INTRODUCTION
At depths of at least 3.2 km, an abundant and diverse microbial biosphere in habitats terrestrial hard rock settings (Pedersen, 1997; Moser et al., 2003; Kovacik et al., 2006; Sahl et al., 2008), but the rock type is likely to exert tremendous control over the geochemically available nutrients and energy sources. In the basaltic-hosted subsurface settings, organisms may be sustained by H2 generated from reactions between water and Fe-rich mafic minerals (Stevens and McKinley, 1995; Lin et al., 2006b). Organisms living in metamorphosed Precambrian sediments likely utilize H2 and SO4\(^{2-}\) derived from the reaction of decaying radionuclides with water and sulfide minerals in a process known as radiolysis (Lin et al., 2005, 2006b; Leficaru et al., 2010). In sedimentary rock, organisms may be energetically supported by organic carbon associated with the deposits (Fredrickson et al., 1997; Kovacik et al., 2006). While most studies have focused on determining the energy sources supporting life in the terrestrial subsurface (see Lovley and Chapelle, 1995; Pedersen, 2000; Amend and Teske, 2005; Fredrickson and Balkwill, 2006 for reviews), the origins of nutrients to subsurface microbial communities have been insufficiently investigated. While field and laboratory experiments have implicated microbes in the release of species such as Ca, Si, P, and Fe from minerals (Rogers et al., 1998; Edwards et al., 2003; Rogers and Bennett, 2004; Wu et al., 2008, 2009; Shelbolina et al., 2010), whether or not geologically sourced nitrogen (i.e., as ammonium substituted into potassic rocks) supports subsurface life is a question that has not yet been posed.

Many forms of nitrogen can be utilized biologically both for biosynthesis and energy metabolism. In subsurface settings, N\(_2\) may be sourced from the mantle (i.e., Crossley et al., 2009). Where oxidized nitrogen is absent, ammonium formed in the subsurface may be the most important form of nitrogen sustaining subsurface communities, but the origin of subsurface ammonium supporting microbial communities is unknown. However, at least two processes are possible. Although few nitrogen-bearing minerals are known, the substitution of NH\(_4^+\) for K\(^+\) in silicate minerals is a widespread process (Holloway and Dahlgren, 2002). Granites, for instance, contain on average ~45 ppm ammonium (Hall, 1999). Another source of ammonium to subsurface environments is biological fixation of subsurface N\(_2\). However, the reduction of N\(_2\) to NH\(_4^+\) by the nitrogenase enzyme requires input of ATP at a high energetic cost (Broda and Paschek, 1980) that may inhibit this process.
process under oligotrophic and anoxic conditions vs. photosynthetically supported surface environments (Lovley and Chapelle, 1995). Despite this, ammonium has been detected in numerous subsurface settings (Lin et al., 2006a; Onstott et al., 2006; Sahl et al., 2008).

Where ammonium is present in the subsurface, it may play a critical role not just as a nutrient, but as an energy source that sustains nitrifying subsurface microbial organisms. Functional gene studies have been used in characterizing nitrification in other subsurface sites where ammonia-oxidizing bacteria (AOB) and/or ammonia-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB; Proteobacteria or Nitrospira) are present (Hirayama et al., 2005; Spear et al., 2007; Weidler et al., 2007). Both archaeal amoA genes and AOA have been detected and commonly exceed the AOB and bacterial amoA genes in a number of oligotrophic settings, indicating that AOA may be adapted to low substrate niches (Martens-Habbena et al., 2009). Nitrite oxidation has also been recognized as a potentially important microbially mediated process in the deep subsurface (Hirayama et al., 2005; Gihring et al., 2006), specifically by the Nitrospira genus of bacteria. Nitrospira appear to be the most diverse and abundant NOB in nitrifying environments, thriving in nitrite and oxygen concentrations that are too low to support proteobacterial NOB (Daims et al., 2001, 2006).

This study evaluates the origin of ammonium in fluids circulating through a granite-hosted molybdenum ore-body at >3000 foot depth. The fluids contain N₂, N₂O, NO₃⁻, NO₂⁻, and ammonium and an estimated 10³–10⁴ cells ml⁻¹ (Sahl et al., 2008), and thermodynamically favorable nitrification reactions may be important in supporting the biomass detected in the fluids (Swanner, 2011). To evaluate possible geologic sources of ammonium, subsurface biotites, and muscovites were screened with Fourier transform infrared (FTIR) microscopy for the presence of NH₄⁺. Concurrently, the potential for the microbial community to fix N₂ into NH₄⁺ was assessed based on the presence of genes for nitrogen fixation in DNA from NH₄⁺-bearing borehole fluids. The DNA was also used to evaluate whether genes for ammonium- and nitrite-oxidation were present. The current work builds on the prior study of Sahl et al. (2008), which reported the presence of archaea and Nitrospira in 16S rRNA clone libraries of the borehole fluids. The new mineralogical, functional gene, and phylogenetic analyses yield insight into the source of ammonium to the subsurface, and how the ecology of the subsurface microbial community may be influenced by the presence of ammonium as an energy source in the oligotrophic granite-hosted environment.

### MATERIALS AND METHODS

#### SAMPLE COLLECTION AND PROCESSING

Fluid samples were collected for geochemistry and molecular biology from boreholes draining at the 7025-foot level (elevation above sea level) within Henderson Mine during sampling trips in March 2006. The collection and processing of these samples has already been reported in Sahl et al. (2008). Samples were taken both before and after expandable packers were inserted to exclude O₂ present in the mine tunnel air from equilibrating with draining fluids. Samples of the borehole fluids are named according to the level within the mine they were collected (e.g., 7025), whether they were collected from boreholes before or after insertion of packing devices (“D” for drain,”P” for packer) and the assigned number of each borehole on that level (1, 2, 3, etc.). Additional geochemical data for borehole 7025-D3 reported here was analyzed according to the methodology described in Sahl et al. (2008).

Mineral samples for NH₄⁺ analysis (biotites and muscovites) in this study were collected from surface sites surrounding the mine, and from a drill core (Table 1). The surface samples were collected from outcrops of Proterozoic biotite gneiss and migmatised (metasediments) intruded by the 1.4 Ga Silver Plume granite within a 10-mile radius of Henderson Mine (Theobald, 1965). Subsurface samples from the ore-bearing stocks, the intruding

### Table 1 | Potassic minerals for FTIR analysis.

| Sample name | Sample type | Location | Mineral | [NH₄⁺] ppm | Reference |
|-------------|-------------|----------|---------|------------|-----------|
| **STANDARD** |             |          |         |            |           |
| MS-14       | Pelitic and banded schists | Moine succession, Scotland | Biotite | 845 | Boyd and Philippot (1998) |
| **SAMPLE** |             |          |         |            |           |
| SP1009      | Biotite gneiss and migmatisita | Vasquez Creek Road, CO | Biotite | ND | This study |
| HS-82-14    | Henderson Urad porphyry | Henderson underground | Biotite | ND | Stein (1985) |
| HS-82-42    | Henderson Urad porphyry | Henderson underground | Biotite | ND | Stein (1985) |
| HS-82-43    | Henderson porphyry | Henderson underground | Biotite | ND | Stein (1985) |
| HS-82-59    | Henderson Urad porphyry | Henderson drill core | Biotite | ND | Stein (1985) |
| HS-82-60    | Henderson primos interior | Henderson drill core | Biotite | ND | Stein (1985) |
| HS-82-61    | Henderson seriate granite | Henderson drill core | Biotite | ND | Stein (1985) |
| HS-82-62    | Henderson Dailey stock | Henderson drill core | Biotite | ND | Stein (1985) |
| HS-82-15    | Henderson, silver plume granite | Henderson underground | Biotite | ND | Stein (1985) |
| HS-82-53    | Silver plume granite, type locality | Silver plume, CO quarry | Muscovite | ND | Stein (1985) |
| 1225 m      | Henderson granite | DUSEL drill core | Muscovite | ND | Jung et al. (2007) |
| 1900 m      | Henderson granite | DUSEL drill core | Muscovite | ND | Jung et al. (2007) |

*Theobald (1965); ND = not detected.
Henderson, Seriate, and Urad granite and the Silver Plume granite were acquired from Holly Stein at Colorado State University (Stein, 1985). Hydrothermally altered Henderson Granite was also acquired from a drill core, and named according to the depth of drilling in feet (Sahl et al., 2008). Samples were crushed, sieved between 100 and 400 μm and biotites were concentrated with a Frantz magnetic separator. Minerals were washed in distilled H2O and dried at 50°C prior to analysis.

**FTIR MICROSCOPY**

Analysis for NH4+ in biotites and muscovites was made by FTIR microscopy as previously described (Busigny et al., 2003, 2004; Papineau et al., 2005; Papineau, 2006). Briefly, individual grains were analyzed using a Thermo Nicolet Continuum microscope linked to a Nexus 670 FTIR spectrometer at the University of Colorado on NaCl plates under CO2-purged air. The infrared beam was collimated to a 100 μm × 100 μm window and focused normal to the c-axis of mica grains. Two hundred transmission IR (4000–650 cm⁻¹) spectra were collected per grain using an MCT/A detector cooled by liquid nitrogen to minimize electronic noise and absorption of water.

**AMPLIFICATION OF FUNCTIONAL GENES**

The DNA used in this study was originally acquired through DNA extraction and amplification procedures reported in Sahl et al. (2008). The DNA was tested here for the presence of functional genes of the nitrogen cycle by PCR amplification, cloning, and sequencing. To evaluate the potential for ammonium to be biochemically fixed in the boreholes, the nifH gene that encodes for part of the nitrogenase enzyme was amplified from DNA of the 7025-P4 borehole using a nested PCR approach with primer sets nifA/nifRev and nifB/nifRev, respectively (Reed et al., 2010). The nifH products were extracted and cleaned from a gel using the EZNA gel extraction kit (Omega Bio-Tek, Inc.). The products were then cloned using a TOPO 1-shot cloning kit (Invitrogen) and sequenced commercially with M13F by SeqWright (Houston, TX, USA).

In addition to the N-fixation genes, two marker genes for nitrification were also assessed by PCR amplification. The primers 301F/302R (Norton et al., 2002) were used to amplify the bacterial ammonium-oxidation gene (amoA) that encodes for part of the ammonium monooxygenase enzyme under previously described cycling conditions (Hirayama et al., 2005). The potential presence of archaeal amoA genes was also assessed with previously described primers (Francis et al., 2005; Spear et al., 2007). Finally, the presence of nitrite oxidation genes from the Nitrospira genus was interrogated with the nxrBF916 and nxrBR1237 primers designed to target a 321 bp fragment of the nxrB gene that encodes for the β subunit of the nitrite oxidoreductase enzyme (Lücker et al., 2010). This gene was previously called norB, which is also the notation for nitric oxide reductase, and so was recently changed to nxrB to eliminate confusion (Starkenburg et al., 2006). As the nxrB gene is related to genes from the nar family that encodes for nitrate reductase in phylogenetically diverse organisms, it was necessary to use primers specific to the nitrite oxidoreductase-encoding genes from the Nitrospira genus. Only one definitive Nitrospira nxrB gene sequence exists in the public database from the metagenome of Ca. *Nitrospira defluvii* (Lücker et al., 2010), and so the primers for that organism were used here. Successfully amplified genes were cloned and sequenced as described above for nifH. Representative functional gene sequences from this study have been submitted to GenBank under accession numbers JN560700–JN560714.

**PHYLGENETIC ANALYSIS OF FUNCTIONAL GENES**

Nucleotide sequences were edited with Sequencher 4.7. Nucleotide sequences of the functional genes nifH and nxrB were translated to protein (amino acid) sequences using the transAlign script (Bininda-Emonds, 2005). Both nucleotide and amino acid sequences of functional genes were aligned using ClustalW. Similar sequences were identified from a blastn or blastx search of the NCBI database, and reference sequences were subsequently downloaded from this database. Phylogenetic trees were assembled using the RAxMLBlackBox with the JTT model of substitution (Stamatakis et al., 2008). The maximum-likelihood search was used to find the best-scoring tree, which was selected and edited in FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Additionally, 16S rRNA sequences of *Nitrospira* from borehole 7025-P4 reported in Sahl et al. (2008) were reanalyzed in order to resolve the genus-level groupings of these organisms. The sequences were aligned using the Greengenes database (Desantis et al., 2006) and RAxMLBlackBox was used for tree construction.

**RESULTS**

**GEOCHEMISTRY OF THE FLUIDS**

The aqueous geochemistry of fluids from boreholes 7025-D1/P1, 7025-D4/P4, 7150-D1, and 7150-D3 were previously reported (Sahl et al., 2008). Geochemistry from an additional borehole, 7025-D3, are reported here to establish that NH4+ increases corresponding to Mn along at least three boreholes (Figure 1). Borehole 7025-D3 had a pH of 6.14 and contained 0.52 mM Mn, 4.2 mM SO4²−, 0.68 mM F, and 3 μM NH4+.

**NH4+ CONTENT OF MICAS**

Biotite samples analyzed for NH4+ content (Table 1) were collected from surface outcrops of biotite gneiss and migmatite (sample SP1009) surrounding Henderson Mine. Biotites were also analyzed from subsurface samples of the Henderson and Seriate granite, the mineralized stocks of the Henderson and Urad ore-bodies, and the Silver Plume granite (sample names beginning with HS-82). Muscovite grains from hydrothermally altered Henderson granite obtained from drill core were also analyzed (samples 1225 and 1900 m). Although reference biotites from the Moine metsediment (MS-14; Boyd and Philippot, 1998) were successfully analyzed as NH4+-bearing standards with a characteristic and quantitative peak for NH4+ bending at 1429 cm under FTIR (data not shown), none of the Henderson subsurface biotite or muscovite samples displayed a peak corresponding to NH4+ bending.

**AMPLIFICATION AND PHYLOGENETIC ANALYSIS OF FUNCTIONAL GENES OF THE NITROGEN CYCLE**

Genomic DNA samples from boreholes for which 16S rRNA clone libraries had been assembled (7025-D1/P1 and 7025-P4; Sahl et al., 2008) were screened for the presence of functional genes of the nitrogen cycle. The nifH gene was successfully amplified only from...
borehole 7025-P4 DNA. Cloning and sequencing of this product resulted in retrieval of 39 nifH sequences of ~370 bp. The two unique nifH phylotypes (defined by >90% amino acid identity) are shown in the phylogenetic tree in Figure 2, along with nifH from other environments, all of which are longer than 300 bp. One phylotype (n = 2) groups with Verrucomicrobia nifH sequences that encode the C-type nitrogenases characteristic of Clostridium, Gram positive bacteria, and β-Proteobacteria (Mehta et al., 2003; Young, 2005). However, there were no Verrucomicrobial 16S rRNA gene sequences detected in this sample. The Gram positive group includes the Paenibacillus that are known to fix nitrogen (Canfield et al., 2005), and Paenibacillus were isolated previously from Henderson Mine (Mayhew et al., 2008). No nifH sequences were amplified from the DNA of the Paenibacillus isolates with the nifH primers used in this study (data not shown), suggesting nifH may not be present in Henderson Mine Paenibacillus. The second phylotype (n = 37) clusters separately, near to nifH sequences from the Nitrospira phylum and encodes B-type nitrogenases (characteristic of Proteobacteria, Cyanobacteria, and Firmicutes; Young, 2005). The only members of the Nitrospirae phylum detected in 7025-P4 DNA sample were from the genus Nitrospira, and none of these organisms are known to contain nifH (Lücker et al., 2010).

The amoA gene for bacterial ammonia oxidation could not be amplified from either of the borehole samples. DNA from a Japanese Gold Mine that contained AOB (Hirayama et al., 2005) was used as a positive control to ensure that this was indeed a negative result and not due to a problem with the amplification protocol. In contrast, 43 archaeal ammonium-oxidation amoA gene sequences of ~700 bp were amplified from 7025-P4 DNA. Four unique phylotypes (>90% nucleotide identity) were recovered. The relationship of 7025-P4 amoA and other environmental and pure culture amoA are shown in Figure 3, where all of the sequences included in the analysis are longer than 500 bp. One phylotype groups together with sequences from a mine adit in Colorado and hot springs clines in China (Spear et al., 2007; Zhang et al., 2008; Jiang et al., 2010), while two other phylotypes fall into a previously described cluster of amoA (Beman and Francis, 2006), determined by a common node with high bootstrap values.

The nxrB primers amplified several differently sized products, and as such are non-specific and should not be used in future amplification of nxrB from environmental DNA. For this reason, and because none of the proteobacterial nitrifying organisms (i.e., Nitrobacter, Nitrococcus, and Nitrospina) were detected in 7025-P1 DNA (Sahl et al., 2008), the nxrB primers were only used on 7025-P4 DNA. Amplification of the nxrB gene with Nitrosospira-specific primers was successful for the Nitrosospira-containing DNA from borehole 7025-P4, retrieving two sequences of ~339 bp that are 93% identical in amino acid to the nxrB of Ca. Nitrospira defluvii (FP929003; Lücker et al., 2010). The 7025-P4 nxrB amino acid sequences were aligned with the nxrB from nitrite-oxidizing Proteobacteria, as well as with sequences of the related nar genes (encoding the nitrate reductase enzyme). All of the sequences included in the analysis were longer than 250 bp. The 7025-P4 nxrB cluster closely with the Ca. Nitrospira defluvii nxrB, a relationship that is supported by high bootstrap values (Figure 4). This cluster is separated from the nar sequences and the nxrB sequences of Proteobacteria.

**DISCUSSION**

**GEOLOGICAL CONTRIBUTIONS TO THE SUBSURFACE NITROGEN CYCLE**

The aqueous geochemistry of two of the borehole fluids at the 7025 level addressed in this study have been previously reported (Sahl et al., 2008). The ammonium concentrations of boreholes P1 and P4 were 5 and 112 μM, respectively 2 weeks after being fitted with packing devices, and the concentrations are very similar to those reported before packer insertion (9 and 113 μM, respectively). Sahl et al. (2008) noted geochemical trends from this dataset signifying the mixing of different fluids, a rock-reacted brine, and meteoric water, via the covariance in F or SO42− and the dissolved Mn concentration across fluids sampled from five boreholes at two levels within Henderson Mine. In this study we note that the fluid NH4+ concentration also appears to increase corresponding with the greater degree of water-rock interaction across boreholes of the 7025 level, when data from a third borehole, 7025-D3 (reported here) is included (Figure 1). This trend suggests that NH4+ is sourced from the breakdown of NH4+-bearing minerals at depth. From the mineral assemblage present at Henderson (Theobald, 1965; Stein, 1985), the phases most likely to contain NH4+ are biotite and muscovite, where NH4+ can be substituted for K+ (Honma and Itihara, 1981; Hall, 1987).

Geological nitrogen as NH4+ substituted for K+ could not be detected in biotite collected from Precambrian metasedimentary rocks surrounding Henderson Mine. Thus it is unlikely that the Henderson granite or stocks would have taken up NH4+ directly...
from the metasediments when they were emplaced (i.e., Hall et al., 1991). However, the Tertiary Henderson granite and ore-bearing stocks (e.g., variably containing molybdenite, quartz, K-feldspar, biotite, fluorite, pyrite, or magnetite) are not directly hosted by the metasediments, but rather by the Precambrian Silver Plume granite. For this reason, biotites from the Silver Plume granite were also screened for the presence of ammonium. The Silver Plume granite biotites also do not show evidence of NH$_4^+$ incorporation. Even if Silver Plume granite did contain ammonium, it is unlikely to have been transferred to the Henderson granite and ore-bearing stocks, because previous isotopic studies indicate little chemical interaction between the Henderson ore-body and surrounding wall rocks (Stein and Hannah, 1985).

Another possible mechanism that could supply ammonium to biotites of the Tertiary Henderson granite and the ore-bearing stocks would be from interaction with the Proterozoic basement, if it contains NH$_4^+$ (Honma and Itihara, 1981; Hall, 1988). This type of transfer is possible based on the $\varepsilon_{Nd}$ and $\varepsilon_{Sr}$ values of Henderson granite, which sources its Mo from the mid- to lower crust (Farmer and Depaolo, 1984). Although an appropriate mid- or lower-crust sample was not available for FTIR analysis to screen for NH$_4^+$-content, none of the biotites of the Henderson granite and stocks contained NH$_4^+$. While other potassic phases were not analyzed (i.e., muscovite and potassium feldspar), biotites generally have the highest NH$_4^+$ contents of silicate minerals (Honma and Itihara, 1981; Hall, 1987). However, muscovites that formed

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The phylotypes from this study fall into four distinct sub-clusters within the previously defined A and B clusters (Beman and Francis, 2006; Zhang et al., 2008). The bacterial amoA outgroup has been removed for visual clarity. The scale represents 0.4 substitutions per site.

The Henderson nxrB amino acid sequences retrieved in this study, clones 74 and 122 (bold) are highly similar to the sequence of nxrB in Ca. Nitrospira defluvi, and this relationship is supported by high bootstrap values. The other nxrB sequences belong to organisms of the Proteobacteria, except for the Japanese Au mine clones (Hirayama et al., 2005), whose phylogenetic affiliation is unknown. Scale bar represents 0.6 substitutions per site.
after hydrothermal alteration of the Henderson granite were analyzed for NH$_4^+$ because hydrothermal alteration of granites with fluids that have interacted with NH$_4^+$-bearing crustal rocks has been observed for intrusive granites in Italy (Hall et al., 1991). The hydrothermal muscovites did not contain NH$_4^+$, which is consistent with the geological interpretation that the suite of hydrothermally altered minerals at Henderson formed due to the introduction of juvenile, magmatic fluids that did not interact with the surrounding metasediments, granite, or groundwater (Seedorff and Einaudi, 2004).

There are other potential sources of non-biological NH$_4^+$ that could not be evaluated for several reasons. For example, nitrogen compounds could have been introduced to subsurface fluids through ammonium nitrate fuel oil (ANFO) that was used as a blasting agent at Henderson (Jensen et al., 1983). However, the boreholes had flushed for almost a year prior to sampling and only the rock-reacted fluids being dewatered from the mine contained high NH$_4^+$ (i.e., borehole 7025-P4; 112 μM). A meteoric source was unlikely as well, as the most dilute, meteoric-dominated waters contained the lowest levels of N (as NH$_4^+$, NO$_3^-$, and NO$_2^-$; Figure 1). Modern precipitation in Colorado can exhibit high levels of nitrate (up to ~20μM) in the mountains from industrial activities (Faure and Mensing, 2005) that could be transported to the subsurface in meteoric water. However, the dilute borehole waters at Henderson have an average residence time of 13,000 years based on $^{14}$C dating, and is dominantly pre-1950s based on tritium content (Sahl et al., 2008), further underscoring that this modern meteoric water is not likely to be the nitrogen source to subsurface fluids.

**BIOLOGICAL FIXATION AS A SOURCE OF SUBSURFACE AMMONIUM**

The presence of nifH genes in DNA in borehole 7025-P4 (Figure 2; 112 μM NH$_4^+$), and the lack of detectable nifH genes in boreholes 7025-D1/P1 (5–9 μM NH$_4^+$) suggests that microbes inhabiting 7025-P4 fluids are capable of fixing N$_2$ gas to NH$_4^+$. This finding of a biological source of nitrogen specific to 7025-P4 is consistent with the lack of evidence for a general subsurface nitrogen source from the breakdown of NH$_4^+$-bearing potassic minerals (Table 1).

However, the presence of functional genes does not necessarily signify expression or activity of the encoded enzymes, and so further experiments are needed to evaluate whether nitrogen fixation is occurring. Fresh samples would be needed to measure $^{15}$N uptake or acetylene reduction as evidence for nitrogen fixation, and we have been unable to access Henderson Mine for further sampling of these boreholes, and so the further geochemical assays could not be undertaken to augment the dataset in the current study.

If organisms in 7025-P4 fluids do fix nitrogen, they would need to be utilizing a metabolic reaction capable of generating enough ATP and electrons to fuel nitrogen fixation. Although specific N$_2$-fixing organisms cannot be identified in 7025-P4 based solely on the detected 16S rRNA and nifH genes, the geochemical dataset of Sahl et al. (2008) can be used to infer which chemolithoautotrophic metabolisms could support the subsurface community thermodynamically. The reaction of H$_2$S oxidation with electron acceptors such as O$_2$, NO$_3^-$, Fe(OH)$_3$, and MnO$_2$, or S$^0$ and Fe$^{2+}$ oxidation with NO$_2^-$, are more thermodynamically favorable in borehole 7025-P4 relative to 7025-P1 (Swanner, 2011). There is a greater than ~10 kJ mol$^{-1}$ of electrons transferred increase between 7025-P1 and 7025–P4 for all of these reactions, and all yield at least ~20 kJ mol$^{-1}$ of electrons transferred in borehole 7025-P4. Thus nitrogen fixation may be fueled preferentially in the more rock-reacted borehole fluid by any of these reactions. Given the fact that the borehole fluid microbial community compositions are modulated by the degree of mixing between meteoric and rock-reacted fluids (Sahl et al., 2008), we suggest that the potential for nitrogen fixation is strongly tied to the aqueous geochemistry. Furthermore, despite this energetic strain of nitrogen fixation, it has been documented for deep-sea sulfate-reducing methanotroph consortia, whose syntrophic metabolisms provides one of the lowest energetic yields known to sustain microbial life (~40 kJ mol$^{-1}$ electron transferred to support two organisms; Dekas et al., 2009), suggesting that the energetic yields of reactions possible in 7025-P4 fluids could support nitrogen fixation.

The inference that ammonium is biologically fixed in Henderson fluids raises another question about why nitrogen-fixing organisms would fix nitrogen to levels as high as 112 μM NH$_4^+$ in an environment where total numbers of microbes are only $10^3$–$10^4$ cells ml, and only half of the nitrogen is converted into organic forms (Sahl et al., 2008). Intriguingly, nitrogen fixation occurs in deep-sea methane-oxidizing archaeal/bacteria consortia in sediments that already contain ammonium (Dekas et al., 2009), suggesting either that localized depletions of ammonium within the consortium drive fixation, or that nitrogenase activity is not well regulated by NH$_4^+$ concentrations. A lack of regulation on N$_2$-fixation by NH$_4^+$ concentrations up to 94 mM has been recently documented for the verrucomicrobial methanotroph *Methylacidiphilum fumariciolicum* strain SoLV (Khadem et al., 2010), and some proteobacterial nitrogenases also appear to be unregulated by NH$_4^+$ (Rudnick et al., 1997). One other reason for nitrogen fixation in the presence of abundant ammonium may be that the transfer of ammonium between nitrogen-fixing and ammonium-utilizing microbes may not be very efficient. Nitrogen-fixing cyanobacteria from the Baltic Sea have been observed to lose up to 30% of their fixed nitrogen to bulk surroundings (Ploug et al., 2010). Further culture and assay-based studies are needed to determine if and why excess nitrogen is being fixed in 7025-P4, but such experiments were not undertaken in the current work due to the sampling limitations discussed above.

It is well established that nifH tree topology often mirrors topology of the 16S rRNA gene tree (with some variation due to lateral gene transfer; Hennecke et al., 1985; Normand and Bouquet, 1989; Young, 2005). Although not definitive evidence of phylogenetic affiliation, the nifH sequences detected in borehole 7025-P4 DNA may be useful in informing the classes of potential nitrogen-fixing organisms. In Figure 2 the C-type nifH phylotype (n = 2) groups with *Verrucomicrobia* nifH, but no 16S rRNA from *Verrucomicrobia* were detected in the DNA of 7025-P4. This phylotype of nifH may source from the *Chlorobi* in 7025-P4 DNA, which are phylogenetically close to *Verrucomicrobia* by 16S rRNA and nifH measures. *Chlorobi* 16S rRNA genes were detected in this sample (Sahl et al., 2008), although the Henderson nifH and *Chlorobi* nifH protein sequences were only 91% similar. A more likely possibility is that this nif operon was horizontally transferred (Hirsch et al., 1993) and may not reflect the true phylogeny of organisms.
The B-type \textit{nifH} phylotype \((n = 37)\) was much more abundant than the C-type phylotype in 7025-P4 DNA, implying the organisms from which these sequences came from must be much more abundant in the sample. In fact, the most dominant organism in this sample by the measure of 16S rRNA gene sequences (37\%) are the novel phylum Henderson candidate division that were first detected in the Henderson environment (Sahl et al., 2008), and subsequently detected in seafloor basalt (Omoregie et al., 2008; Santelli et al., 2008). The phylogenetic analysis of Sahl et al. (2008) demonstrates that the closest related phyla to Henderson candidate division are the Acidobacteria and the Nitrospirae. In Figure 2, the B-type \textit{nifH} phylotype is similarly related to the \textit{Nitrospira} sequence from \textit{T. yellowstonii}. There were \textit{Nitrospira} 16S rRNA sequences detected in this sample (Sahl et al., 2008), but they belong specifically to the \textit{Nitrospira} genus, whose only representative sequenced genome does not contain any homologs to the \textit{nifH} gene, suggesting this genus is unlikely to fix nitrogen (Lücker et al., 2010). There were also Acidobacteria 16S rRNA sequences detected in the 7025-P4 DNA sample, but of the three sequenced Acidobacteria genomes, none possess any \textit{nif} genes (Ward et al., 2009). The B-type \textit{nifH} phylotype may source from Henderson candidate division bacteria, but this phylotype of \textit{nifH} was not amplified from the DNA of borehole 7025-P1, which contained 56\% Henderson candidate division 16S rRNA sequences. Without further investigation of the genome of Henderson candidate division or other organisms in borehole 7025-P4, it is unclear which organisms the B-type or C-type \textit{nifH} phylotype source from.

### Henderson Fluids Support Nitrification

The presence of high \(\text{NH}_4^+\), \(\text{NO}_2^-\), and \(\text{NO}_3^-\) and low levels of \(\text{O}_2\) \((1.25 \text{ mM})\) in borehole 7025-P4 suggest that ammonium and nitrite oxidation may be occurring. In fact, both of the reactions of nitrification, summarized by Eq. 1 and 2 below, are thermodynamically favorable in borehole 7025-P4 (Swanner, 2011).

\[
\text{NH}_4^+ + 1.5\text{O}_2 \rightarrow \text{NO}_2^- + \text{H}_2\text{O} + 2\text{H}^+ \quad (1)
\]

\[
\text{NO}_2^- + 0.5\text{O}_2 \rightarrow \text{NO}_3^- \quad (2)
\]

Reactions 1 and 2 yield \(-40\) and \(-35 \text{ kJ mol electron transferred}\), respectively. In addition, micromolar quantities of gaseous \(\text{N}_2\) were detected in 7025-P4. \(\text{N}_2\) is an intermediate in both microbial nitrification/denitrification, and as such is a hallmark of biological nitrogen cycling. One abiotic mechanism for \(\text{N}_2\) production involves reaction of \(\text{NO}_2^-\) or \(\text{NO}_3^-\) with (ultra)mafic rocks and minerals (dolerite, augite, and olivine; Samarkin et al., 2010), but none of these phases are present at Henderson and so this mechanism is unlikely. \(\text{N}_2\) can also be produced from \(\text{NO}_2^-\) in the presence of \(\text{Fe}^{2+}\) and \(\text{Cu}^{2+}\) (both observed in the borehole fluids) within 24 h at \(p\text{H} 6\) (Moraghan and Buresh, 1977). Therefore, even if \(\text{N}_2\) is abiotically produced, the additional presence of \(\text{NO}_2^-\) strongly implicates either nitrification or denitrification in 7025-P4 fluids as a mechanism for continued \(\text{NO}_2^-\) production. Because of the presence of \(\text{N}_2\), it is also worth noting that nitrate reduction coupled to inorganic electron donors is thermodynamically favorable; in contrast, microbially mediated reactions such as anammox, the anaerobic oxidation of ammonium with nitrite is less likely in Henderson, due to predicted unfavorable thermodynamics under \textit{insitu} conditions (Swanner, 2011). In addition, no organisms belonging to the phylum \textit{Planctomycetes} were detected at Henderson, and only these organisms are currently known to carry out anammox (Kuenen, 2008). A final consideration on the favorability of nitrification in the borehole fluids is that the \(C: N\) is near unity, which should drive the consumption of ammonium by nitrification relative to the assimilation of ammonium by heterotrophs, because \(C: N\) values generally need to be \(>20\) for heterotrophic ammonium uptake to predominate (Strauss and Lambert, 2000). Additionally, inorganic phosphate levels were below detection (Sahl et al., 2008), further suggesting that the borehole fluids are oligotrophic.

The organisms most likely to be carrying out ammonium-oxidation in borehole 7025-P4 are AOA, as no AOB were detected in 7025-P4 DNA by 16S rRNA (Sahl et al., 2008). The archaeal organisms previously detected in borehole 7025-P4 belong to the phylum \textit{Crenarchaeota} (Brochier-Armanet et al., 2008; Spang et al., 2010) as reported in Figure 6 of Sahl et al. (2008). Our realignment of these previously retrieved 16S rRNA sequences with sequences from other subsurface mines (data not shown) reveal that they are closely related to other subsurface \textit{Crenarchaeota}, some of which have been implicated in archaeal ammonium oxidation (Takai et al., 2001; Nunoura et al., 2005; Spear et al., 2007; Rastogi et al., 2010). Four of the six archaeal sequences retrieved from 7025-P4 are 97\% identical to \textit{Ca. Nitrosopumilus garginsi}, an ammonium-oxidizing (AOA) isolate (Hatzenbihler et al., 2008), and \textit{amoA} genes have been detected and implicated in ammonium oxidation for other closely related organisms within the \textit{Crenarchaeota} (Treusch et al., 2004). Our finding of \textit{amoA} in DNA of the 7025-P4 borehole that contains high \(\text{NH}_4^+\) and organisms closely related to AOA suggests, in conjunction with the geochemical evidence for nitrification, that AOA may be active in subsurface fluids at Henderson Mine. Furthermore, this study is at least the third to amplify archaeal \textit{amoA} from a terrestrial subsurface setting (Spear et al., 2007; Weidler et al., 2007), and our phylogenetic analysis in Figure 3 suggests that there may be sub-clusters of \textit{amoA} defined by subsurface and hot spring clones (Beman and Francis, 2006; Zhang et al., 2008). Future subsurface studies may reveal further AOA and \textit{amoA} diversity, and will establish the contribution of these processes at depth to the global nitrogen cycle.

The presence of archaeal \textit{amoA} in the 7025-P4 DNA sample and absence of the bacterial version of this gene may be explained by the geochemical conditions. The sub-millimolar concentration of \(\text{NH}_4^+\) in borehole 7025-P4 is ideal for AOA who are inhibited by higher concentrations of \(\text{NH}_4^+\) (i.e., 2–3 mM). One cultivated AOA, \textit{Nitrosopumilus maritimus} has an ammonia monooxygenase enzyme that reaches maximal activity at \(\text{NH}_4^+\) concentrations that are 100-fold less than those observed for AOB enzymes (Martens-Habbena et al., 2009). The low-\text{O}_2 concentrations in 7025-P4 may favor AOA who thrive when dissolved \text{O}_2 is 3–20 \text{ \mu M} (Ergruder et al., 2009). The archaea were only detected after packer insertion, further supporting their adaptation to thrive in low-\text{O}_2 niches. The presence of sulfide in wastewater reactors and estuary sediments has an inhibitory effect on the activity of AOB relative to AOA (Sears et al., 2004; Caffrey et al., 2007), and a similar phenomenon has been observed in cultivated AOB (Hooper and Terry, 2010).
Dissolved sulfide was not measured in Henderson fluids, but is likely present due to the extensive sulfide mineralization at Henderson, and the formation of elemental sulfur where borehole fluids are released into oxic mine tunnels (Templeton and Swanner, unpublished data).

While the *Crenarchaeota* have only recently been characterized as AOA (Könnecke et al., 2005), the ensuing widespread detection of AOA in soils, estuaries and marine environments and their abundance worldwide relative to AOB signifies that these organisms likely do account for a large portion of both terrestrial and marine nitrogen cycling (Francis et al., 2007). AOA are also preferentially found in low-nutrient environments (Ergruder et al., 2009), and they seem to be adapted to higher salinity (Bernhard et al., 2010), although AOB appear to outnumber AOA in estuarine environments regardless of varying nutrient fluxes (Wankel et al., 2011). AOA are also predominant in environments above 40°C (Zhao et al., 2011), which is the temperature of Henderson fluids. Furthermore, AOA are phylogenetically diverse, and this diversity may underscore their adaptation to diverse environmental conditions relative to the phylogenetically restricted AOB (Francis et al., 2005).

AOB and AOA are not the only organisms capable of oxidizing ammonia. Methanotrophs can also oxidize ammonia due to the broad substrate specificity of the enzyme methane monooxygenase. However, methanotrophs cannot grow using ammonia as a substrate, nor can ammonia oxidizers grow using methane as a substrate (Bédard and Knowles, 1989; Hanson and Hanson, 1996). Bacterial methanotrophs reside within the α- and γ-Proteobacteria. Both of these Proteobacterial divisions were detected in borehole 7025-P4 DNA via 16S rRNA, and the fluids from this sample contained submicromolar levels of methane (Sahl et al., 2008). However, the closest isolated methanotrophs were only 92% related to the 7025-P4 α-Proteobacteria sequence, and only 86% identical to the γ-Proteobacteria sequences. Although ammonium-oxidation by methanotrophs may not be significant in Henderson borehole fluids, it could be an important process in other sites were ammonium and methanotrophs are both abundant, such as a Japanese gold mine (Hirayama et al., 2005). Methanotrophs could contribute to subsurface nitrogen cycling by fixing nitrogen as well (Hanson and Hanson, 1996; Auman et al., 2001). The ability to fix nitrogen may confer a selective advantage for methanotrophs inhabiting low-nutrient subsurface habitats, as is hypothesized for a Japanese uranium mine (Mills et al., 2010).

The nitrification pathway may be carried to completion by the nitrite-oxidizing *Nitrospira* genus (Daims et al., 2001). 16S rRNA sequences belonging to the *Nitrospira* genus were detected in borehole 7025-P4 DNA (1% of the microbial community; Sahl et al., 2008). As was the case with AOA, *Nitrospira* were not detected in the DNA of borehole 7025-D1/P1. The *Nitrospira* sequences from 7025-P4 are distinct at the phylum level from the Henderson candidate division proposed for unique sequences detected in DNA from both boreholes at the Henderson 7025 level (D1/P1 and P4; Sahl et al., 2008). In the current analysis in Figure 5, the *Nitrospira* sequences detected from 7025-P4 DNA group closely with a clone from a deep gold mine (Gihring et al., 2006), but apart from other identified clusters of *Nitrospira* (Daims et al., 2001; Lebedeva et al., 2011). The bootstrap values that support this topology are all >90%, suggesting that Henderson sequences may define a novel cluster of subsurface *Nitrospira*. All sequences included in this analysis were longer than 800 bp.

Despite the limited sequence data available for the *Nitrospira nxrB* gene, which encodes the nitrite oxidoreductase, the primers of Lücke et al. (2010) did successfully amplify *nxrB* from the DNA sample of 7025-P4. As 7025-P4 DNA did not contain any 16S rRNA sequences corresponding to Proteobacterial nitrite-oxidizers (i.e., *Nitrobacter*, *Nitrococcus*, and *Nitrospira*), it is possible the *nxrB* sequences we amplified are from *Nitrospira* spp. Because of the high similarity of the amino acid sequence of the amplified genes to that of the Ca. *Nitrospira defluvii nxrB* and the high bootstrap values supporting this relationship, it is likely 7025-P4 *nxrB* clones 74 and 122 are actual *Nitrospira nxrB* sequences (Figure 4). Alternatively, this sequence could encode a related *nar* gene or a *nxrB* gene from *Proteobacteria*, but this is unlikely as no nitrifying *Proteobacteria* were detected in this sample. However, without direct evidence from longer sequences of the *Nitrospira* genome in borehole 7025-P4 DNA, it cannot be established that the *nxrB* genes from this study belong to *Nitrospira* genus, and the sample limitations discussed above prohibit further genetic analysis. The *nxrB* sequences retrieved in this study may be useful in future primer design and acquisition of quality environmental sequence data for *Nitrospira*, which will aid in understanding the evolutionary history of the enzymes involved in nitrite/nitrate oxidation and reduction and the organisms that possess them.

The presence of *Nitrospira* in borehole 7025-P4 offers insight into the ecology of these organisms. Three pure cultures consist of obligately lithothrophic nitrite-oxidizers from the genus *Nitrospira* (Watson et al., 1986; Ehrich et al., 1995; Lebedeva et al., 2008, 2011). The cultures were isolated out of both freshwater and marine habitats (Koops and Pommerening-Röser, 2001), as well as hot springs (Lebedeva et al., 2011), suggesting the ecology of this genus is not limited to one type of geochemical environment. The abundance of *Nitrospira* in nitrite-oxidizing environments in comparison to nitrite-oxidizers of the *Proteobacterial* nitrite-oxidizers (i.e., *Nitrobacter*) is evidence for their contribution to nitrite oxidation in a number of environments (Daims et al., 2001).

The *Nitrospira* genus seems to be inhibited by higher concentrations of nitrite (i.e., >5–15 mM; Ehrich et al., 1995; Lebedeva et al., 2011) and O2 (Altmann et al., 2003) than are preferred by *Nitrobacter* species (Ehrich et al., 1995; Daims et al., 2006), suggesting *Nitrospira* may be preferentially adapted to oligotrophic conditions such as are experienced in borehole 7025-P4. *Nitrospira* have now been detected in three subsurface settings where O2 was <10 µM and NO3− did not exceed 120 mM (i.e., borehole 7025-P4; Hirayama et al., 2005; Weidler et al., 2007), although they have also been detected in subsurface fluids with millimolar concentrations of NO3− but low-O2 (Gihring et al., 2006). *Nitrospira* may well be quite common in subsurface settings that contain NO3−, and future investigations may uncover more phylogenetic and physiological knowledge about this important genus.

**CONCLUSION**

This study demonstrates that the deep subsurface microbial communities in the borehole fluids of Henderson Mine have the
genetic capacity to fix nitrogen. This fixed nitrogen, as ammonium, is likely to be the primary nitrogen source for organisms living in the subsurface, as no evidence was found that ammonium was sourced geologically from NH$_4^+$-bearing potassic minerals such as biotites. Although the presence of nifH genes in borehole 7025-P4 is only indirect evidence that nitrogen fixation is occurring, it is also correlates to elevated NH$_4^+$ concentrations and the lack of nifH in boreholes with lower NH$_4^+$ concentrations. If nitrogen fixation is occurring at Henderson it highlights the phenomenon of excess nitrogen fixation under energy–poor conditions. The availability of ammonium, regardless of the ultimate source, also has important consequences for the subsurface microbial community not only as an essential nutrient but because it can also be used as an energy source. There is evidence for ammonium and nitrite oxidation in borehole 7025-P4 where ammonium concentrations are highest based on the detection of archaeal ammonia mono-oxygenase (amoA) and nitrite oxido-reductase (nirB) functional genes. While other subsurface studies have detected genes encoding for both steps of nitrification (Hirayama et al., 2005; Weidler et al., 2007), neither study addressed the source of ammonium fueling this process. Moreover, the nirB sequences detected from borehole 7025-P4 are the first amplified from the Nitrospira genus in environmental samples. The presence of nitrifiers 3000 feet below the surface at Henderson also reinforces the idea that Crenarchaea and Nitrospira may be adapted to low nutrient and substrate environments and could be key players in nitrogen cycling in similar settings. Thus this also work highlights the potential that biological nitrogen fixation and nitrification may be ubiquitous biogeochemical processes in the deep subsurface and maybe important to consider in accounts of global nitrogen cycling.

ACKNOWLEDGMENTS

This work was supported by grants from the David and Lucille Packard Foundation and the National Science Foundation (NSF-BIO 0623815) to Alexis S. Templeton. The investigation was also
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