses.

The *Thiothrix* clade contains strains that are capable of both autotrophic and heterotrophic growth. To determine the trophic nature of *N. ictus* epibionts, we exposed live amphipods to sulfidic cave water enriched in ^{13}C bicarbonate, separated epibiont filaments from the exoskeletons, and analyzed them using a combination of FISH and SIMS. Epibiotic *Thiothrix* filaments from *Niphargus* exposed to ^{13}C bicarbonate were on average ~550% enriched in ^{13}C in relation to control filaments (Figure 3), showing that they rapidly incorporated ^{13}C into their cells during the experiment. These results are consistent with the *N. ictus* symbionts being chemoautotrophic, and thus they are unlikely to be colonizing *N. ictus* exoskeletons to derive organic carbon from their hosts.

Epibiotic growth of chemoautotrophic sulfur-oxidizing bacteria on invertebrates is extremely common in sulfidic marine environments and has so far been described in six eukaryotic phyla, including invertebrates and protists (Polz *et al.*, 2000). Whereas free-living sulfur-oxidizing bacteria are restricted to a narrow interface where sulfide and oxygen co-exist, epibiotic bacteria can achieve high growth rates by 'hitch-hiking' on mobile invertebrates that travel between oxic and anoxic microenvironments (Cavanaugh, 1994; Polz *et al.*, 2000). *N. ictus* appears to confer a similar benefit to its epibiotic bacteria. In Frasassi waters, free-living *Thiothrix* are numerically dominant microbial populations only in turbulent, high oxygen, low sulfide niches (Macalady *et al.*, 2008). In contrast, the *N. ictus* epibionts flourish along with their hosts in streams and lakes with a much broader range of sulfide and oxygen concentrations and water flow characteristics. These habitats include stagnant lakes with no conspicuous biofilm development, slow-moving pools dominated by *Beggiatoa* mats and *Thiovulum* veils, and turbulent streams with high sulfide-to-oxygen ratios dominated by filamentous ε-proteobacteria (Table 1). We observed *N. ictus* both in their natural environment and in captivity, and found that they regularly move back and forth between oxic water at the air–water interface and anoxic water at the water–sediment interface. The *N. ictus* exoskeleton is therefore an ideal niche for sulfur-oxidizing bacteria, and the *Thiothrix* epibionts presumably benefit from having a vehicle for dispersal as well as reliable, alternating access to both sulfide and oxygen. The epibiotic *Thiothrix* is closely related by 16S rDNA phylogeny to some phylotypes in the bacterial biofilms in Frasassi cave waters (Figure 1). It is possible that the epibiotic *Thiothrix* is the only strain within Frasassi waters capable of attaching to a chitin surface using chitinase activity, but this remains to be examined in future studies.

We found the epibiotic *Thiothrix* phylotype on all juvenile and adult *N. ictus* across the full range of sizes observed in their natural cave population, collected over a 2-year period. As all amphipods periodically shed their exoskeleton during growth, our data imply that *N. ictus* reacquire their epibionts after each molting stage and maintain a specific epibiotic phylotype of *Thiothrix* between generations. Moreover, *N. ictus* maintained for 3 months in an aquarium supplied with sulfidic cave water retained a dense cover of the epibiotic *Thiothrix* phylotype. These observations imply that the epibiont filaments are maintained on individual amphipods for long periods, rather than being continuously inoculated from the environment. Like all amphipods, *N. ictus* females brood their young in pouches located ventrally between their anterior walking legs. Thus, the epibionts could transfer from female to offspring (vertical transmission), ensuring the maintenance of the symbiosis between generations.

Earlier studies of invertebrates with sulfur-oxidizing epibiotic bacteria have suggested that the bacteria provide a ready source of food for their hosts (Polz *et al.*, 1998, 2000; Bright and Giere, 2005). Amphipods are known for their grooming behavior and ingestion of material scraped from the exoskeleton is common (Holmquist, 1985). The gnathopods of *N. ictus*, which are claw-like appendages used in amphipod species for grooming, are covered with a dense growth of *Thiothrix* filaments (Supplementary Figure 3). Moreover, *Thiothrix* filaments attached to *N. ictus* typically appear to be trimmed to a short and uniform length (Supplementary Figure 7) compared with biofilm-forming *Thiothrix* filaments we observe in cave streams (Macalady *et al.*, 2006, 2008). Thus, it is likely that the *Thiothrix* epibionts form at least one component of the *N. ictus* diet. Furthermore, the *Thiothrix* epibionts may serve to protect their amphipod hosts from sulfide toxicity. Sulfide is a potent inhibitor of aerobic respiration, and earlier studies have proposed that sulfide-oxidizing epibionts assist their eukaryotic hosts by detoxifying sulfide (Somero *et al.*, 1989; Bright and Giere, 2005). Frasassi cave waters contain up to 550 micromolar sulfide. We have observed that *N. ictus* thrives in Frasassi waters containing the highest sulfide levels, whereas other macroinvertebrates are restricted to cave waters with less than 250 micromolar sulfide. The gills of amphipods, where sulfide could

permeate easily, are attached at the base of thoracic appendages (gnathopods and pereopods; see Supplementary Figure 3). In most *N. ictus* individuals we studied, these appendages contained the highest density of sulfur-oxidizing *Thiothrix* epibionts compared with the rest of the exoskeleton.

Amphipods of the genus *Niphargus* are widely distributed across Europe and most species are specialized for living in freshwater subterranean environments (Holsinger, 1993; Fišer *et al.*, 2008). They are known to be tolerant to hypoxia (Hervant *et al.*, 1999) and are thus pre-adapted to hypoxic conditions in sulfidic waters. However, sulfide toxicity could have been a potential barrier to colonization from surrounding, non-sulfidic aquifers, and the acquisition of sulfur-oxidizing symbionts may have facilitated the successful colonization of the sulfidic cave environment. *N. ictus* is distributed throughout the Frasassi cave system and is the numerically dominant macroinvertebrate of the ecosystem. This is reminiscent of deep-sea vents and seeps, where numerically dominant invertebrates are often symbiotic with chemoautotrophic bacteria. The discovery of the *N. ictus-Thiothrix* symbiosis on land thus highlights the importance and apparently ubiquitous nature of animal-bacterial symbioses in sulfide-rich environments.

Epibiotic associations have been proposed as the initial step toward more integrated, intracellular symbioses (Smith, 1979; Cavanaugh, 1994; Wahl and Mark, 1999), leading eventually to the development of obligate endosymbioses and organelles. In the marine environment, symbioses between animals and chemoautotrophic bacteria are an ancient phenomenon, often involving obligate, intracellular microbial partners. Fossil records indicate that some shallow marine bivalve lineages with endosymbiotic chemoautotrophic bacteria are almost half a billion years old (Distel, 1998). Symbiotic taxa from vents and seeps diversified between 10 to 100 million years ago, and many of them may have been derived from symbiotic ancestors in other sulfide-rich marine environments (Van Dover *et al.*, 2002; Little and Vrijenhoek, 2003). The symbiosis between *Thiothrix* and *N. ictus* is likely very young in comparison to these marine examples. The northeastern Apennine region including the Frasassi area began to emerge above sea level approximately 3 million years ago. Continuing tectonic uplift and erosion of the 3 km-thick, Jurassic to Miocene sedimentary succession subsequently removed rock layers above the Jurassic limestone hosting the cave system, most recently leaving surface and karst features that record the history of cave development at Frasassi (Mazzanti and Trevisan, 1978; Alvarez, 1999). Geomorphological studies of these features constrain the age of the oldest sulfuric acid-derived cave level in the Frasassi system between 350 000 and 1 million years (Mariani *et al.*, 2007; Cyr and Granger, 2008).

N. ictus is endemic to the Frasassi cave system. We considered the possibility that *N. ictus* or its ancestor colonized the Frasassi cave system after acquiring *Thiothrix* epibionts in a sulfidic environment elsewhere. In this case, the symbiosis could be up to several million years old. This is unlikely for several reasons. The dispersal of groundwater fauna is typically very restricted due to the discontinuous nature of macroscopic pores in the terrestrial subsurface (Lefébure *et al.*, 2006). Moreover, the high relief and structural complexity of the geology in central Italy present additional barriers to the dispersal of groundwater taxa. Adjacent sulfidic karst areas are separated from the Frasassi cave system by major thrust faults that interrupt the continuity of groundwater aquifers (Alvarez, 1999) making it extremely unlikely that *N. ictus* diversified from an ancestor already symbiotic with sulfur-oxidizing *Thiothrix* epibionts. Thus, we contend that the *N. ictus-Thiothrix* symbiosis originated within the Frasassi cave system less than 1 million years ago and is a unique example of a chemoautotroph-animal symbiosis in the early stages of evolution.

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