Anaerobic carboxydotrophic bacteria in geothermal springs identified using stable isotope probing

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Carbon monoxide (CO) is a potential energy and carbon source for thermophilic bacteria in geothermal environments. Geothermal sites ranging in temperature from 45 to 65°C were investigated for the presence and activity of anaerobic CO-oxidizing bacteria. Anaerobic CO oxidation potentials were measured at up to 48.9 μmoles CO g−1 (wet weight) day−1 within five selected sites. Active anaerobic carboxydotrophic bacteria were identified using 13CO DNA stable isotope probing (SIP) combined with pyrosequencing of 16S rRNA genes amplified from labeled DNA. Bacterial communities identified in heavy DNA fractions were predominated by Firmicutes, which comprised up to 95% of all sequences in 13CO incubations. The predominant bacteria that assimilated 13C derived from CO were closely related (>98% 16S rRNA gene sequence identity) to genera of known carboxydotrophs including Thermincola, Desulfotomaculum, Thermolithobacter, and Carboxydocella, although a few species with lower similarity to known bacteria were also found that may represent previously unconfirmed CO-oxidizers. While the distribution was variable, many of the same OTUs were identified across sample sites from different temperature regimes. These results show that bacteria capable of using CO as a carbon source are common in geothermal springs, and that thermophilic carboxydotrophs are probably already quite well known from cultivation studies.

Keywords: carboxydotrophs, stable isotope probing, geothermal, carbon monoxide (CO), thermophile

Introduction

Carbon monoxide (CO) is an odorless gas toxic to many animals due to its competitive binding to hemoglobin (Haab, 1990). It has been estimated that about 3.3 × 10⁹ metric tons of CO are released annually to the atmosphere (Conrad, 1996). There are numerous natural biogenic and abiogenic sources of CO. Thermal decomposition and photochemical degradation of organic compounds are important sources of abiotic CO (Sipma et al., 2006). CO is also a component of volcanic emissions, which may contain as much as 1–2% of CO per volume of total gas (Giggenbach, 1980; Svetlichny et al., 1991a; Sokolova et al., 2009 and references therein). Biogenic CO may also be produced in microbial ecosystems, and net CO production has been reported for marine algae (Conrad, 1988) and hypersaline cyanobacterial mats (Hoehler et al., 2001) during photosynthesis. Sulfate-reducing bacteria (SRB) have also been shown to produce CO during fermentation (Voordouw, 2002). Some microbes growing in high temperature environments are likely capable of growth at low concentrations of CO (Sokolova et al., 2009). It has also been suggested that CO-oxidizing microbes...
may occupy micro-niches in which biogenic CO locally accumulates to high levels (Techtmann et al., 2009).

Microorganisms that have the ability to oxidize CO are termed “carboxydotrophs” (King and Weber, 2007). A number of aerobic and anaerobic bacteria as well as some anaerobic archaea (e.g., methanogens) are capable of using CO as a source of energy and/or carbon (e.g., Mörsdorf et al., 1992; Oelgeschläger and Rother, 2008; Sokolova et al., 2009). Carboxydotrophic energy generation employs the enzyme CO dehydrogenase (CODH) that oxidizes CO to CO$_2$, generating electrons. The aerobic and anaerobic versions of this enzyme differ. Anaerobic CODH in bacteria is encoded by coo genes (Techtmann et al., 2011) and contains nickel in the active site, while aerobic CODH is encoded by cox genes and contains molybdenum (e.g., Dobbeck et al., 2001; King and Weber, 2007). Within the Domain Bacteria, anaerobic carboxydotrophs are typically found within the phylum Firmicutes and some in the class Alphaproteobacteria of the Proteobacteria (see Techtmann et al., 2009). Purple non-sulfur bacteria (i.e., phototrophic Alphaproteobacteria) exist among the known anaerobic CO oxidizers and were among the first discovered (Uffen, 1981; Kerby et al., 1995). However, an increasing number have been identified that are strictly anaerobic thermophiles belonging to the phylum Firmicutes (e.g., Svetlichny et al., 1991b; Sokolova et al., 2002). Hydrothermal systems have been proposed as early ecosystems supporting chemolithotrophic life, including thermophilic anaerobic bacteria and archaea using CO as an energy and carbon source (e.g., Cavicchioli, 2002; Wächtershäuser, 2006; King and Weber, 2007). Examples of thermophilic archaea that use CO include Thermococcus sp. NA1, capable of both heterotrophic and carboxydotrophic growth (Lee et al., 2008) and Archaeoglobus fulgidus capable of using CO as an autotrophic growth substrate (Henstra et al., 2007a).

While the anaerobic oxidation of CO may be coupled to a variety of respiratory processes such as sulfate reduction and acetogenesis (Oelgeschläger and Rother, 2008), hydrogenogenic carboxydotrophs make up the majority of thermophilic CO oxidizing microbes that have been identified in geothermal environments (e.g., Svetlichny et al., 1991a,b; Sokolova et al., 2004, 2005; Slepova et al., 2006). These bacteria oxidize CO via the water-gas-shift reaction (Uffen, 1981; Sipma et al., 2006):

$$\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2 \quad (\Delta G^0 = -20\text{kJ}) \quad (1)$$

Thermophilic bacteria and archaea with the capacity for hydrogenogenic carboxydotrophy have been isolated from various locations around the world including the Kunashir Island, Russia (Svetlichny et al., 1991a), Kamchatka (Sokolova et al., 2002; Slepova et al., 2006), Yellowstone National Park (Sokolova et al., 2004), and Iceland (Novikov et al., 2011). The isolates share similar ranges of optimal pH (ca. 6.8–7.0) and temperature (ca. 55–83°C) (see Henstra et al., 2007b; Techtmann et al., 2009) but are phylogenetically divergent (Techtmann et al., 2009). Unlike mesophilic carboxydotrophs, the thermophilic hydrogenogenic species isolated so far do not show growth inhibition by high levels of CO. In fact, most grow under atmospheres of 100% CO, far above natural CO concentrations in geothermal systems (e.g., Svetlichny et al., 1991a,b).

**Materials and Methods**

**Geothermal Spring Sample Collection**

Five geothermal springs were selected for CO-SIP investigations from among geothermal sites in Western Canada (Grasby et al., 2000; Sharp et al., 2012). Soil or sediment or biomat samples were collected at various times of the year between fall 2010 and fall 2012 into sterile screw-cap tubes (Table 1). Samples were kept cold as soon as possible to minimize changes in the microbial community during transport. Collected material was sub-sampled within approximately 5 days of sampling for DNA extraction, and the remainder was stored at 4°C for 1–2 d prior to incubation studies.

**Soil Microcosms and CO Oxidation**

Approximately 2–5 g of sample material (wet weight) was added to 120-ml serum bottles and crimp-sealed with sterile blue butyl rubber stoppers. An anoxic environment was created by repeated (3×) evacuation and refilling with N$_2$ gas. CO was
16S rRNA gene copies were selected for pyrosequencing (e.g., DCm2010) (Supplementary Table S1). However, in some cases the amount of DNA present was too low to obtain enough for pyrosequencing (e.g., DCm2010) (Supplementary Figure S1). Samples were prepared for sequencing analysis as described previously (Grasby et al., 2013; Sharp et al., 2014a) using FLX Titanium amplicon primers 454T_RA_X and 454T_F, which contain 16S rRNA gene targeted primers 926fw (5′-aaactYaaaKgaattgRcgg-3′) and 1392r (5′-acggcgggtgRc-3′) designed to target both bacteria and archaea (Ramos-Padrón et al., 2011). PCR reactions and purification were performed as described in Sharp et al. (2014a). Purified PCR products (ca. 150 ng total DNA) were analyzed at the Genome Quebec and McGill University Innovation Centre, Montreal, Quebec on a 454 Life Sciences Genome Sequencer FLX (Roche) machine running the Titanium chemistry.

**Sequence Data Processing**

Quantitative Insights Into Microbial Ecology (QIIME) pipeline version 1.8 (Caporaso et al., 2010) was used to process raw sequence data as in Sharp et al. (2014a). A minimum quality score of 25 was used and sequences were screened using ChimeraSlayer (Haas et al., 2011). Taxonomic identification of a representative sequence (most common) for each phylotype (clustered at 97% similarity) was determined using nucleotide
Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) against the Silva 111 reference database (Pruesse et al., 2007). Eukaryotic and chloroplast sequences were removed from further analysis. Final numbers ranged from 2195 to 21118 sequences per sample (Table S1). A phylogenetic tree was constructed using the parsimony-add function in ARB (Ludwig et al., 2004). 16S rRNA gene sequences obtained from this study have been deposited in the SRA database under accession numbers SRP028305 and SRP059036. Representative sequences of identified OTUs present at 25 fold enrichment are provided in the Supplementary Material.

Results

Biodegradation of Carbon Monoxide

Anaerobic oxidation of carbon monoxide was detected via monitoring of the CO mixing ratio in serum bottle headspaces after adding CO. Communities that showed evidence for CO consumption were selected for further investigation using stable isotope probing (SIP). The average values for anaerobic CO oxidation potentials for each sample is listed in Table 1. Rates ranged from 13.6 to 48.9 nmol mol g\(^{-1}\) d\(^{-1}\). The fastest rates were observed in samples from the Dewar Creek hot spring. Despite the anaerobic conditions, no methane production was observed in samples from the Dewar Creek hot spring. Despite other Dewar Creek and Lakelse samples, with estimated CH\(_4\) production rates of 0.013–11.4 nmol mol day\(^{-1}\), no added CO always showed very minor density shifts in DNA and 16S rRNA gene counts. This indicated that other autotrophs

Identification of Active CO Consuming Bacteria Using SIP Combined with 16S rRNA Gene Sequencing

Density profiles of DNA extracted from samples after incubation with \(^{13}\)CO generally ranged from 1.660 to 1.800 g ml\(^{-1}\). Shifts in DNA density compared to un-amended control samples were often subtle (Supplementary Figure S1). Therefore, quantitative real-time PCR (qPCR) counts of total bacterial 16S rRNA gene abundance were used to identify density fractions that had no assimilation of the \(^{13}\)C from \(^{13}\)CO into DNA. In contrast, no significant shift in DNA density or 16S rRNA gene copies was observed over multiple incubations of the PB1 sample under \(^{13}\)CO. Although this sample also oxidized CO, no assimilation of \(^{13}\)C was evident. These two samples were representative of the two major patterns observed. Other samples analyzed are shown in Supplementary Figures S1, S2.

A fundamental issue with interpreting SIP results is cross-labeling of other bacteria via metabolic products, especially CO\(_2\). The severity of this problem can be estimated via several controls. In GR1 (Figure 1A), as well as in several other samples tested (Supplementary Figures S1, S2) incubations with only \(^{13}\)CO and no added CO always showed very minor density shifts in DNA and 16S rRNA gene counts. This indicated that other autotrophs...
growing on substrates such as sulfur and ammonia were of minor importance. CO was therefore the primary energy source in the incubations, and the food webs detected were ultimately based on CO oxidation.

In samples like GR1, microcosms containing $^{13}$CO as the sole carbon and energy source (with no CO$_2$ addition) displayed the greatest shift in density (Figure 1). Less of a shift in density was observed in incubation with $^{13}$CO + $^{12}$CO$_2$, probably because assimilation of $^{12}$C from the added CO$_2$ diluted the labeling effect. Incubation with $^{12}$CO + $^{13}$CO$_2$ showed some increase in 16S rRNA gene copies compared to the un-incubated control, albeit to a lesser extent than in 13CO. The apparent assimilation of C preferably from $^{13}$CO but also from 13CO$_2$ indicates that CO$_2$ was probably the primary C source of the CO oxidizers, but that there may be a diffusion effect whereby the 13CO$_2$ produced directly by a carboxydotroph from 13CO is preferentially assimilated compared to exogenous $^{12}$CO$_2$ supplied in the atmosphere.

In the majority of cases, the “heavy” fractions that showed the greatest increase in 16S rRNA gene copies were at a density of ca. 1.730 g ml$^{-1}$ (Figure 1, Supplementary Figure S2). Heavy fractions that contained a large increase in DNA amounts and/or 16S rRNA gene copies as compared to an un-incubated, non-labeled control were selected for 16S rRNA gene sequencing. In some cases (e.g., DCm2010), the amount of DNA present in the corresponding high-density fractions of the unlabeled controls was too low to obtain enough for successful pyrotag sequencing despite inputs of similar total amounts of DNA (see Supplementary Figure S1).

Most predominant operational taxonomic units (OTUs) in the heavy fractions of all samples (i.e., most putative carboxydotrophs) belonged to the phylum Firmicutes, in particular to the class Clostridia. In heavy fractions recovered from $^{13}$CO incubations that showed observable shifts in 16S rRNA gene copies, members of the phylum Firmicutes accounted for 31–95% of the reads (Table S1). One of the lowest % of Firmicutes was from GR1 which had a very high proportion of Crenarchaeota in both the original community and in all heavy fractions. However, of bacterial sequences only, Firmicutes accounted for 81.5% in the heavy fraction of GR1-13CO. The proportions of top phyla (>1% of sequences) of both the un-amended environmental samples and the heavy fractions for two sites that showed strong DNA labeling are shown in Figure 2.

The taxonomic identifications of OTUs recovered from $^{13}$CO microcosm heavy fractions that were present at 25 fold enrichment compared to the original environmental sample are shown in Table 2. A 16S rRNA gene phylogenetic tree was constructed using the top OTUs from each $^{13}$CO heavy fraction showing an observable shift compared to reference sequences (Figure 3). Taxonomic identifications of OTUs present at >1% of all sequences are presented in Supplementary Table S2. Most of the putative carboxydotrophs identified belonged with >98% sequence identity to genera that include known CO-oxidizers, such as Thermincola, Desulfotomaculum, Carboxydocella, and Thermolithobacter (Sokolova et al., 2002, 2005, 2007; Parshina et al., 2005a,b). For example, the most abundant OTU (OTU_17948) recovered from $^{13}$CO heavy fractions from Dewar Creek (DCm2010 and DCmN11) and Lakelse springs showed 99% sequence identity to both Thermincola potens and to Thermincola carboxydiphila, known CO-oxidizing bacteria (Sokolova et al., 2005; Byrne-Bailey et al., 2010) (Table 2). Members of the genus Carboxydocella were

![Figure 2](image-url)
also detected in the majority of heavy fractions across all sites. This genus was represented by multiple OTUs, however the top OTU_7600 identified in most samples corresponded to Carboxydocella thermoautotrophica (98% similarity). Members of the genus Desulfotomaculum were most predominant in DCs9, a sediment sample collected from Dewar Creek. In DCs9, 31.9% of sequences were attributed to the genus Desulfotomaculum. 30.0% of sequences were in OTU_3148, 15.7% in OTU_20883 representing 8.1% of the total for DCs9_13 CO, and 7.6% in OTU_17986 which showed 99% sequence identity to D. kuznetsovii and D. luciae.

Some OTUs identified may reflect bacteria with as yet unknown or unconfirmed CO-oxidizing capabilities. One such cluster, OTU_20883, present in both the GR1_13 CO and GR1_12 CO + 13 CO + 12 CO heavy fraction at 15.1 and 5.3% of sequences respectively, is 93% similar to Candidatus “Desulforudis audaxviator.” Neither this OTU nor any others that showed any similarity to this bacterium were detected in the control environmental sample from this site. BLAST results for OTU_17986 representing 8.1% of the total for DCs9_13 CO returned equal results for Thermincola potens and Thermincola carboxydiphila. However, the sequence identity to both was only 90%, and it branches distantly from Thermincola in the phylogenetic tree (Figure 3). This may represent another related CO-oxidizing genus, the exact nature of which requires further study.

While PB1 and Liard2 sediments did oxidize CO, incubations with 13 CO did not show any observable shift in 16S rRNA gene density profiles. Nevertheless, we did for comparison analyze the heavy fractions from these incubations. The heavy fraction of PB1 showed a slight increase in Firmicutes as compared to the proportion present in the original community (Table S1), however no OTUs with sequences >1% were associated with known CO-oxidizing bacteria (Table S2). In comparison, the heavy fraction from Liard2_13 CO had a number of OTUs associated with CO-oxidizing bacteria. Only one OTU was present at 25 fold enrichment and represented the top OTU of this fraction (Table 2). OTU_7600 had a 98% BLAST identity to Carboxydocella thermoautotrophica. It represented 28.0% of sequences in the same OTU in the original environmental sample. n.d., not detected.

TABLE 2 | The percent of total sequences associated with top OTUs identified in heavy fractions of 13 CO incubations.

| OTU   | Phylum         | BLAST identification               | Identity (%) | DCm2010 g ml−1 | DCMN11 g ml−1 | DCs9 g ml−1 | L3 g ml−1 | GR1 g ml−1 | Liard2 g ml−1 | PB1 g ml−1 |
|-------|----------------|-----------------------------------|--------------|----------------|----------------|-------------|------------|------------|--------------|------------|
| 3148  | Firmicutes     | Desulfotomaculum kuznetsovii/D. luciae | 99           | 30.0 (n.d.)    |                |             |            |            |              |            |
| 3442  | Firmicutes     | Thermolithobacter carboxydiphilans | 100          | 8.9 (n.d.)     |                |             |            |            |              |            |
| 7600  | Firmicutes     | Carboxydocella thermoautotrophica | 98           | 7.6 (n.d.)     | 2.9 (n.d.)     | 17.9 (0.0)  | 17.0 (n.d.)| 28.0 (n.d.)|              |            |
| 9076  | Firmicutes     | Sporomusa sphaeroides             | 94           |                |                |             |            |            |              |            |
| 12486 | Firmicutes     | Caloramator australicus           | 99           |                |                |             |            |            |              |            |
| 14221 | Firmicutes     | Desulfurispora thermophilia       | 97           |                |                |             |            |            |              |            |
| 17597 | Firmicutes     | Streptococcus thermophilus         | 100          |                |                |             |            |            |              |            |
| 17948 | Firmicutes     | Thermincola potens/ T. carboxydiphila | 99           | 68.2 (n.d.)    | 65.5 (n.d.)    | 48.4 (n.d.) | 3.9 (n.d.) |            |              |            |
| 17986 | Firmicutes     | Thermincola potens/ T. carboxydiphila | 90           |                |                |             |            |            |              |            |
| 18478 | Deinococcus-Thermincola potens/ T. carboxydiphila | 100 |                |                |             |            |            |              |            |
| 20883 | Firmicutes     | Candidatus Desulfurispora audaxviator | 93           |                |                |             |            |            |              |            |
| 21098 | Proteobacteria | Azonexus caeni                      | 98           |                |                |             |            |            |              |            |

The density of the heavy fraction analyzed is reported in g ml−1. OTUs reported are those with a 25 fold enrichment compared to the original community in at least one sample site. The percent of total sequences for each OTU is reported with BLAST identification and percent sequence identity to the top cultured BLAST hit. Numbers in brackets represent the proportion (%) of sequences present in the same OTU in the original environmental sample. n.d., not detected.
While obtained following different methodology, these in vitro rate estimates are 2–4 orders of magnitude lower than those measured in the present study, and show that potential rates of CO oxidation may vary greatly between environments. The observation of little to no methane production in most samples was consistent with the negligible proportions of *Euryarchaeota* detected in the original environmental communities and the lack of archaean sequences detected in heavy SIP fractions (e.g., Figure 2A).

Distinct differences were noted between the original microbial communities and the communities detected in $^{13}$C-labeled heavy DNA fractions for each sample. In most cases, *Firmicutes* were present at <1% in the original communities but increased in abundance in $^{13}$CO incubated heavy fractions, reaching up to ca. 95% of all 16S rRNA gene reads (Table S1). These results indicate that CO-metabolizing bacteria make up a relatively minor component of the overall population within these geothermal systems but are still present and may become active if CO is provided. Most of the bacteria identified in heavy fractions showed high identities (>98%) to known CO-oxidizing bacteria described from other geothermal springs, particularly *Carboxydocella* and *Thermincola* species (Table 2). This finding suggests not only that the CO-SIP procedure was successful in identifying primary carboxydotrophs without cross-feeding artifacts, but also that the predominant carboxydotrophic bacteria in geothermal environments may in fact already be well described from cultivation studies. This is a rather unusual finding for SIP experiments (e.g., Redmond et al., 2010). Geographically, it also indicates that anaerobic thermophilic carboxydotrophic bacteria are highly cosmopolitan (at least at the species/genus level), since most described isolates have been obtained from Russian geothermal sites. Aerobic carboxydotrophy is taxonomically diverse (e.g., King and Weber, 2007). And while the presence of CO in geothermal spring emissions may suggest the potential for wide-spread CO metabolism, the current study supports the notion that the capacity for anaerobic carboxydotrophy among thermophiles is more limited.

While the most predominant bacteria identified via SIP were similar to carboxydotrophs isolated from other geothermal springs, there were a few exceptions. Among the predominant OTUs detected in heavy DNA fractions (Table 2), three OTUs showed <95% 16S rRNA gene sequence identity to any described species. For example, an OTU making up 15% of the heavy DNA fraction in sample GR1 had only a moderate similarity to the proposed genus “Desulfuridis.” *Candidatus “Desulfuridis audaxvator”* was identified in the fracture water of a South African gold mine. This isolate has components of the Wood-Ljungdahl pathway and may be capable of CO oxidation and assimilation (Chivian et al., 2008). However, the low identity (93%) of our OTU indicates a genus-level divergence to Ca. “D. audaxvator.”

While the use of 16S rRNA gene qPCR greatly improved the detection of shifts in density within the CsCl gradients, in general the observed shifts were subtle. Approximately 0.47 mmol of total $^{13}$C was added to each of the $^{13}$CO SIP incubations. However, previous studies show that relatively little of the CO oxidized microbially in geothermal habitats is incorporated into $^{13}$C incorporation (Schildkraut et al., 1962). However, the high proportions in un-amended and $^{12}$C controls of *Crenarchaeota* in the case of GR1 suggest that these microbes were not carboxydotrophs but rather are naturally present at that density due to relatively high G + C content.

**Discussion**

In this study, bacteria potentially involved in the anaerobic oxidation of carbon monoxide were identified from hot spring environments using DNA-SIP. Five geographically diverse geological settings were identified in which potential anaerobic CO-oxidation was detectable. These five locations are dispersed geographically over an area of approximately 1 million km$^2$. The measured CO-oxidation potentials were variable between geothermal springs. Comparative rates from other environments are rarely reported, however CO-oxidation rates of 120 $\mu$mol l$^{-1}$ of sediment d$^{-1}$ were estimated in slurries from Uzon Caldera, Kamchatka (Kochetkova et al., 2011), and a $^{14}$CO tracer was used to estimate a rate of 40.75 nmol CO cm$^{-3}$ sediment d$^{-1}$ for another anaerobic hot spring community in Kamchatka (Slepova et al., 2007). While obtained following different methodology, these in vitro
biomass. Using radioisotope tracers to examine a hot spring community from Kamchatka, it was estimated that 85% of the 14CO was oxidized to CO2 while only 0.5% was used for cell biomass production. The remainder was distributed between dissolved organic matter and minor (0.001%) amounts of methane (Slepova et al., 2007). At 0.5% incorporation, a maximum of ca. 2.35 µmol of 13C would have been incorporated into the bacteria identified in the current study and may explain why shifts in the density of labeled DNA were minor compared to SIP experiments using substrates such as 13CH4 where more C is incorporated into the cells over a short incubation period (Dumont et al., 2011). The use of qPCR and the examination of the 16S rRNA gene copy profiles provided a means by which to identify subtle shifts in DNA density (Lueders et al., 2004; Sharp et al., 2012). The lack of observable shifts in 16S rRNA gene copies in one of our samples (13CO-incubated Portage Brûlé) indicated that some geothermal communities may contain bacteria that are using CO as an energy source to maintain the population but are perhaps growing slowly, maybe due to other nutrient limitations, or more likely are incorporating other, possibly organic, C sources into biomass (Figure 1B). This does indicate limitations in the 13CO-SIP technique. While it appeared to be effective in identifying some carboxydrotrophs, it cannot identify all of these metabolically diverse microorganisms including potential carboxydoheterotrophs.

The 13CO-SIP technique is also challenging because carboxydrotrophs may incorporate CO2 rather than CO directly, and because the initial products of CO-oxidation, H2 and CO2, may lead to labeling of other autotrophs. Our experiments included controls suggesting that these problems were minor. The greatest shifts in density were observed when 13CO was provided as the sole carbon source (i.e., no extra CO2 was added), but incubations with 13CO + 12CO2 showed smaller shifts in DNA density compared to 13CO alone. Most likely, an initial oxidation of 13CO to 13CO2 via the gas-water shift reaction is followed by assimilation of the produced 13CO2. Incubations with 13CO2 alone (and no CO added) showed little or no apparent shifts in DNA density, indicating that labeling of autotrophs growing on substrates already present in the samples, such as sulfur or ammonia, was not an issue, and that CO was ultimately the primary energy source in the incubations. However, there is the distinct possibility of hydrogenotrophic organisms using the H2 and 13CO2 produced via the gas-water shift reaction. This particular form of cross-feeding cannot be eliminated from the current results, but lines of evidence suggest that it was minor: (1) Cross-feeding with H2 may occur only in addition to primary CO oxidation- i.e., it can only be a secondary process given that CO was the major energy source available; (2) almost all of the detected bacteria in the present study were closely related to known carboxydrotrophs; and (3) potentially hydrogenotrophic but non-carboxydrotrophic bacteria such as the members of the phylum Deinococcus-Thermus were detected in some heavy DNA fractions, but were always of minor importance compared to known carboxydrotrophs (e.g., Thermus scotoductus in DCs9, Table 2; Table S2). Complete genomes of both T. scotoductus and T. antrantikianii (Table S2) lack genes related to CO metabolism (http://img.jgi.doe.gov/).

Many bacterial species that possess cooS genes have a primary metabolism that does not focus on CO, however their presence may imply a potential underlying or backup CO-dependent physiology should conditions vary and become more optimal for CO-oxidation (Techtmann et al., 2011). While challenging to measure in situ, localized accumulations of CO may create microniches within geothermal systems in which low abundance carboxydrotrophic population members may thrive. For example, the species composition of the heavy fractions was similar for two biomat samples DCm2010 and DCmN11 with a dominance of Thermicola potens in both cases. The presence of CO oxidizing bacteria in these samples is perhaps not surprising given the observation of net CO production within microbial mats. Saline and intertidal sand flat photosynthetic microbial mats exhibited a net production of CO (3.1–5.4 µmol m−2 d−1) during daylight hours and were also observed to have a net production of H2 (Hoehler et al., 2001). As many of the Dewar Creek samples that showed evidence of CO-oxidation were comprised of biomats with relatively high proportions of cyanobacteria (Figure 2B, Table S1), these results support CO as a potentially important carbon and energy source in microenvironments within geothermal systems. Variation in CO metabolizing bacteria was observed between samples collected from the same geothermal system but under different temperature regimes. OTU_3148 was 99% similar to both Desulfitomaculum kuznetsovi and Desulfotomaculum luciae, detected in 13CO incubations of DCs9, another site from the Dewar Creek hot spring. Desulfotomaculum kuznetsovi is an obligate anaerobe and is capable of growth with CO as the sole carbon and energy source (Parshina et al., 2005a). Thermolithobacter carboxydivorans was also detected in this particular sample site but was not detected in any other microcosm. The optimum growth temperature for T. carboxydivorans of 70°C may also explain the presence of this bacterium in DCs9 with an environmental and incubation temperature of 65°C as opposed to other Dewar Creek samples with lower in situ temperatures incubated at 55°C (Sokolova et al., 2005).

The dominance of Firmicutes in heavy-density DNA fractions of geothermal samples incubated under 13CO confirms that representatives of this phylum may play a predominant role relative to other phyla in anaerobic oxidation of CO in geothermal environments. In particular, they may reflect minor populations within geothermal microenvironments where localized CO concentrations may be high. While the detection of such microenvironments in situ is challenging, the hypothesized presence of these localized CO-rich niches (e.g., Techtmann et al., 2009) suggests a mechanism by which these carboxydrotrophs may exist. Despite comprising a relatively small proportion of in situ communities, the CO oxidizing bacteria are active and show some variation across geothermal environments. Oxidation potentials are higher than the few previously reported rates for mixed geothermal communities. Despite geographical differences, thermophilic bacteria associated with anaerobic CO-oxidation are widely distributed geographically and the predominant species are well-described from cultivation studies. The presence of a few OTUs that do not show high degrees of similarity to any known cultured representatives indicates that a
few new lithoautotrophs that have not been previously identified as CO-oxidizers may also be present in the geothermal springs tested and require further study.

The detection of sequences associated with known CO-oxidizing bacteria in high abundance supports the applicability of the CO-SIP technique. SIP can be applied to target autotrophs by adding an energy substrate along with $^{13}$CO$_2$. This works as long as the added substrate is the primary energy source for the autotrophic community. We have previously used this approach to identify autotrophic methanotrophs (Sharp et al., 2012). We therefore conclude that the CO-SIP technique, which works in a similar way to identify autotrophic carboxydontrophs, does have some value, although of course the results still need to be interpreted with caution. Controls are necessary to demonstrate a low rate of assimilation of $^{13}$CO$_2$ by other autotrophs. It should also be stressed that heterotrophic carboxydontrophs will be missed - this was a possible explanation for the failure of some of the samples assayed in this study. Cross feeding via H$_2$ + CO$_2$ produced via the gas-water shift reaction is also a potential issue, although this appeared to be minor in this particular study. This study appeared to work because the bacteria identified were primarily known carboxydontrophs, however identification of a new potential carboxydontroph should only be taken as initial evidence that requires verification with other methods including sequencing of potential cooS genes.

Author Contributions

AB, CS and SG collected samples; AB performed SIP incubations with input from PD; AB with aid from CS extracted DNA, prepared samples for sequencing and carried out data processing. AB and PD wrote the initial draft of the paper; all authors designed the study, discussed the results and commented on the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00897

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