We performed a microbial community analysis of biofilms inhabiting thermal (35 to 50°C) waters more than 60 m below the ground surface near Acquasanta Terme, Italy. The groundwater hosting the biofilms has 400 to 830 μM sulfide, <10 μM O₂, pH of 6.3 to 6.7, and specific conductivity of 8,500 to 10,500 μS/cm. Based on the results of 16S rRNA gene cloning and fluorescent in situ hybridization (FISH), the biofilms have low species richness, and lithoautotrophic (or possibly mixotrophic) Gammaproteobacteria and Epsilonproteobacteria are the principle biofilm architects. Deltaproteobacteria sequences retrieved from the biofilms have <90% 16S rRNA similarity to their closest relatives in public databases and may represent novel sulfate-reducing bacteria. The Acquasanta biofilms share few species in common with Frasassi cave biofilms (13°C, 80 km distant) but have a similar community structure, with representatives in the same major clades. The ecological success of Sulfurovumales-group Epsilonproteobacteria in the Acquasanta biofilms is consistent with previous observations of their dominance in sulfidic cave waters with turbulent water flow and high dissolved sulfide/oxygen ratios.

Despite rapid progress in the past decade, the deep subsurface remains one of the least explored microbial habitats on earth. Recent studies illustrate the presence of significant spatial heterogeneity (13, 53) and the strong influence of mineralogy and fluid flow on subsurface microbial biodiversity (9, 16, 31, 61). Data obtained by drilling are complemented by an increasing number of studies that exploit subsurface passages navigable by humans (17). These subsurface passages include caves (14, 46) and mines (22, 32, 47, 52, 57). Approximately 10% of known caves (49) and perhaps more (33) are formed where reduced, sulfidic groundwaters interact with oxidized water descending from surface environments. Limestone dissolution in these groundwater mixing zones results in deep caves that receive few organic inputs from the surface. Due to the presence of both sulfide and oxidants where the groundwaters mix, lithoautotrophic microorganisms thrive and supply the primary productivity for food chains that may include invertebrate and vertebrate animals (11, 23). Interest in these isolated terrestrial chemosynthetic microbial communities is fueled by their potential as model systems for microbial biogeography and as analogs for oxygen-poor, sulfur-rich environments prevalent early in Earth history or on other planets.

The sulfidic caves studied by microbiologists to date include Lower Kane Cave in Wyoming (15), Cueva de Villa Luz in Mexico (5, 23), Movile Cave in Romania (7, 28), Parker Cave in Kentucky (4), and the Frasassi caves in Italy (38, 39). The average water temperatures of previously studied sulfidic caves range from 12 to 28°C. In contrast, the groundwater in the Grotta Nuova di Rio Garrafo and associated caves near Acquasanta Terme (Italy) reaches temperatures up to 50°C (M. Mainiero, unpublished results). The Acquasanta caves host conspicuous microbial biofilms that have not been previously investigated, presenting an opportunity to compare subsurface environments with similar energy resources but large differences in temperature.

A reconnaissance of the Acquasanta caves (Fig. 1) showed that they contain biofilm types also reported in other sulfidic caves, including viscous snottites on walls above the water table (see Fig. S1a, b, and c in the supplemental material), reddish clay-rich deposits (“ragu”) similar to those on walls in Cueva de Villa Luz (see Fig. S1e in the supplemental material), and white biofilms covering sediments below the water table (see Fig. S1d and f in the supplemental material). As in Cueva de Villa Luz, subaerial surfaces in areas with high sulfur gas concentrations are covered with elemental sulfur deposits (see Fig. S1c in the supplemental material). Here, we describe the diversity and community structure of biofilms in the thermal Acquasanta groundwater. In addition, we investigate whether the Acquasanta stream biofilms have a structure consistent with a simple ecological niche model developed for sulfur-oxidizing clades inhabiting nonthermal sulfidic caves (37). The niche model considers aqueous sulfide and oxygen concentrations and hydrodynamic shear and suggests that in turbulently flowing (high shear) waters with high sulfide/oxygen ratios, Epsilonproteobacteria should outcompete filamentous Gammaproteobacteria, such as Beggiatia and Thiothrix. We find that Acquasanta cave stream biofilms share very few phylotypes in common with other sulfidic caves studied to date and are dominated by Epsilonproteobacteria as predicted by the niche model.

**MATERIALS AND METHODS**

**Site description and field geochemistry.** Grotta Nuova di Rio Garrafo (Fig. 1) is located approximately 2 km south of Acquasanta Terme, Italy (13°25′E, 42°45′N). The cave entrance is 15 m above the western bank of the Rio Garrafo (Garrafo River), and the sulfidic water table in the cave is approximately 50 m below the level of the perched river. Grotta Nuova di Rio Garrafo contains more than 1 km of passages with strong vertical development in marly Scaglia Rossa limestone (18, 21). The water table is accessible via vertical caving routes. Sam-
pling teams were equipped with gas monitors (O₂, CO₂, SO₂, and CH₄) for safety reasons. Gas masks were necessary to prevent the inhalation of toxic concentrations of sulfur gases while sampling. The sulfidic groundwater in the cave has conductivity values as high as 10.5 mS/cm and contains up to 825 μM H₂S. The waters are near-neutral (pH 6.3 to 6.8), and the major ion constituents are Na⁺, Ca²⁺, Cl⁻, HCO₃⁻, and SO₄²⁻ (18). The ammonium concentrations are ≤400 μM, the dissolved organic carbon concentrations are ≤4.7 mg/liter, and nitrate and nitrite were detected in situ by spectrophotometric analysis. The results of replicate oxygen analyses were within 20% of each other. No sulfide and oxygen concentration data were obtained in 2005 due to a spectrophotometer malfunction. Nitrate, nitrite, ammonium, and sulfate were measured on water filtered (0.2-μm pore size) into sterile bottles at the point of collection and transported on ice. Measurements were made at the Osservatorio Geologico di Coldigioco Geomicrobiology laboratory using a portable spectrophotometer within 12 h of collection according to the manufacturer’s instructions (Hach Co., Loveland CO). Light microscopy of live biofilm samples (stored 4°C) and RNA-later-preserved samples was performed within 24 h of collection on a Zeiss model 47-30-12 microscope (1,250× magnification) at the Osservatorio Geologico di Coldigioco Geomicrobiology laboratory. The presence of elemental sulfur particles and inclusions was assessed using an alcohol dissolution test as described in reference 35.

**FISH.** Clones retrieved in 16S rRNA gene libraries were checked against the probe sequences listed in Table 1 to ensure probe specificity and membership in target groups. FISH experiments were carried out as described in reference 3 on 10-well Teflon-coated slides using the probes listed in Table 1. Oligonucleotide probes were synthesized and labeled at the 5’ ends with fluorescent dyes (Cy3

**TABLE 1. FISH probes used in this study**

<table>
<thead>
<tr>
<th>Probe</th>
<th>Sequence (5’–3’)</th>
<th>% Formamide</th>
<th>Label</th>
<th>Target specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338ᵃ</td>
<td>GCT GCC TCC CGT AGG AGT</td>
<td>0–50</td>
<td>FITC</td>
<td>Most Bacteria</td>
<td>2</td>
</tr>
<tr>
<td>EUB38-IIIᵇ</td>
<td>GCA GCC ACC CGT AGG TGT</td>
<td>0–50</td>
<td>FITC</td>
<td>Planctomycetes</td>
<td>10</td>
</tr>
<tr>
<td>EUB338-IIIᵇ</td>
<td>GCT GCC ACC CGT AGG TGT</td>
<td>0–50</td>
<td>FITC</td>
<td>Verrucomicrobiales</td>
<td>10</td>
</tr>
<tr>
<td>ARCH915</td>
<td>GTG CTC CCC CCG CAA TTC CT</td>
<td>20</td>
<td>Cy3</td>
<td>Most Archaea</td>
<td>38</td>
</tr>
<tr>
<td>EP404</td>
<td>A.AA KGY GTC ATC CTC CA</td>
<td>30</td>
<td>Cy3</td>
<td>Most Epsilonproteobacteria</td>
<td>39</td>
</tr>
<tr>
<td>GAM42ᵃᵇ</td>
<td>GCC TTC CCA CAT CGT TT</td>
<td>35</td>
<td>Cy3</td>
<td>Most Gammaproteobacteria</td>
<td>40</td>
</tr>
<tr>
<td>DELTA495ᵃᶜ</td>
<td>AGT TAG CCG GTG CTT CCT</td>
<td>45</td>
<td>Cy3</td>
<td>Most Deltaproteobacteria, some Gemmatimonas group</td>
<td>35</td>
</tr>
<tr>
<td>SRB385</td>
<td>CGG CGT CGC TGG GTG AGG</td>
<td>35</td>
<td>Cy3</td>
<td>Some Deltaproteobacteria, some Actinobacteria, and Gemmatimonas group</td>
<td>3</td>
</tr>
</tbody>
</table>

ᵃ EUBMIX is a mixture of EUB338, EUB38-III, and EUB338-III.
ᵇ Requires competitor oligonucleotide cGam42ᵃ: GCCCTCCCACTCTCGTTT.
ᶜ Requires competitor oligonucleotide cDELTA495ᵃ: AGTACCGGTTGCCTCTT.
and fluorescein isothiocyanate (FITC) at Sigma-Genosys (United States). Cells were stained after hybridization with 4′,6-diamidino-2-phenylindole (DAPI), mounted with Vectashield (Vectashield Laboratories, United States) and viewed on a Nikon E800 epifluorescence microscope. Images were captured with a Nikon charge-coupled device (CCD) camera using NIS Elements AR 2.30 image analysis software. The fluorescence coming from DNA probes in Fig. S2 in the supplemental material is shown in false color, and an autolevel function was applied to each image shown in the figure. For population estimates based on FISH, the biofilm matrix was easily disrupted by shaking the sample tube. At least 3 slide wells were examined for each probe combination. At least 10 images were collected for each sample and probe combination, taking care to represent the sample variability, and a total DAPI-stained area of at least 3 × 10^5 mm² (equivalent to the area of 5 × 10^5 Escherichia coli cells) was analyzed in each case. The presence of cells ranging from small rods to large filaments precluded simple counting that does not take into account differences in biomass between the cell types. A comparison between visual area estimates and area counts obtained using the object count tool of NIS Elements AR 2.30 image analysis software indicated that visual estimation gave the same results within error in less time. Since semiquantitative area counts are sufficient to support the conclusions obtained in this study, a visual estimation approach was employed.

**Clone library construction.** Environmental DNA was extracted, amplified, and cloned using archaeal- and bacterial-domain-specific primers as described in reference 37. Briefly, environmental DNA was extracted from approximately 0.5 g of biofilm using phenol-chloroform extraction. Each 50-μl PCR mixture contained environmental DNA template (1 to 150 ng), 1.25 U Ex Taq polymerase (TaKaRa Bio, Inc., Shiga, Japan), 0.2 mM each deoxynucleoside triphosphate (dNTP), 1× PCR buffer, 0.2 μM 1492r universal reverse primer, and 0.2 μM 27f bacterial domain primer (34). Thermal cycling was as follows: initial denaturation for 5 min at 94°C and 25 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. For archaeal PCRs, the primers used were 21f (5′-TTCGCGTTGATCCYGCCGGA) and 977r (5′-YCCCGCCTGATTGACCAATTT), and thermal cycling was performed as described above except that the annealing temperature was 58°C. PCR products were cloned into the pCR4-TOPO plasmid and used to transform chemically competent OneShot MACH1 T1 Escherichia coli cells (TOPO TA cloning kit, Invitrogen, Carlsbad, CA). The plasmid inserts were screened using colony PCR with M13 primers. Colony PCR products of the correct size were purified using a QIAquick PCR purification kit (Qiagen, Inc., United States).

**Sequencing and phylogenetic analyses.** Clones were sequenced at the Penn State University Biotechnology Center with Sanger technology (ABI Hitachi 3730XL DNA analyzer with BigDye fluorescent terminator chemistry) using T3 and T7 plasmid-specific primers. Sequences were assembled with Phred base calling using CodonCode Aligner version 1.2.4 (CodonCode Corp., United States) and manually checked for ambiguities. The nearly full-length gene sequences (all >1,400 bp) were compared against sequences in public databases using BLAST (1) and submitted to the online analyses CHIMERA_CHECK version 2.7 (8) and Bellcorethon 3 (24). Putative chimeras were excluded from subsequent analyses. Sequences were aligned to the 7,682-character Hugenholtz alignment using the NAST aligner at greengenes (12). NAST-aligned sequences were loaded into an ARB database (36) containing over 230,000 full-length sequences. Alignments were edited manually in ARB using the ARB Editseq editor. Only sequences longer than 1,350 bp were used for phylogenetic analyses, with the exception of short Gammaproteobacteria clones from Parker Cave’s Sulfur River (4). The analyses included the top BLAST hits, the top five closest relatives in the ARB database, and representatives of major divisions within each of the targeted groups (e.g., *Sulfurovumales*), with an emphasis on sulfidic cave sequences. The alignments were trimmed so that all sequences were of equal length (the final alignment lengths were 1,153, 1,420, and 1,257 positions for analyses of *Sulfurovumales*, Thiofaba/Thiovogus, and Deltaproteobacteria clades, respectively), and nucleotide positions with less than 50% base pair conservation were masked (calculated separately from all sequences in each analysis, exclusive of outgroups). Shorter-length sequences (Parker Cave clones AF047617 and AF047623) were included in phylogenetic analyses with their missing data coded as missing and treated according to PAUP* defaults for each analysis type (59). Maximum-likelihood, maximum-parsimony, and neighbor-joining analyses were performed using PAUP* version 4b10 (59). Maximum-likelihood analyses used the general time-reversible (GTR) substitution model with gamma-distributed among-site variation, at least five random-addition-sequence replicates, and tree bisection-reconnection (TBR) branch swapping. Base frequency, substitution rates, and shape parameter were estimated from the data. Maximum-parsimony bootstrap analyses (2,000 replicates) were performed via heuristic search with 100 random-addition-sequence replicates and TBR branch swapping. Neighbor-joining bootstrap analyses (2,000 replicates) were performed with a Jukes-Cantor (JC)-corrected distance matrix.

**Diversity analyses.** Rarefaction analyses were calculated based on the frequency of operational taxonomic units (OTUs) defined by the program DOTUR, version 1.53 (54). OTUs were defined at 98% sequence similarity, using the DOTUR “furthest neighbor” algorithm, based on ARB distance matrices.

**Nucleotide sequence accession numbers.** The 16S rRNA gene sequences determined in this study were submitted to GenBank and were assigned accession numbers GU390708 to GU390880.

## RESULTS

**Field observations and geochemistry.** Water and biofilm samples were collected at site AS1 (Fig. 1) in 2005, 2007, and 2008. The stream at site AS1 was fast flowing and turbulent for the entire length of the passage, and nearly every surface in the channel was covered with biofilms of “streamer” morphology (see Fig. S1d and f in the supplemental material). Other biofilm morphologies were rare and limited in areal extent. The streamers were 1 to 2 cm in length on average (see Fig. S1d and f in the supplemental material) and covered both fine gray sediment and exposed limestone surfaces underwater. Microscopic examination of both live and RNA-later-preserved streamers within 24 h of collection revealed that none of the cells contained intracellular sulfur inclusions. All biofilm samples had abundant elemental sulfur particles outside the cells in the biofilm matrix. The streamers collected in 2007 and 2008 were dominated by filamentous bacteria, with rod-shaped and coccoid cells also present. Filamentous bacteria were less common in the 2005 samples (AS05-2 and AS05-3), which were dominated by long thin rods.

The geochemical data for waters collected at sample site AS1 (Table 2) reflected changes in the degree of dilution of the thermal groundwater by surface water recharge (18). The conductivity

### Table 2. FISH cell area abundances and geochemical data for Acquasanta cave stream biofilm samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Date collected</th>
<th>Percent hybridization by each FISH probe</th>
<th>Temp °C</th>
<th>pH</th>
<th>Sp. cond (mS/cm)</th>
<th>SO₄²⁻ (mM)</th>
<th>O₂ (μM)</th>
<th>H₂S (μM)</th>
<th>H₂S (g) ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS05-3</td>
<td>18/08/2001</td>
<td>++++ +++++ +++++ +++++ +++++ +++++</td>
<td>40.4</td>
<td>6.37</td>
<td>8.4</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>801</td>
</tr>
<tr>
<td>AS05-2</td>
<td>18/08/2001</td>
<td>+++++ +++++ +++++ +++++ +++++ +++++</td>
<td>40.4</td>
<td>6.37</td>
<td>8.4</td>
<td>NM</td>
<td>NM</td>
<td>NM</td>
<td>801</td>
</tr>
<tr>
<td>AS07-6</td>
<td>31/05/2003</td>
<td>+++++ +++++ +++++ +++++ +++++ +++++</td>
<td>44.1</td>
<td>6.38</td>
<td>10.6</td>
<td>9.92</td>
<td>3.06</td>
<td>801</td>
<td>100</td>
</tr>
<tr>
<td>AS07-7</td>
<td>31/05/2003</td>
<td>+++++ +++++ +++++ +++++ +++++ +++++</td>
<td>44.1</td>
<td>6.38</td>
<td>10.6</td>
<td>9.92</td>
<td>3.06</td>
<td>801</td>
<td>100</td>
</tr>
<tr>
<td>AS08-2</td>
<td>13/06/2004</td>
<td>+++++ +++++ +++++ +++++ +++++ +++++</td>
<td>42.7</td>
<td>6.31</td>
<td>10.5</td>
<td>12.71</td>
<td>6.69</td>
<td>415</td>
<td>15</td>
</tr>
<tr>
<td>AS08-3</td>
<td>13/06/2004</td>
<td>+++++ +++++ +++++ +++++ +++++ +++++</td>
<td>42.7</td>
<td>6.31</td>
<td>10.5</td>
<td>12.71</td>
<td>6.69</td>
<td>415</td>
<td>15</td>
</tr>
</tbody>
</table>

* Estimated proportion of total cells. ND, none detected; ++, <30%; +++, 30 to 15%; ++++, 15 to 35%; +++++, 35 to 75%; ++++++, 75 to 100%; NM, not measured. No ARCH915-positive cells were detected.

<table>
<thead>
<tr>
<th></th>
<th>Sp. cond</th>
<th>SO₄²⁻</th>
<th>O₂</th>
<th>H₂S</th>
<th>H₂S (g) ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS05-2</td>
<td>15</td>
<td>3.06</td>
<td>801</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>AS08-3</td>
<td>15</td>
<td>3.06</td>
<td>801</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
was substantially lower in 2005 than in 2007 and 2008. The conductivity and major ion concentrations were similar for samples collected in 2007 and 2008, but the hydrogen sulfide concentrations in both the stream and the cave atmosphere were much higher in 2007 (Table 2). These differences in geochemistry were not accompanied by any noticeable changes in the density or macroscopic morphology of the microbial streamers covering the stream sediments.

**16S rRNA clone libraries.** Attempts to amplify archaeal 16S rRNA genes from the biofilm samples were unsuccessful, whereas positive-control DNA yielded archaeal PCR products of the expected length in the same PCR runs (data not shown). A total of 174 nearly full-length bacterial 16S rRNA gene sequences were retrieved from samples AS05-3 (80 clones) and AS07-7 (94 clones) (Fig. 2A). The results of rarefaction analyses (Fig. 2B) suggested that the major bacterial populations present in the biofilms have been adequately sampled. The most abundant phyletypy in both libraries fell within the Sulfurovumales clade in the Epsilonproteobacteria. Sulfurovumales clones (Fig. 3) constituted 40% and 67% of libraries AS05-3 and AS07-7, respectively. Most of the Sulfurovumales sequences were 98% similar to clones AS05-3-B2 and AS07-7-B27 (Fig. 3) and formed a clade with a single Frasassi cave clone. The AS05-3 library also included several clones (6.3%) of a second Sulfurovumales phyleotypy. The second most abundant phyletypy in both libraries (10.6% in AS05-3 and 26.3% in AS07-7) fell within a clade containing the isolates “Candidatus Thiobacillus baregensis” and *Thiofaba tepidiphila* in the Gammaproteobacteria (Fig. 4). The Deltaproteobacteria sequences in the libraries were very distantly
related (<90%) to sequences in public databases (Fig. 5). Both libraries contained representatives of *Bacteroidetes*, *Elusimicrobia*, and MVP-15, in addition to rare phylotypes (Fig. 2). The bacterial diversity in both samples was very low, even compared with that of sulfur-oxidizing biofilms from other sulfidic cave environments (Fig. 2).

**Epifluorescence microscopy.** Fluorescent in situ hybridization (FISH) was used to evaluate the relative abundances of major microbial populations in the biofilms (Table 2). The FISH data were consistent with the clone libraries, suggesting little PCR or DNA extraction bias for major populations. No hybridization to archaeal-domain-specific probe ARCH915 was detected. More than 95% of DAPI-stained cells hybridized with the bacterial-domain-specific probe EUBMIX in all samples. The EUBMIX-negative populations were small cocci that did not appear to hybridize with any probes used in this study (see Fig. S2 in the supplemental material). No nucleated cells (i.e., protists or fungi) were observed. Large holdfast structures uniting many filaments, such as those observed for *Thiothrix* spp. or filamentous *Epsilonproteobacteria* “rosettes” in Frasassi cave streamers (37, 39), were not observed in the Acquasanta biofilms.

Samples AS07-6, AS07-7, AS08-2, and AS08-3 were dominated by EP404-positive filaments (Table 2; also see Fig. S2 in the supplemental material). Samples AS05-2 and AS05-3 contained long, thin EP404-positive rods, as well as a population of large EP404-positive cocci (see Fig. S2 in the supplemental material). GAM42a-positive cells were present in all samples and were short rods with uniform morphology across samples (see Fig. S2 in the supplemental material). A newly designed FISH probe developed to identify close relatives of “Ca. Thiobacillus baregensis” in biofilms from other caves hybridized to the same set of short rods visualized using GAM42a (data not shown). Consistent with the results of FISH experiments, *Gammaproteobacteria* sequences retrieved in both Acquasanta clone libraries consisted of a single phylotype closely related to “Ca. Thiobacillus baregensis” (Fig. 4). FISH experiments using SRB385 and Delta495 probes yielded similar area estimates and cell morphologies in all samples. SRB385- and Delta495-positive cells were predominantly large rods, with smaller populations of smaller rods and cocci (see Fig. S2 in the supplemental material).

**DISCUSSION**

**Biofilm community composition.** Based on a full-cycle rRNA approach, Acquasanta stream biofilms are dominated by *Sulfurovumales* (*Epsilonproteobacteria*) populations, along with important and sometimes equally large populations of *Gammaproteobacteria* related to “Ca. Thiobacillus baregensis”
Table 2 and Fig. 2; also see Fig. S2 in the supplemental material). Minor populations include Deltaproteobacteria, Bacteroidetes, Spirochaetales, Elusimicrobia, MVP-15, and rare taxa. Although archaea were not detected using FISH or PCR, small cells that did not hybridize with either archaeal or bacterial domain-specific probes made up 5% of the community and may prove to be archaeal cells.

The Sulfurovumales (Fig. 3) are a monophyletic clade with few isolates and large numbers of environmental sequences and have been retrieved from sulfidic caves and springs worldwide (6). Based on the results of FISH experiments, the most abundant Epsilonproteobacteria populations are filamentous in 2007 and 2008 biofilms and nonfilamentous in 2005 biofilms. Cells in these populations have indistinguishable morphologies (long thin rods) but different arrangements (see Fig. S2 in the supplemental material). Because the most abundant Epsilonproteobacteria clones are nearly genetically identical in samples from 2005 and 2007 (Fig. 3, clones AS053-B2 and AS077-B27), we hypothesize that they represent highly related species capable of both filamentous and nonfilamentous habits. We recognize that this hypothesis is speculative and remains to be tested using strain-specific probes. Filamentous Sulfurovumales have not been cultured or investigated using metagenomics to date, and thus, there are few unassailable constraints on their metabolism. The genome of Sulfurovum sp. strain NBC37-1, distantly related to Acquasanta clones (<91.5% similarity) but their closest cultivated relative, has recently been sequenced (44). Sulfurovum sp. NBC37-1 is a lithoautotrophic hydrothermal vent symbiont that can use hydrogen and reduced sulfur species as electron donors. Based on the predominance of sulfur-based lithotrophic metabolisms in hydrothermal vent Epsilonproteobacteria (6, 43) and the importance of Epsilonproteobacteria in terrestrial chemoautotrophic ecosystems where reduced sulfur species contribute the bulk of the available chemical energy (37, 51), it is likely that Sulfurovumales clones retrieved from Acquasanta biofilms will prove to be sulfur-oxidizing lithoautotrophs, sulfur-reducing lithoautotrophs, or possibly, mixotrophs that can reduce sulfur using organic compounds, depending on environmental conditions.

The Gammaproteobacteria clones from Acquasanta clone libraries belong to a single phylotype represented by clones AS053-B125 and AS077-B32 (Fig. 4). Related isolates include the sulfur-oxidizing lithoautotrophs “Ca. Thiobacillus baregensis” (25, 26), Thiofaba tepidiphila (42), and Thiovirga sulfuroxidans (29, 30). Representatives of the clade defined by these isolates (Fig. 4) are important constituents of stream biofilms in other sulfidic caves. For example, close relatives of “Ca. Thiobacillus baregensis” were found in six out of six Frasassi stream biofilm clone libraries (37). Clones in this clade have also been retrieved from Parker Cave in Kentucky (4) and Movile Cave in Romania (7). Thus, both the metabolisms of cultivated relatives and the distribution of related clones in the environment suggest that Acquasanta “Ca. Thiobacillus baregensis” relatives are sulfur-oxidizing lithoautotrophs.

The Deltaproteobacteria 16S rRNA clones are very distantly related (<95%) to publicly available sequences, including environmental clones (Fig. 5). With the exception of the AS053-B137 phylotype (3 clones), the clones belong to a large environmental clone group with no cultivated representatives. The nearest relatives of these phylotypes are from marine sediment
and methane seep environments, including Eel River sediments ("Eel-2" clade) (48), where sulfate reduction is an important process. Acquasanta Deltaproteobacteria may thus represent novel sulfate-reducing bacteria. However, because of their dissimilarity to characterized isolates, inferences about their metabolism remain somewhat speculative. It is interesting to note that none of the diverse Deltaproteobacteria clones from the Frasassi cave system fall within the Acquasanta environmental clone groups. Conversely, Acquasanta clones are not represented in the Desulfocapsa clade, which contains the vast majority of Frasassi Deltaproteobacteria clones (37, 39).

The Acquasanta caves and the cooler Frasassi caves located ~80 km away provide an interesting geochemical and physical context for comparing microbial communities. The hydrogen sulfide in Acquasanta and Frassasi cave waters is thought to have a similar source, namely, partial reduction of sulfate in gypsum-bearing evaporite rocks in the underlying Triassic Burano Formation (18, 20). The conductivity of the sulfidic water at Acquasanta is somewhat higher than at Frasassi (~2,000 versus ~9,000 μS/cm), although dissolved ions are present in similar ratios (18, 19). The temperature of the sulfidic water in the Frasassi cave system is 13 to 14°C, compared to 35 to 50°C at Acquasanta.

The most abundant Acquasanta clones belong to clades also containing Frasassi phylotypes (i.e., Sulfurovumales in the Epsilonproteobacteria and "Ca. Thiobacillus baregensis" relatives in the Gammaproteobacteria) (37). However, the Acquasanta phylotypes are distinct (Fig. 3 and 4), and Frasassi clones are often more closely related to phylotypes from geographically distant sulfidic caves, such as Movile Cave (Romania), Parker Cave (Kentucky), and Lower Kane Caves (Wyoming), than to clones from the geographically nearby Acquasanta caves. Phylogeographical patterns can arise via several unrelated mechanisms, including selective pressures imposed by the environment, dispersal limitations, and chance historical occurrences, such as colonization events (41). Because the stream waters in the Acquasanta caves have a significantly higher temperature than previously studied sulfidic cave waters, it is worth considering whether environmental selection based on temperature could explain the phylogeographical pattern we observe. However, clades within the Sulfurovumales containing Frasassi and other nonthermal cave clones also contain sequences from hot sulfidic springs, suggesting that temperature alone cannot account for the large genetic distances between Frasassi and Acquasanta phylotypes.

**Sulfur oxidizer niches.** We previously proposed a simple niche model for sulfur-oxidizing bacteria in cave streams of the nonthermal Frasassi cave system (37). In the model, two niche dimensions (hydrodynamic shear and aqueous sulfide/oxygen ratio) controlled the biofilm population structures. The model described the distribution of three major biofilm types named after their dominant populations: (i) *Beggiatoa* spp. forming...
sulfide/oxygen ratio. These relationships are depicted in Fig. S3 in the supplemental material, with the addition of Acquasanta samples from Table 2. The niche model correctly predicts that *Epsilonproteobacteria* are the dominant biofilm populations in Acquasanta waters (high shear and a high sulfide/oxygen ratio).

Sulfidic caves have important similarities with nonthermal and moderately thermal zones around hydrothermal vents, including complete darkness, high sulfide concentrations, and food chains based on bacterial chemosynthesis. We could not identify any directly comparable quantitative phylogenetic studies of moderately thermal vent microbial community composition in the literature. However, the niche model emerging from studies of sulfidic caves is at least consistent with what is known about niches of cultivable mesophilic and moderately thermophilic vent chemosynthetic bacteria (6, 43, 55). Based on the physiology and genome content of hydrothermal vent isolates from mixing-zone habitats, *Gammaproteobacteria* are facultatively aerobic, whereas *Epsilonproteobacteria* are strictly anaerobic to facultatively aerobic. These trends parallel the high sulfide/oxygen niche of filamentous *Epsilonproteobacteria* and low sulfide/oxygen niche of *Thiotrix* spp. (*Gammaproteobacteria*) in turbulently mixed sulfidic cave streams.

Implications and future work. Niche separation has been documented for microbial clades at a variety of taxonomic levels. For example, environmental specialization correlates with marine bacterioplankton clades within the *Vibrionaceae* based on *hsp60* sequencing (27), with *Bacillus simplex* strain groups typed by randomly amplified polymorphic DNA (RAPD)-PCR (56), with subgroups of *Actinobacteria* clade acI based on 16S rRNA sequences (45), with bacterial phyla and classes in pasture soils (50), and more speculatively, with phylum- or subphylum-level clades present in metagenomic data sets (60). Members of sulfur-oxidizing clades, such as the *Sulfurovumales*, appear to show fidelity to a relatively narrow geochemical niche, outcompeting coexisting representatives of other major sulfur-oxidizing clades when conditions are correct. Further effort is warranted to establish the evolutionary history of environmental specialization and niche differentiation among sulfur oxidizers and other microbial functional guilds.

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