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Diversity and activity of aerobic thermophilic carbon monoxide-oxidizing bacteria on Kilauea Volcano, Hawaii

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DIVERSITY AND ACTIVITY OF AEROBIC THERMOPHILIC CARBON MONOXIDE-OXIDIZING BACTERIA ON KILAUEA VOLCANO, HAWAI'I

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by

Caitlin E. King
B.S., Louisiana State University, 2008
December 2013
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ABSTRACT

Aerobic carbon monoxide (CO) oxidation is used by phylogenetically and physiologically diverse microorganisms inhabiting a variety of terrestrial and aquatic ecosystems. Activity assays, culture-based studies, and molecular-based approaches targeting the coxL gene, encoding the large subunit of CO dehydrogenase, were used to investigate the role of temperature in structuring CO-oxidizing communities at Kilauea Volcano, Hawai‘i. CO uptake activity was assessed for unvegetated and vegetated temperate volcanic deposits that experience different temperature regimes due to plant development during ecosystem succession. Both CO-oxidizing communities had similar short-term responses to temperature; however, results from extended incubations (30 d) at elevated temperature (55 °C) indicate that succession expanded the capacity of the vegetated community to adapt to high temperature. Aerobic CO uptake was also examined for geothermal sites including two soils and two microbial biofilms at the Puhimau geothermal area and Kilauea Iki crater. CO oxidation occurred at elevated temperatures for all sites assessed; however, cardinal temperatures for CO activity were not strongly correlated to in situ temperatures. These results also extended the known upper temperature limit (80 °C) for aerobic CO oxidation.

Culture-based methods targeting thermophiles at these sites yielded 31 newly isolated thermophilic CO-oxidizing strains in 8 genera. Two strains were formally described as novel species. The isolation of multiple Thermogemmatispora strains (Class: Ktedonobacteria) led to exploration of CO oxidation in this group; CO oxidation was found to be a common trait among Ktedonobacteria. Additionally, geothermally-heated biofilms at Puhimau were dominated by Ktedonobacteria as determined from coxL clone libraries, 16S rRNA gene pyrosequencing, and analysis of coxL fragments from a biofilm metagenome.
Thermophiles are known to exist in temperate environments, but their maintenance and activity remain unclear. We examined the activity of thermophilic CO-oxidizing bacteria under different temperature regimes including 25 °C, 55 °C, and an oscillating temperature regime (20 – 55 °C). Three hours per day above 45 °C was sufficient for growth and CO oxidation activity. CO oxidation at moderate temperatures could contribute to maintenance metabolism and survival of thermophiles under suboptimal conditions. Collectively these studies show that thermophilic CO oxidizers are active and abundant in thermal systems on Kilauea Volcano.
CHAPTER 1.
INTRODUCTION

The Significance of Carbon Monoxide

While notorious as a toxic gas, carbon monoxide (CO) is actually one of the most versatile molecules on the planet. CO participates in important reactions that affect atmospheric chemistry, microbial energetics, heme turnover, and cell signaling (King and Weber, 2007). CO concentrations in the troposphere range from 50–350 ppb (Crutzen and Gidel, 1983), and the atmospheric lifetime of CO is relatively short (10 – 52 d; Daniel and Solomon, 1998; Holloway et al., 2000). Even though it is present at low concentrations in the atmosphere, CO is an important trace gas that directly and indirectly contributes to climate change through its removal of atmospheric hydroxyl radicals (OH∙), creation of tropospheric ozone (O₃), and direct radiative forcing.

The greatest influence of CO on atmospheric chemistry occurs through its rapid reaction with OH∙, a major tropospheric oxidant (Taylor et al., 1996; King, 2003b). Hydroxyl radicals are responsible for cleansing the atmosphere of reactive gases and serve as the dominant greenhouse gas sink (Guthrie, 1989). CO and other traces gases control the concentration of OH∙ available to react with greenhouse gases such as methane (CH₄) and carbon dioxide (CO₂). Increases in these trace gases decrease OH∙ levels resulting in higher concentrations and extended decay times for greenhouse gases (Lu and Khalil, 1993). CO provides the largest direct sink for atmospheric OH∙, consuming twice as much OH∙ as CH₄ (Lu and Khalil, 1993). In addition to extending the decay time of CH₄ by decreasing available OH∙, CO also inhibits CH₄ uptake by methanotrophs due to competitive interaction with methane monooxygenase (Bender and Conrad, 1994) further exacerbating CH₄ increases.
CO also influences atmospheric chemistry through tropospheric ozone (O₃) formation which traps solar radiation at the surface of the Earth (King, 1999a). Adding CO to a system with sufficient available NO results in the photochemical production and accumulation of O₃ (Daniel and Solomon, 1998). Since ground-level O₃ is a pollutant, CO also has a secondary effect on air quality. Tropospheric O₃ can lower crop yields (Chameides et al., 1994) and exacerbate respiratory issues in humans, which sometimes leads to death (Bell et al., 2004).

Lastly, CO absorbs and emits low intensity infrared thermal radiation in the wavelength range from 1800–2300 cm⁻¹ in the troposphere (Daniel and Solomon, 1998). The direct radiation of CO (0.06–0.11 W m⁻²; Evans and Puckrin, 1995) is comparable to that of N₂O (0.16 W m⁻²; Hauglustaine et al., 1994) and is significant enough to warrant inclusion in climate change models. Overall, the short-term (< 15 years) cumulative global warming potential (GWP) due to anthropogenic emissions of CO is estimated to exceed that of the greenhouse gas nitrous oxide (N₂O) (Daniel and Solomon, 1998). The climate forcing of CO is due primarily to relatively high annual emissions. Nearly twice as much CO is emitted annually than CH₄, and over 85-fold more CO is emitted from anthropogenic sources than N₂O (Daniel and Solomon, 1998).

**Sources of Carbon Monoxide**

Carbon monoxide is produced from anthropogenic, abiotic, and biotic sources. Abiological oxidation of CH₄ in the atmosphere is the largest source of CO producing 744–924 Tg CO annually (Monson and Holland, 2001), followed by anthropogenic sources (Daniel and Solomon, 1998; King, 1999b; Table 1.1). The partial combustion of petroleum fuels, particularly from motor vehicles, and biomass burning are the greatest man-made sources of CO (Taylor et al., 1996). Photolysis of dissolved organic matter in the ocean is another source of CO; although, the ocean contributes < 10% of the CO produced by anthropogenic sources (Taylor et al., 1996;
Stubbins et al., 2006). A small amount of CO is also produced from volcanic emissions (Martinez-Alonso et al., 2012)

Table 1.1. Sources of CO to the atmosphere.

<table>
<thead>
<tr>
<th>Source</th>
<th>CO Tg yr$^{-1}$</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_4$ reaction with OH·</td>
<td>744 - 924</td>
<td>Monson and Holland, 2000; Holloway et al., 2000</td>
</tr>
<tr>
<td>Oxidation of biogenic hydrocarbons</td>
<td>748</td>
<td>Bergamaschi et al., 2000; Holloway et al., 2000</td>
</tr>
<tr>
<td>Biomass burning</td>
<td>370 - 1280</td>
<td>Bergamaschi et al., 2000; Holloway et al., 2000</td>
</tr>
<tr>
<td>Fossil fuel combustion</td>
<td>300 - 478</td>
<td>Oliver et al., 1996; Holloway et al., 2000</td>
</tr>
<tr>
<td>Degradation of soil organic matter</td>
<td>100</td>
<td>Schade and Crutzen, 1999</td>
</tr>
<tr>
<td>Photolysis of DOM* in the oceans</td>
<td>13 – 50</td>
<td>Bates et al., 1995; Berntsen and Jackson, 1997</td>
</tr>
<tr>
<td>Volcanic emissions</td>
<td>5.5</td>
<td>Martínez-Alono et al., 2012</td>
</tr>
</tbody>
</table>

*DOM=dissolved organic matter

Thermochemical and photochemical reactions release CO from organic matter in soil. The illumination of plant litter and mineral soils by sunlight in the ≤ 400 nm wavelength range results in CO production (Schade et al., 1999). Biomass burning during deforestation produces CO from organic matter (Zepp, 1994). Additionally, recently burned sites are transformed from CO sinks to CO sources because the removal of tree cover allows more light to reach the soil resulting in increased photochemical and thermal CO production (Kuhlbusch et al., 1998). A large amount of CO is also released during the oxidation of biogenic hydrocarbons (i.e. isoprenes, terpenes, and other volatile organic compounds produced by plants) (Bergamschi et al., 2000).

Carbon monoxide is a byproduct of many biological reactions. Bacteria, fungi, plants, and animals can produce CO through heme oxidation, lipid peroxidation, and aromatic amino acid degradation (King and Weber, 2007). CO is also formed by anaerobic bacteria living in aquatic environments (Schmidt and Conrad, 1993). Biological oxidation of CH$_4$ to CO occurs in
methanotrophs (Taylor et al., 1996; Manning et al., 2005), but this is negligible ecologically. Plant roots provide a belowground source of CO producing 170–260 Tg yr\(^{-1}\) (King and Crosby, 2002). A small amount of CO is also derived from enzymatic and photochemical reactions occurring within macroalgal bladders (King, 2001).

An estimated 2,500–2,600 CO Tg yr\(^{-1}\) is added to the atmosphere from these combined natural and anthropogenic sources (Holloway et al., 2000; King and Weber, 2007). CO concentrations have increased over 60 ppb over the last 200 years (Haan et al. 1996). The multitude sources of CO provide numerous local hot spots of CO for bacteria to exploit, which may contribute to the diversity and ubiquity of CO oxidizers. This widespread functional group of microorganisms plays an important role in mitigating atmospheric CO increases.

**Microbial Carbon Monoxide Oxidation**

Both anaerobic and aerobic bacteria use CO as an energy source. CO might have been utilized first by thermophilic or hyperthermophilic anaerobes. The evolution of anaerobic CO-oxidization may have been favored early in Earth’s history by relatively high CO concentrations (> 100 ppm) that have been proposed for the Archean atmosphere (Miyakawa et al., 2002). The history of aerobic CO oxidation is uncertain, but is linked to evolution of aerobic respiration and oxygen accumulation in the atmosphere due to the development of oxygenic photosynthesis (King and Weber, 2007).

Aerobic CO metabolism is used by physiologically diverse microorganisms including obligate and facultative chemolithoautotrophs and facultative chemolithoheterotrophs, some of which are capable of mixotrophic metabolism (King and Weber, 2007). Most of the pioneering work on aerobic CO oxidizers focuses on carboxydotrophs (Meyer and Schlegel, 1983; Meyer and Krueger, 1986), microbes which grow under elevated CO concentrations (> 1%) using CO as
a sole energy and carbon source. Most carboxydotrophs use CO oxidation as an alternate energy source, which is coupled to CO₂ assimilation by the Calvin-Benson-Bassham cycle. Carboxydotrophy is usually stimulated by nutrient limitation (King and Weber, 2007). Growth on elevated CO (> 1%) may be a metabolic anomaly limited to a few species with unknown ecological relevance. In contrast, carboxydovores use lower levels of CO (< 1000 ppm) as an energy source, and use organic matter as a carbon source (King and Weber, 2007). In situ CO uptake is more likely attributed to carboxydovores utilizing mixotrophic metabolism, simultaneous use of atmospheric concentrations of CO and organic substrates. Carboxydovores have greater ecological significance than carboxydotrophs since the former are often capable of CO uptake at atmospheric concentrations (King and Weber, 2007).

In the general equation for microbial CO oxidation: \( \text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^- \), carbon dioxide and two reducing equivalents are produced. Reducing equivalents can be transferred to electron transport proteins or coupled to other reactions such as oxygen reduction, dissimilatory nitrate reduction, or denitrification (King and Weber, 2007). Carboxydotrophs use the conserved energy from CO oxidation to fix CO₂ into biomass typically through the Calvin-Benson-Bassham cycle which uses the enzyme ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) encoded by the gene \text{cbbl} (King and Weber, 2007).

Microbial aerobic CO oxidation is catalyzed by the enzyme carbon monoxide dehydrogenase (CODH), encoded by the \text{cox} operon. CODH is part of the molybdenum hydroxylase protein family, consists of a dimer of heterotrimers (Dobbek \textit{et al.}, 1999), and is often found bound to the cytoplasmic membrane (Morsdorf \textit{et al.}, 1992). The three structural CODH protein subunits, small, medium, and large, are encoded by the genes \text{coxS}, \text{coxM}, and \text{coxL}, respectively (King and Weber, 2007). The \text{coxS} subunit is an iron-sulfur protein involved
in electron transport. The coxM subunit is a flavin adenine dinucleotide (FAD) containing protein. The coxL subunit contains the active site of the enzyme where CO is oxidized at the molybdenum ion of a CuSMoO₂ cluster (Gnida et al., 2003). Aerobic CODH contains a molybdenum cytosine dinucleotide (MCD) cofactor that coordinates the molybdenum atom at the active site. The active site contains a cysteine, serine, phenylalanine, and arginine motif with a copper atom linking the molybdenum atom to the sulfur atom of the cysteine residue. Electrons are transferred to [2Fe-2S] clusters in the small subunit and FAD in the medium subunit (Dobbek et al., 1999). While the final electron acceptor is typically oxygen, nitrate can be substituted under anaerobic conditions (King and Weber, 2007).

The genes encoding the structural proteins for aerobic CODH are arranged in the transcriptional order coxM, coxS, and coxL (King and Weber, 2007). In addition to these three structural genes, several accessory genes are often present in the cox operon. For example, the cox operon of Oligotropha carboxidovorans OM5 contains 9 accessory genes in addition to the 3 structural genes (Fuhrmann et al., 2003). The accessory genes coxD, coxE, and coxF are involved in biosynthesis of the CuSMoO₂ center and were considered essential (Santiago et al., 1999); however, CO oxidation has been recently observed in the thermophile Thermomicrobium roseum DSM 5159 (Wu et al., 2009) which only contains coxMSLF (http://img.jgi.doe.gov/cgi-bin/w/main.cgi?section=GeneDetail&page=geneDetail&gene_oid=643626032). Variation among taxa in cox operon gene content might be related to differential regulation of cox expression and perhaps different CO uptake rates.

Genome annotations have revealed putative cox genes (termed form II) that are phylogenetically similar to authentic cox genes (form I), but whose true function is unknown (King and Weber, 2007). Form I CO oxidation is well documented, but the CO-oxidizing activity
of form II has been documented only for *Bradyrhizobium japonicum USDA 110*, which oxidizes CO at an extremely low rate (Lorite, 2000) and whose form II CODH likely has a different preferential substrate. Form II cox operons are also typically found in a different order, as *coxS*, *coxM*, and *coxL*, than form I genes. Authentic form I CODH has a unique \((A/S)Y(R/A/S)CSFR\) amino acid motif at the active site that aids in binding and reacting CO at the MCD cofactor, while putative form II CODH has an AYRGAGR motif (King and Weber, 2007). Differentiating these motifs is a helpful diagnostic tool for properly annotating true form I *coxL* sequences. CO-oxidizing isolates may possess only form I, multiple copies of form I, or form I and form II, and they may or may not possess RubisCO. Previous research suggests that CO-oxidizing bacteria lacking RubisCO use CO only as a supplemental energy source (King and Weber, 2007).

**Diversity and Evolution of Carbon Monoxide-Oxidizing Bacteria**

Bacteria, Archaea, and Eukarya represent the three domains of life. While bacteria and archaea are both prokaryotic (organisms which lack nuclei), they are not closely-related phylogenetically. Bacteria represent the most deeply-branching lineage with Archaea and Eukarya splitting off later from a shared common ancestor based on rRNA sequence comparisons (Woese *et al.*, 1990). There are currently 30 bacterial phyla and 5 archaeal phyla in the List of Prokaryotic Names with Standing Nomenclature (LPSN, 2013). The phylogeny of microorganisms is primarily based on molecular sequences because phenotypes are not always indicative of evolutionary relationships. Based on current species definitions, bacterial strains which share \(\geq 97\%\) 16S rRNA gene similarity (Stackebrandt and Goebel, 1994) or \(\geq 70\%\) whole genome DNA similarity (Wayne *et al.*, 1987) are considered to be the same species. 16S rRNA is part of the small subunit of prokaryotic ribosomes; its primary structure is highly conserved but also contains hypervariable regions. Differences in 16S rRNA gene sequences have been
used to measure phylogenetic distance between bacterial strains and inform taxonomy for over 30 years (Woese and Fox, 1977). Based on the existing species definition of 97% 16S rRNA similarity, King and Weber (2008) determined that coxL sequences sharing > 90% identity represented distinct taxonomic units. Diversity assessments of CO oxidizers are based on these phylogenetic guidelines.

Molecular approaches have made it possible to study the diversity of CO-oxidizing microorganisms without relying on culture-based methods. Primers were designed to amplify a 1260 to 1290 bp segment encompassing the active site of the large subunit of the coxL gene (Dunfield and King, 2004). A wide array of CO-oxidizing functional groups from phylogenetically diverse lineages have been found using these primers. Form I CODH genes have identified numerous aerobic CO-oxidizing bacteria in the α, β, and γ Proteobacteria, Actinobacteria, and Firmicutes lineages inhabiting soils, volcanic deposits, the marine water column, and other habitats (Tolli et al., 2006; King and Weber, 2007). Several coxL sequences were reported that do not cluster within any known phyla. In fact, the majority of the sequences found at several unvegetated volcanic sites were from unknown organisms in these novel CO-oxidizing lineages (Dunfield and King, 2004).

Prior to 2009, CO oxidizers had only been identified in the phyla Actinobacteria, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, and Firmicutes (King and Weber, 2007; Weber, 2009). Since that time, cox operons have been identified in the genomes of several additional phyla, including δ-Proteobacteria, Chloroflexi, Deinococcus-Thermus, Bacteroidetes, Crenarchaeota, Euryarchaeota, and Geoarchaeota (http://img.jgi.doe.gov/). coxL sequences containing the diagnostic CSFR motif were identified in available genomes in the IMG database by performing a BLASTP with a form I coxL query sequence (King and King, unpublished)
results). Based on these results, aerobic CO oxidizers constitute approximately 2.5% of the ~5900 bacterial genomes and 2.5% of the ~200 archaeal genomes in IMG database. In comparison, anaerobic CODH, a nickel containing enzyme encoded by the coo operon, has been identified in 6% of all microbial genomes (Techtman et al., 2012), indicating that the utilization of CO is widespread among microbes.

Some of the newly identified aerobic CO oxidizers include thermophiles, which are defined as organisms with optimal growth temperatures > 50 °C. At present, form I coxL genes have been documented in 79 genera belonging to 9 bacterial phyla and 3 archaeal phyla (Figure 1.1, Table A.1; King and King, unpublished results). Thermophilic and thermotolerant (defined as organisms that grow optimally temperatures < 50 °C, but are able to grow at higher temperatures) CO-oxidizing taxa, include 19 genera and represent > 20% of all identified CO oxidizer strains (Figure 1.2, Table A.1). cox operons were also present in 3 single cell amplified genomes from uncultured thermophilic Crenarchaeota from iron-oxidizing biofilms at hot springs at Yellowstone National Park (GenBank accession numbers: WP_018031948, WP_018032234, WP_018033649). Relative to mesophiles, thermophilic CO oxidizers appear phyla-rich but species-poor, which might be related to the lack of sampling in hot environments.

Prior to the development of bacterial genome sequencing and genomic databases, recognition of CO-oxidizing bacteria depended on cultivation, which led to the description of a small number of thermophiles including ‘Pseudomonas thermocarboxydovorans’ (Lyons et al., 1984), ‘Bacillus schlegelii’ (Kruger and Meyer, 1984), and several Streptomyces (Gadkari et al., 1990; Kim et al., 1998; Kim and Goodfellow, 2002). The moderate thermophile ‘Streptomyces thermoautotrophicum’ represents the only known obligately chemolithotrophic carboxydotroph (Gadkari et al., 1990; Hugeldieck and Meyer, 1992), but the type strain has since been lost.
Other CO oxidizers capable of growth on CO include *Pseudomonas carboxydoflava*, *Pseudomonas carboxydohydrogena*, *Bacillus schlegelii*, *Streptomyces thermoautotrophicum*.

**Figure 1.1.** Phylum composition (%) of CO-oxidizing Bacteria and Archaea. Data derived from Table A.1.

**Figure 1.2.** Relative percentage of mesophilic, thermophilic, and thermotolerant CO-oxidizing strains. Data derived from Table A.1.
*Mycobacterium smegmatis, Mycobacterium gordonae, Mycobacterium tuberculosis,* and *Carboxyphilus carboxydus* (King and Weber, 2007). Carboxydotrophs have been isolated from a variety of environments including wastewater (Meyer and Schlegel, 1983), freshwater sediment (Kruger and Meyer, 1984), human lungs (King, 2003b), compost (Lyons *et al.*, 1984), and soil (Park *et al.*, 2003), but the ecological significance of this unusual group of organisms remains unclear. Notably, recent 16S rRNA analyses suggest that *Bacillus schlegelii* belongs to a novel genus for which the name *Hydrogenibacillus* was proposed (Kämpfer *et al.*, 2012). Recent 16S rRNA gene analyses from ‘*Pseudomonas thermocarboxydovorans*’ suggest that this species also belongs in a different genus, *Tepidimonas* (King and King, unpublished results). Insights from genomes suggest that thermophilic environments may represent untapped reservoirs of novel CO oxidizers. However at present, *Thermomicrobium roseum* represents the only aerobe from a naturally hot habitat (Jackson, 1973) with described CO uptake activity (Wu *et al.*, 2009).

Genome-enabled research has expanded the diversity of CO oxidizers and provided new insights about the evolutionary history of aerobic CO oxidation. Although, *coxL* phylogeny largely parallels 16S rRNA gene phylogeny (Figures A.1 and A.4), *coxL* genes from Euryarchaeota and Actinobacteria appear to be the most deeply-branching sequences (Figure 1.3). Although bootstrap support varies, the tree topologies produced using maximum-likelihood and neighbor-joining tree-building methods based on amino acid alignments and nucleotide alignments (Figure 1.3, Figure A.2, Figure A.3), all place Euryarchaeota (*Natronorubrum*) as the deepest-branching *coxL* clade. However, multiple attempts to measure CO uptake in *Natronorubrum* have failed (King and King, unpublished results). The divergence of *coxL* sequences from *Natronorubrum* strains could possibly be due to accumulation of mutations in unexpressed genes, rather than due to its ancestral history; however, their *coxL* sequences appear
to have all required motifs for functional CODH. If *Natronorubrum* CODH is indeed active, aerobic CO oxidation may have arisen in Euryarchaeota.

Figure 1.3. Maximum-likelihood based tree (100 bootstrap replicates) depicting phylogeny of full-length *coxL* predicted amino acid sequences obtained from available genome. Alignment and tree created using ClustalW and MEGA5.1, respectively. Subtrees are collapsed with phyla labeled. Numbers at nodes indicate bootstrap support. Form II sequences from *Sinorhizobium fredii* (YP_002827560) and *Roseobacter denitrificans* (YP_683179) were used as an outgroup. Expanded tree provided in Figure A.4.
Figure 1.4. Maximum-likelihood based phylogeny (100 bootstrap replicates) of 16S rRNA gene sequences of selected CO-oxidizing taxa. Aligned 16S rRNA gene sequences were obtained from the GreenGenes 16S rRNA database (http://greengenes.lbl.gov). Numbers at nodes indicate bootstrap support. Subtrees are collapsed with phyla labeled. Expanded tree provided in Figure A.1.

Phylogenetic analyses of coxL also suggest a common ancestor for Proteobacteria and a polyphyletic clade containing Firmicutes, Bacteroidetes, Chloroflexi, Deinococcus/Thermus, and, perhaps most surprising, Crenarchaeota and the recently described (Kozubal et al., 2012) novel phylum Geoarchaeota. The phylogenetic placement of these archaeal coxL genes compared to the 16S rRNA phylogeny suggests a horizontal gene transfer (HGT) event may have conferred CODH to the Creanarchaeota/Geoarchaeota (Figures 1.3, 1.4). Within each clade, however, coxL relationships are mostly congruent with 16S rRNA gene phylogeny (Figure A.1) which allows environmental sequences to be classified with reasonable confidence. Targeting thermophilic CO oxidizers for isolations could add more strains to the species-poor phyla which could help improve phylogenetic analyses. Since the origin of life is thought to be thermophilic (DiGuilio,
thermophilic CO oxidizers might provide insights into the evolutionary history of aerobic CODH.

Some CO oxidizers have more than one cox operon. For example, *Burkholderia xenovorans* LB400 has two phylogenetically similar cox operons on different chromosomes. *Nocardioides* sp. JS614, *Thermocrispum agrest* DSM 44070, *Thermocrispum municipale* DSM 44069, and *Pseudonocardia dioxanivorans* CB1190 have two copies of coxMSL in tandem, but in opposite orientations for the latter three. The two copies of the coxL gene are phylogenetically distinct from one another. One copy clusters with other actinobacterial coxL, while the other forms a divergent clade with the δ-proteobacterium *Haliangium ochraceum* DSM 14365 (Figure 1.3, Figure A.3). *Solirubrobacter soli* DSM 22325 and *Solirubrobacter* sp. URHD0082 have two copies of coxL in tandem, but only one copy of coxM and coxS located within the same operon; both sequences cluster with Actinobacteria (Figure A.3). While CO uptake has been well documented for several Actinobacteria (King, 2003), CO uptake by *Haliangium, Nocardioides, Pseudonocardia*, and *Thermocrispum* has not yet been confirmed. The significance of having multiple form I coxL genes and whether both copies are actively transcribed is unknown. Isolates harboring multiple copies of form I coxL genes raise questions about the regulation and evolution of cox genes found in tandem, in opposite orientations, or on separate chromosomes.

Due to genome-enabled analyses, the phylogenetic distribution of CO oxidation and physiological diversity of CO oxidizer has been greatly expanded. However, while cox genes have been identified in numerous new lineages (Table A.1), many organisms need CO uptake assays to confirm activity. Notably, *Natronorubrum tibetense, Ktedonobacter racemifer*, and *Niastella koreensis* have not oxidized CO under multiple conditions tested (King and King,
unpublished). However, the conditions that activate CODH expression are not well understood. Determining whether these CODH are active will inform evolutionary analyses of cox.

**Ecology of Carbon Monoxide-Oxidizing Bacteria**

While OH∙ removes ~80% of annual CO emissions (Crutzen and Gidel, 1983; Khalil, 1999), microbial CO oxidation represents another significant CO sink (Table 1.2). Soil microbes are estimated to consume ~10% of the global CO emissions from the combined anthropogenic, biological, and abiological sources (Daniel and Solomon, 1998; King, 1999b). CO-oxidizing bacteria are not limited to soil environments; marine habitats, legume plants, and volcanic deposits have all been found to contain abundant and diverse CO-oxidizing communities (King and Weber, 2007). If the CO uptake activities from these environments are combined with soil estimates, CO-oxidizing microbes could consume up to 20% of the total CO emissions to the atmosphere each year (King and Weber, 2007).

**Table 1.2. CO sinks.**

<table>
<thead>
<tr>
<th>Sinks</th>
<th>CO Tg yr⁻¹</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO reaction with OH∙</td>
<td>2491</td>
<td>Holloway <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Soil bacteria (removal from atmosphere)</td>
<td>300</td>
<td>King, 1999b; King and Hungria, 2002</td>
</tr>
<tr>
<td>Soil bacteria (removal from plant roots)</td>
<td>200</td>
<td>King and Crosby, 2002</td>
</tr>
<tr>
<td>Marine bacteria</td>
<td>50</td>
<td>Zafiriou <em>et al.</em>, 2003</td>
</tr>
</tbody>
</table>

Aerobic CO oxidation by soil microbes has been observed in soil from tropical and deciduous forests, bogs, fields, loam, volcanic ash, silt, clay, croplands, and grasslands (King, 1999b). Aerobic CO oxidizers are thought to play a more important role in soil CO uptake than anaerobic CO oxidizers since oxic environments have higher CO uptake rates than anoxic environments (King, 1999b). The majority of soil CO oxidizers fall within the α, β, and γ-Proteobacteria, Actinobacteria, or unresolved lineages (King, 2007b).
Marine CO oxidizers are critical to attenuating CO emission from the ocean and are estimated to consume 86% of the CO produced in the ocean (Zafiriou et al., 2003). Marine CO-oxidizing communities are dominated by α-Proteobacteria and γ-Proteobacteria and include *Halomonas, Cyclobacterium* (Tolli et al., 2006), *Cytophaga, Flexibacter*, and *Bacteriodes* genera among others (King and Weber, 2007). Studies of CO uptake in coastal waters estimate that α-Proteobacteria account for roughly 15% of the total CO-oxidizing activity in coastal environments (Tolli and Taylor, 2005). Some of the active CO-oxidizing marine genera include *Ruegeria, Roseobacter, Stappia*, and *Labrenzia* (Tolli et al., 2006; Weber and King, 2007; Table A.1). While several strains possess RubisCO and may be capable of carboxydotrophy, most marine isolates have come from heterotrophic enrichments and are therefore considered to be facultative chemolithoautotrophs (Weber and King, 2007).

The environmental significance of aerobic CO-oxidizing microbes lies partially in their ability to reduce CO concentrations in the atmosphere. Additionally, some groups of CO oxidizers have clinical and agricultural significance. The animal pathogens *Mycobacterium tuberculosis* and *Mycobacterium bovis* oxidize CO using the form I CODH (King, 2003c). Multiple strains of the human and animal pathogens *Mycobacterium tuberculosis* and *Mycobacterium bovis* harbor *cox* genes (Table A.1), and CO-metabolism is thought to contribute to latent infections (Zacharia and Shiloh, 2012). CO oxidizers may exist in other pulmonary pathogens since CO concentrations are elevated in the lungs, especially during infection (2-50 ppm; Zacharia and Shiloh, 2012). Indeed, a complete form I *cox* operon exists in the genome of the opportunistic pathogen *Rhodococcus equi* (Weinstock and Brown, 2002; Table A.1).

CO oxidizers also have an active agricultural role as plant-symbionts. Plant roots produce CO, which supports the growth of CO oxidizers in the rhizosphere and surrounding soil (King...
and Crosby, 2002). Nitrogen-fixing, CO-oxidizing bacteria include several *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium* sp. (Table A.1) (Lorite *et al.*, 2000; King, 2003b; King and Weber, 2007). These plant symbionts live within specialized nodules within the vascular tissue of plant roots. Legume plant roots, which are well known for their association with N$_2$-fixing rhizobia, produce more CO than non-legume plants (King and Crosby, 2002). Rhizobia provide fixed nitrogen for the plant while the plant supplies the microbes with organic substrates and CO as a supplemental energy source. Increased CO concentrations released by the roots inhibit nitrogenase activity, and CO oxidation by bacteria could diminish this inhibitory effect. Plant roots represent a substantial source of CO belowground, and may harbor a diverse community of CO oxidizers upon further examination (King and Crosby, 2002; King and Weber, 2007).

CO oxidation also plays an important role in bacterial succession of young volcanic deposits. CO uptake activity has been confirmed using a combination of molecular approaches and *in situ* uptake assays for several volcanic sites including Kilauea and Mauna Loa in Hawaii and Miyake-jima in Japan (Dunfield and King, 2004; King *et al.*, 2008). The CO uptake rates in these volcanic systems rival CO oxidation rates for mature terrestrial soil sites. Young unvegetated volcanic ecosystems are characterized as organic matter-poor basalt deposits with high concentrations of reduced inorganic substrates from the atmosphere (King, 2003a; King and Weber, 2008). Facultative CO oxidizers are thought to be some of the earliest colonizers of these organic carbon- and nitrogen-limited deposits (King, 2007). Many cultured CO oxidizers also have genes for carbon fixation and nitrogen fixation; therefore, they provide a carbon and nitrogen source to the barren environment and allow further ecosystem succession. The importance of CO oxidation in volcanic ecosystems is further supported by recent evidence that
ammonia and nitrite oxidizers do not significantly contribute to the production of biologically available nitrogen at these volcanic sites (King, 2007).

Most of the CO oxidizers found in young volcanic deposits belong to Actinobacteria, Firmicutes, and unknown lineages. At volcanic sites with reduced water availability and little plant growth, novel sequences constituted 81–97% of the total $\text{coxL}$ sequences retrieved (Dunfield and King, 2004). Forested areas in comparison were dominated by Proteobacteria with approximately 77% of the sequences falling into this lineage. The $\text{coxL}$ environmental sequences at these volcanic sites were related to several facultative carboxydovores within the genera *Bradyrhizobium, Stappia, Bacillus, Burkholderia, Silicibacter, Stenotrophomonas*, and *Mycobacterium*, in addition to a few facultative carboxydotrophic bacteria such as *Oligotropha carboxydorans* and *Pseudomonas thermocarboxydomovans* (Dunfield and King, 2004).

Recent molecular ecological analyses (Weber and King, 2010) found that the CO oxidizer community at an unvegetated volcanic deposit consisted primarily of taxa most closely related to *Ktedonobacter racemifer*, a member of a novel class of Chloroflexi known to harbor multiple thermophiles (Cavaletti *et al.*, 2006; Stott *et al.*, 2008; Yabe *et al.*, 2010; Yabe *et al.*, 2011), suggesting that temperature might govern CO oxidizing communities at this site.

**Objectives of This Study**

The over-arching goal of this study was to understand the role of temperature as a factor shaping the structure of aerobic CO-oxidizing communities at temperate and geothermally-heated sites at the Hawai‘i National Volcanoes Park. A second major goal was to isolate and characterize novel thermophilic CO oxidizers that would promote a greater understanding of CO oxidizer physiology, ecology, and evolution. The research included field, *ex situ*, and culture-based studies coupled with molecular ecological methods to assess the diversity and properties of
thermophilic CO oxidizers. Specific research questions included the following: 1. What effect does temperature have on CO-oxidizing communities in volcanic deposits? 2. What is the activity of CO oxidation for naturally-heated systems? 3. What differences appear in cultivable thermophilic CO oxidizers from thermal and temperate sites? 4. Do temperature regimes affect OTU richness and population structure of thermophilic CO oxidizers? 5. How are thermophilic CO oxidizers maintained at temperate sites?
CHAPTER 2.
TEMPERATURE RESPONSES OF CARBON MONOXIDE- AND HYDROGEN-
OXIDIZING BACTERIA ASSOCIATED WITH TEMPERATE VOLCANIC DEPOSITS*

Introduction

Bacteria actively consume carbon monoxide (CO) and hydrogen (H₂) in both young, organic matter-poor volcanic deposits and in older, more mature deposits with well-developed plant communities (King, 2003a; King and Weber, 2008). Although changes in uptake rates and in the significance of the atmosphere as sources of these gases during ecosystem succession have been addressed previously (King, 2003a; King and Weber, 2008), specific responses of CO and H₂ uptake to environmental variables that change during succession, e.g., organic carbon, soil texture, pH, and temperature, have not been documented (Weber and King, 2009). More generally, the responses of CO and H₂ uptake by soils to these and other variables have received limited attention, even though it is clear that soils play significant roles in the global budgets of both atmospheric CO and H₂ (Conrad, 1996; King and Weber, 2007).

The study presented here documents responses to temperature of CO- and H₂-oxidizing communities at unvegetated (Bare) and vegetated (Canopy) volcanic deposits that experience distinctly different temperature regimes due to plant community development during ecosystem succession. The two sites represent “end members” in a mosaic of plant succession occurring on a single, large cinder deposit emplaced in 1959. Bare surface cinders, which are unshaded and uncolonized by plants, experience dramatic diurnal temperature fluctuations with maxima up to 55 °C. At a contiguous site supporting a closed canopy tree stand, surface material is shaded and experiences only moderate temperature changes (about 15–25 °C). Nonetheless, Bare cinders

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actively consume atmospheric CO \textit{in situ}, and previous studies have shown that the heated surface material (0–2 cm depth) consumes CO more rapidly than cooler sub-surface material (King and Weber, 2008).

Previous molecular ecological analyses have also shown that Bare cinder CO oxidizer communities consist primarily of taxa most closely related to \textit{Ktedonobacteria} (Weber and King, 2010), a group of largely thermophilic or thermotolerant strains isolated from hot compost and geothermally-heated soils (Cavaletti \textit{et al.}, 2006; Stott \textit{et al.}, 2008; Yabe \textit{et al.}, 2010; Yabe \textit{et al.}, 2011). The presence of \textit{Ktedonobacteria}-like taxa in Bare cinders is consistent with results from Ranneklev and Bååth (2001), which indicate that fluctuating thermal regimes can select for and maintain thermophilic or thermotolerant communities simultaneously with mesophilic communities.

In contrast to Bare cinders, molecular ecological analyses have shown that Canopy surface material is dominated by proteobacterial CO oxidizers with little or no indication of thermotolerant taxa (Weber and King, 2010). These differences in CO oxidizer community composition have been attributed to patterns in organic matter accumulation and plant development (Weber and King, 2010), but the presence of \textit{Ktedonobacteria}-like taxa in Bare cinders suggests that temperature regimes might also play a role.

To determine if CO uptake responded to changes in temperature regimes resulting from the effects (e.g., shading) of plant community development during succession, we measured short-term impacts of varied temperatures on uptake rates; we also assessed with an extended incubation (30 d) the capacity for adaption to elevated temperatures (55 °C) that might be favored by thermotolerant or thermophilic taxa maintained by fluctuating temperatures, especially at the Bare site. Since many CO oxidizers also consume H\textsubscript{2} (Hudson \textit{et al.}, 1988;
King, 2003b), and since H₂ uptake by various material on Kilauea volcano has been previously attributed primarily to bacteria (King, 2003a), we also measured responses of H₂ consumption to varied temperature regimes and to an extended incubation at 55 °C.

**Materials and Methods**

**Site Description**

The Pu’u Puai volcanic deposit (GPS coordinates: 19° 24’ 22.5” N x 155° 15’ 18.2” W) in Hawaii National Volcanoes Park (Kilauea Volcano, Hawaii, USA) occurs downwind of an extensive 1959 lava fountain eruption. A mosaic of closed canopy forest patches (> 10 m radius) containing *Meterosideros polymorpha* (Ohia lehua) and *Morella faya* (fire tree) is interspersed with unvegetated cinder patches. Canopy site surface material is characterized by an organic rich peat-like material, while Bare site material consists of organic poor cinders approximately 1 cm in diameter. Numerous details of this system have been described previously (King, 2003; King and Weber, 2008; Weber and King, 2009). Temperature regimes for Bare and Canopy sites differ significantly. Unvegetated Bare site cinders experience substantial diurnal oscillations with maxima to 55 °C (average 24.6 ± 0.4), while the Canopy site experiences a more narrow temperature range with maxima up to 25 °C (average 18.1 ± 0.1; Figure 2.1). Average daily temperatures for surface material (0–2.5 cm) were determined over a 3 d period in August 2008, during which samples were collected using HOBO Data Loggers (Onset Computer Corp.; Pocasset, MA, USA) with TMC 20 sensors. Surface temperatures monitored regularly for more than 18 months at both sites show similar results. Differences in temperature reflect the direct insolation received by Bare cinders and the shaded conditions at the Canopy site.

Bare cinders (0–2 cm depth) and the upper 2 cm of Canopy material beneath the litter layer were collected during August, 2008 using ethanol-sterilized trowels and transferred to
triplicate freezer storage bags. The samples were shipped to a laboratory at Louisiana State University (Baton Rouge, LA) at ambient temperature and processed upon arrival. Canopy material was homogenized by sifting through a sterile 2-mm sieve to remove fine roots and cinders. Bare cinders were partially crushed to reduce the size range to about 5 mm. Samples were used immediately or stored in zip-top bags at ambient temperature (about 25 °C) in the dark until use. Material used to assay short-term responses of CO uptake to temperature was stored for 2 d prior to analysis. Assays for hydrogen uptake and for analysis of responses of CO uptake to long-term elevated temperatures were conducted over a period of 4–12 weeks; prior observations have indicated that activity remains relatively stable during storage, but some changes in communities might occur.

**Figure 2.1.** Surface (0–2.5 cm depth interval) temperature profiles of Canopy and Bare sites determined over 3 d period in August 2008 during which samples were collected.

**Gas Flux Analyses**
Maximum potential CO and H$_2$ uptake rates were determined by adding rate-saturating concentrations of CO and H$_2$ (100 ppm) to samples in gastight containers (King, 1999b). Headspace sub-samples were obtained at intervals using a sterile needle and syringe and analyzed by gas chromatography (RGA reduced gas analyzer; Trace Analytical Instruments; Columbia, MD, USA) as described previously (King, 1999b). All assays were conducted in triplicate; rates were expressed per gram dry weight (gdw) of material based on water contents determined by drying samples at 100 °C.

**Short-term Response of CO and H$_2$ Uptake to Temperature**

Canopy material (0.5 gram fresh weight, gfw) and Bare cinders (2.0 gfw) were transferred into sterile 30 cm$^3$ tubes that were sealed with neoprene stoppers. Two sets of samples were incubated at temperatures from 5–65 °C (5 °C steps) using custom-built heating blocks. After a 10 min delay for temperature equilibration, gases were added to sample headspaces. CO and H$_2$ uptake were measured separately using triplicates for each temperature as described above. Sample dry weights were determined after the assays by drying samples overnight at 100 °C. Arrhenius plots were created by plotting the log-transformed rate constants of CO or H$_2$ uptake over the inverse temperature (Kelvin). Activation energy, the minimal amount of energy for a chemical reaction to occur, was calculated from the y-intercept and slope of a linear regression fit to this data.

**Response of CO and H$_2$ Uptake to Extended Incubations at Elevated Temperature**

Canopy material (25 gfw) and Bare cinders (30 gfw) were transferred into sterile 500 cm$^3$ jars with gastight lids fitted with a neoprene stopper. Triplicate samples with and without addition of a 0.05% yeast extract solution (50 µl [g sample]$^{-1}$) were incubated in the dark at 25 °C and 55 °C for 30 d. The yeast extract solution was applied by uniformly distributing small
droplets on the samples on day 0 and day 14 of the incubation, then air-drying the samples briefly in a laminar flow hood to maintain the original water contents. Samples were supplemented with yeast extract, since the decrease in readily available carbon at higher temperatures (Bárcenas-Moreno et al., 2009) and the limited carbon availability at the Bare site might have prevented adaptation to elevated temperatures. During the 30 d incubation, a 22 gauge needle fitted with a sterile 25 mm syringe filter (0.22 µm pore size) was inserted through the neoprene stopper of each jar to allow atmospheric gas exchange while preventing water loss. This vent was removed prior to uptake assays. Killed controls were created by autoclaving samples for 25 min.

CO and H$_2$ uptake rates were determined as previously described on day 0, after allowing an initial 25 min temperature equilibration, and on day 30. After the day 30 assay, samples previously incubated at 55 °C were incubated at 25 °C. Following a 1 h equilibration period, CO and H$_2$ assays were conducted on the samples to determine residual activity at 25 °C. After the assays, samples were dried for 2 d in an oven at 100 °C to determine dry weights.

Differences in net consumption rates among treatments were analyzed using a two-way analysis of variance with a general mixed model with multiple treatments and repeated measures using SAS software 9.2 (SAS Institute, Cary, NC, USA). Means were distinguished by Tukey’s honestly significant difference test ($\alpha = 0.05$). Differences in net production rates were determined using a two-tailed unpaired T-test ($\alpha = 0.05$).

**Results**

**Short-term Responses of CO and H$_2$ Uptake to Varied Temperature**

Mean Canopy material CO and H$_2$ uptake rates were approximately 23-fold and 17-fold greater than Bare cinder rates, respectively, over the temperature ranges examined. Canopy
material H₂ uptake rates increased with temperature with a plateau from 30 °C to 50 °C followed by a sharp decline in activity at 55 °C (Figure 2.2, graph a). H₂ uptake by Bare cinders also increased with temperature, rising to a distinct peak or optimum at 35 °C with a small decrease at 40 °C and a sharp decline at 55 °C (Figure 2.2, graph b). The Canopy material H₂ uptake activation energy determined from an Arrhenius analysis (50.8 kJ mol⁻¹) was considerably lower than that for Bare cinders (82.9 kJ mol⁻¹; Figure 2.3, graphs a, b). CO uptake rates for both sites increased with temperature from 5 °C to 35 °C followed by a small decline at 40 °C (Figure 2.2, graphs a, b). At temperatures > 45 °C, slight net CO production was observed. Activation energies for CO uptake were similar for Canopy material and Bare cinders (78.9 kJ mol⁻¹ and 71.8 kJ mol⁻¹, respectively; Figure 2.3, graphs c, d).

Response of H₂ Uptake to Extended Incubations at Elevated Temperature

During a 30 d incubation at 25 °C, Canopy material H₂ uptake was unchanged in untreated samples (P = 0.320), but decreased significantly (P = 0.003) by about 50% in soils amended with yeast extract (Figure 2.4, graph a). For Bare cinders incubated at 25 °C, H₂ uptake decreased significantly (about 74%; P < 0.009) in both untreated and yeast extract-amended samples (Figure 2.4, graph b). After 30 d incubations at 55 °C, H₂ uptake was completely inhibited for both sites with or without added yeast extract (Figure 2.4). Neither Bare cinders nor Canopy material previously incubated at 55 °C consumed H₂ during recovery assays at 25 °C. There was no evidence for H₂ uptake by killed controls.
Figure 2.2. Maximum potential uptake rates as a function of temperature for (a) Canopy H$_2$ uptake (b) Bare H$_2$ uptake (c) Canopy CO uptake and (d) Bare CO uptake. All data are means of triplicates ± 1 standard error (s.e.). Values less than zero indicate net CO production.
Figure 2.3. Arrhenius plots for determining activation energy for (a) Canopy H$_2$ uptake ($y = -6.11x + 31.0, r^2 = 0.857; E_a = 50.8$ kJ/mol); (b) Bare H$_2$ uptake ($y = -9.97x + 38.2, r^2 = 0.955; E_a = 82.9$ kJ/mol); (c) Canopy CO uptake ($y = -9.49x + 40.1, r^2 = 0.918; E_a = 78.9$ kJ/mol); and (d) Bare CO uptake ($y = -8.63x + 34.2, r^2 = 0.970; E_a = 71.8$ kJ/mol).
Response of CO Uptake to Extended Incubations at Elevated Temperature

For Canopy material incubated at 25 °C, CO uptake rates after 30 d were not significantly different for samples with and without yeast extract ($P > 0.170$, Figure 2.5, graph a). For Bare cinders incubated at 25 °C, CO uptake rates after 30 d decreased significantly ($P < 0.0001$) relative to initial values for material with or without yeast extract (Figure 2.5, graph b). The extent of the decreases, 73% and 58%, respectively, were similar to those observed for H$_2$ uptake (74%, Figure 2.5, graph b). At 55 °C, CO was initially produced by Canopy material, with no net uptake observed; CO production rates were equivalent in the absence and presence of yeast extract (two-tailed unpaired t-test, $P = 0.735$, Figure 2.5, graph a). After 30 d at 55 °C, CO was consumed rather than produced at similar rates in the absence and presence of yeast extract ($P = 0.986$), and activity was equivalent to about 45% and 55%, respectively, of the levels observed at 25 °C. For Bare cinders incubated at 55 °C, slight CO production occurred in the absence of yeast extract; CO was neither produced nor consumed initially in the presence of yeast extract (Figure 2.5, graph b). After 30 d, no significant net CO consumption or production occurred in the absence or presence of yeast extract. CO uptake was not recovered at 25 °C for samples previously incubated at 55 °C for either site. CO was not consumed in any killed controls, but CO production was observed for killed Canopy material incubated at 55 °C.

Additional analyses of CO and H$_2$ uptake after incubation for 30 d at 25 °C and 55 °C were conducted using material collected in December, 2008. Results were comparable to those presented here (Figure A.5, Figure A.6).
Figure 2.4. Mean maximum potential H$_2$ uptake rates for (a) Canopy and (b) Bare samples incubated at either 25 °C or 55 °C with or without added yeast extract (YE) for 30 d. All data are means of triplicates ± 1 s.e.
Figure 2.5. Mean maximum potential CO uptake rates for (a) Canopy and (b) Bare samples incubated at either 25 °C or 55 °C with or without added yeast extract (YE) for 30 d. All data are means of triplicates ± 1 s.e. Values less than zero indicate net CO production.
Discussion

Responses to temperature by CO and H₂-oxidizing communities offer a number of insights about each process and the impacts of plant development on them. We show, for example, that H₂ uptake activation energies for Bare cinders (82.9 kJ mol⁻¹) and Canopy material (50.8 kJ mol⁻¹) fall within the range reported for bacterial activity (about 50–140 kJ mol⁻¹) and markedly exceed values for exoenzymatic activity (10–30 kJ mol⁻¹; Schuler and Conrad, 1991). This is consistent with the outcome of a previous inhibitor study (King, 2003a), which demonstrated that H₂ uptake was largely bacterial rather than exoenzymatic (Conrad, 1996). Thus, the relative importance of bacteria versus exoenzymes as a hydrogen sink does not appear to be greatly affected by vegetation due to ecosystem succession, at least over the short-term (i.e., decades).

The difference in activation energies between Bare and Canopy sites also indicates that Bare cinder H₂ uptake is more temperature sensitive than that for Canopy material. Indeed, Canopy material, which experiences lower average temperatures and is not subjected to daily extremes, shows greater thermal tolerance through a broader temperature optimum (30–50 °C for Canopy versus 30–35 °C for Bare cinders). Nonetheless, these results indicate that succession does not lead to a lower temperature optimum for Canopy H₂ uptake as an adaptive response to lower temperatures.

The lack of adaptive responses for H₂ uptake is also evident in results from 30 d incubations of Bare cinders and Canopy material with and without yeast extract at 25 °C and 55 °C (Figure 2.4). At 25 °C, Canopy H₂ uptake remained unchanged in the absence of yeast extract and decreased when yeast extract was added, possibly due to substrate-induced repression of hydrogenase production. At 25 °C, Bare cinder H₂ uptake declined regardless of yeast extract
treatment, which suggests that even though Bare and Canopy systems are capable of active H$_2$ consumption, the process is regulated differently at the two sites. Incubation at 55 °C for 30 d resulted in complete inhibition of H$_2$ uptake for both sites regardless of yeast extract availability. Additionally, heated samples were not able to recover H$_2$ uptake at 25 °C, which indicates that the original H$_2$-oxidizers had been effectively lost. This agrees with results of Chowdhury and Conrad (2010), who showed that temperatures of 50 °C or even less rapidly deactivated H$_2$ uptake by a variety of soils, including desert sands. Recovery apparently required synthesis of new enzymes, which appears to have not occurred for Bare cinders or Canopy material incubated at 55 °C over a 30 d period.

The short-term responses of CO uptake to temperature differ little between Bare cinders and Canopy material (Figure 2.2, graphs c, d). Activity for both sites rises to an optimum of 30–35 °C, and then declines sharply with net CO production at temperatures ≥ 45 °C. Activation energies for both sets of samples are also equivalent indicating similar sensitivities to temperature change. These results suggest little, if any, adaptation by Canopy material CO uptake to lower temperature regimes, an observation consistent with results for H$_2$ uptake.

Interestingly, Bare cinder and Canopy material temperature optima and activation energies both exceed values measured for atmospheric CO uptake by a temperate continental forest soil (33.2 kJ mol$^{-1}$, Maine, USA; King, 1999a). The lower temperature optima and temperature sensitivity observed for a continental soil might reflect adaptations to the generally colder climate at the site. This suggests that adaptations to lower temperature regimes resulting from plant community development on Hawaiian volcanic deposits might occur, but require longer time spans than represented by the sites in this study (about 50 years).
However, results from extended incubations of Bare cinders and Canopy material at 55 °C indicate that succession may also expand temperature response capacities (Figure 2.5). In particular, after 30 d incubation at 55 °C, CO uptake by Bare cinders remains undetectable with or without yeast extract (Figure 2.5, graph b). In contrast, CO uptake by Canopy material increased dramatically, with a change from initial net CO production to net CO consumption (Figure 2.5, graph a). Since CO production is largely an abiological process (in this case resulting from thermal degradation of organic matter) while CO uptake is a microbial process (Conrad and Seiler, 1985; King and Weber, 2007), the observed shift from production to consumption indicates that the Canopy site harbors thermophilic CO oxidizers that are either activated (e.g. spore germination) or capable of significant growth and CO uptake when temperature regimes are suitable. Since yeast extract did not enhance activity after 30 d relative to no additions (Figure 2.5), endogenous substrates appear sufficient to fuel activation or growth.

Several previous studies (Ranneklev and Bååth, 2001; Pettersson and Bååth, 2003; Hartley et al., 2008; Bárcenas-Moreno et al., 2009) have demonstrated that soil microbial communities can adapt to elevated temperature regimes. For example, using growth rate as a variable, Ranneklev and Bååth (2001) have shown that heating peat soil from 25 °C to 55 °C resulted in community adaptation with optimal growth at 55 °C in only 3 d. Heating resulted in rapid growth of thermophiles due to limited substrate competition and additional resources from mesophilic bacterial necromass (Bárcenas-Moreno, 2009). Although bulk heterotrophic communities at Bare and Canopy sites might have adapted similarly to elevated temperature, H$_2$-oxidizers at both sites were either inactivated or killed, while only Canopy CO oxidizers were capable of thermal adaptation. These results show that responses to temperature changes vary
between processes (e.g., H$_2$ versus CO uptake) and for the same process at different sites (e.g., Bare versus Canopy).

The results of extended incubations at 55 °C are surprising in at least two respects. H$_2$ oxidation occurs in many bacterial taxa, including thermophiles (Friedrich and Schwartz, 1993). Some thermophiles, e.g., Bacillus schlegelii, also oxidize both CO and H$_2$ (Hudson et al., 1988). Thus, the absence of an adaptive response for H$_2$ uptake is enigmatic when a response is observed for CO. The development of a thermophilic CO uptake capacity by Canopy material suggests that incubation conditions per se did not preclude activation or growth of facultative lithotrophs in general. Whether or not H$_2$-oxidizing communities associated with other soils and volcanic sites lack an adaptive response to elevated temperatures is currently unknown.

Development of a thermophilic CO uptake capacity in Canopy material but not Bare cinders is also surprising. Bare cinders support populations of Ktedonobacteria-like CO oxidizers based on analyses of coxL and 16S rRNA gene sequences (Weber and King, 2010). Indeed, Ktedonobacteria-like sequences dominate Bare cinder coxL libraries (Weber and King, 2010). These sequences, which are most closely related to a class of bacteria consisting of a majority of thermophiles (Stott et al., 2008; Yabe et al., 2010; Yabe et al., 2011), have not been observed in Canopy site coxL or 16S rRNA gene sequence libraries. Thus, one could anticipate an adaptive response to elevated temperature for Bare cinder CO uptake even though some Ktedonobacteria are mesophilic (Cavaletti et al., 2006).

The absence of a Bare cinder response might indicate that incubation conditions were unsuitable for activation or growth of Ktedonobacteria-like populations. The fact that successful enrichments of thermophilic Ktedonobacteria from Bare cinders have required multiple efforts (Chapter 4) supports this possibility. Development of thermophilic CO uptake in Canopy
material but not Bare cinders might also reflect differences in CO oxidizer diversity, population sizes, and resource availability for growth and metabolism, all of which increase from Bare cinders to Canopy material (King and Weber, 2008; Weber and King, 2010). Since thermophiles occur as dormant forms in many soils (Marchant et al. 2002, Rahman et al. 2004, Marchant et al. 2008), higher total microbial and CO oxidizer diversity and population sizes in Canopy material might increase the likelihood that one or more thermophiles becomes active with elevated temperature. Similarly, higher organic matter and nitrogen contents of Canopy material might facilitate activation or growth.

Responses of CO and H₂ uptake to temperature also offer additional insights about the communities involved in these processes. The similarities of short-term and long-term responses of CO and H₂ uptake in Bare cinders suggest that the two processes might be carried out in part by the same bacteria, or at least physiologically similar populations. In contrast, different responses by CO and H₂ uptake in Canopy material suggest that distinct populations carry out the two processes, with little overlap between them.

Although Canopy material and Bare cinders support distinctly different CO-oxidizing communities (Weber and King, 2010) and respond differently to long-term elevated temperatures, differences in temperature regimes between sites, including extremes and mean values, do not result in different temperature optima or temperature sensitivity. Temperature responses and adaptation of Canopy material and Bare cinder H₂ uptake also appear unaffected by differences between the sites in ambient temperature regimes. A comparison of Bare cinder and Canopy material responses to water stress has revealed a similar pattern, with no difference in the impact of imposed water stress on CO oxidation, in spite of dramatic differences between the sites in water regimes in situ (Weber and King, 2009). These results collectively suggest that
during succession, changes in edaphic factors, e.g., temperature and water potential, affect rates of bacterial activities, but do not play a primary role in establishing the ecophysiological properties of bacterial communities on young volcanic systems. Other factors, such as the specific phylogenetic composition of colonists arriving at these sites, their physiological characteristics and survival, and the development of plant communities, likely play more determinative roles over short time periods (perhaps decades).
CHAPTER 3.
CHARACTERIZATION OF CO UPTAKE BY GEOTHERMALLY-HEATED SOILS
AND BIOFILMS

Introduction

CO uptake has been well documented for a variety of temperate soils (King, 1999a; King 1999b; King and Hungria, 2002) and volcanic deposits (King, 2003; King and Weber, 2008; King et al., 2008). CO consumption at thermophilic temperature (55 °C) has also been observed for artificially-heated soil (Chapter 2); however, CO dynamics in naturally-heated systems remain unknown. The only previous investigation, a study of hot springs at Kamchatka (Eastern Russia), failed to demonstrate thermophilic CO oxidation (King and Weber, unpublished results).

CO oxidation has been characterized in the thermophiles ‘Pseudomonas thermocarboxydovorans’ (Lyons et al., 1984), ‘Bacillus schlegelii’ (Kruger and Meyer, 1984), and four Streptomyces species (Gadkari et al., 1990; Kim et al., 1998; Kim and Goodfellow, 2002). Additionally, several recently published genomes of thermophiles contain form I cox genes encoding the potential ability to oxidize CO (Chapter 1). These genomes span a vast physiological and phylogenetic breadth including sulfur-oxidizing Crenarchaeota (Reno et al., 2009), Chloroflexi (Wu et al., 2012), ferrous iron-oxidizing Firmicutes (Li et al., 2011; Anderson et al., 2012), Deinococcus/Thermus (Tindall et al., 2010), and halophilic Bacteroidetes (Nolan et al., 2009). A few thermotolerant species, including extremely halophilic Euryarchaeota (Xu et al. 1999; Cui et al., 2007), methylotrophic Bacilli (Heggeset et al., 2012), and nitrite-oxidizing Chloroflexi (Sorokin et al., 2012), also harbor cox operons in their genomes. Additionally, three thermophiles isolated from natural geothermal sites, Thermomicrobium roseum DSM 5159, Sphaerobacter thermophilus DSM 20745, and Meiothermus ruber DSM
1279, have been recently confirmed to oxidize CO in culture (Wu et al., 2009; Chapter 4) indicating that thermophilic CO oxidizers might be ubiquitous in thermal environments.

Although genomic evidence and limited data from cultures suggest that thermophilic CO oxidation could be a widespread process in thermal systems, no previous studies have demonstrated this capacity in situ or with intact samples ex situ. Thus the purpose of this work was to determine if CO oxidation can be observed for naturally-heated sites and to assess limits of activity. This study examines aerobic CO uptake at four unique geothermal sites at the Hawaii Volcanoes National Park: two soils from the Puhimau geothermal area, microbial biofilms growing near fumaroles at Puhimau, and microbial biofilms on a tumulus within Kilauea Iki Crater. The terms microbial biofilm and biofilm are used interchangeably to describe layers of microorganisms within a matrix adhering to a surface. Microbial biofilms are self-contained ecosystems that depend on carbon autotrophy and have mostly closed biogeochemical cycles (Stal, 1995). These results represent the first CO study of thermal systems and microbial biofilms

**Materials and Methods**

**Site Descriptions**

The Puhimau geothermal area is located at Kilauea Volcano at the Hawaii Volcanoes National Park (GPS coordinates: N 19° 22.2”, W 155° 14’ 56.6”). Originally a *Metrosideros* forest, in 1937–1938 a near-surface magma intrusion killed the vegetation present leaving behind dead tree stumps which now act as fumaroles (Smith, 1981). Steam produced from geothermally-heated groundwater escapes from the base of these tree stumps. Acidic, red to brown biofilms grow on the illuminated side of these tree stumps (Figure 3.1, graph a). A baked white crust forms at the surface of the surrounding soil due to the underlying magma, and steam escapes when the porous, rocky soil is disturbed.
Kilauea Iki Crater was created from the 1959 eruption of Kilauea Volcano. The crater floor, once a lava lake, contains many steaming fissures and large piles of rock that serve as vent sites. Multilayered green, pink, and white microbial biofilms drip from venting cracks on a tumulus near the center of the crater (GPS coordinates: N 19° 24’ 51.1”, W 155° 14’ 57.4”; Figure 3.1, graph b).

Figure 3.1. (a) Reddish biofilms growing on a dead tree stump at Puhimau. (b) Dripping biofilms upon rocks at Kilauea Iki Crater.

Site Characterization

Instantaneous site temperatures were taken at each sampling time with a digital thermometer inserted ~1 cm into the material. Temperature stability was determined using HOBO Data Loggers (Onset Computer Corp.) equipped with TMC50-HD soil temperature sensors. Temperature profiles for triplicate Puhimau biofilms and soils within the tree stumps were determined over two 5 d periods in September 2011 and February 2012. The temperature profile of a Kilauea Iki biofilm was also determined in September 2011. In situ pH values of
biofilms were determined in December 2010 using a MI-4154 Micro-combination microelectrode (Microelectrodes, Inc., Bedford, NH, USA). pH values at other collection dates were determined from 1:1 slurries using freshly collected samples and deionized H₂O. Water potential was obtained using a WP4-T dewpoint potentiometer (Decagon Devices, Pullman, WA, USA). Water content was determined gravimetrically after drying samples in an 80 °C oven for 2 d.

**Ex situ CO Uptake Rates**

Triplicate samples were aseptically collected on March 20–21, 2010 and processed within 3 h of collection. Triplicate Puhimau microbial biofilm and stump soil samples represent distinct tree stumps. Soil samples were collected from the upper 2 cm of surface soil. Intact samples (2 gram fresh weight (gfw)) were placed in sterile 60 cm³ serum bottles sealed with gastight neoprene stoppers. Puhimau and Kilauea Iki biofilms were incubated in a water bath at 55 °C. Puhimau soil collected from the interior of tree stumps was incubated at 60 °C. Puhimau unvegetated soil, collected from the surrounding barren soil, was initially incubated in an 80 °C water bath and later transferred to 60 °C after 4 d. A second set of triplicate Puhimau unvegetated soil samples were incubated in a 55 °C water bath. Elevated concentrations (100 ppm) of CO were added to the headspace of each bottle to determine maximum potential CO oxidation rates. Gas headspace subsamples were taken at appropriate intervals using a sterile needle and syringe and analyzed by gas chromatography (Peak Performer 1, Peak Laboratories, Mountain View, CA, USA) consistent with previously described methods (King, 1999b). After completing the assay, samples were dried in an oven (2 d) and gram dry weight (gdw) was recorded.
CO Uptake Across Temperature

Triplicate samples were collected aseptically transferred to Whirl-Pak bags on December 14–15, 2010 and on May 2, 2011. Samples were shipped at ambient temperature to a laboratory at Louisiana State University (LSU, Baton Rouge, LA) and processed immediately upon arrival (within 4 d of collection). Sample material (0.5 gfw) was transferred to triplicate sterile 60 cm³ serum bottles sealed with gastight neoprene stoppers. Saturating concentrations (100 ppm) of CO were added to the headspaces of bottles which were incubated at the temperatures 30–80 °C (10 °C steps). Samples were incubated in either a water bath (30 °C), hot-air incubator (40–70 °C), or oven (80 °C). Gas headspace subsamples were analyzed by gas chromatography (RGA3, Trace Analytical, Muskegon, MI, USA) as described above (King, 1999b).

Most Probable Number (MPN) Estimates

Triplicate samples were collected on September 4–5, 2011, shipped to LSU, and processed immediately upon arrival (within 4 d of collection). Triplicate samples (0.5 gfw) were placed into sterile 60 cm³ bottles with 5 ml CoxPPYE medium. CoxPPYE medium had the following composition (per L deionized H₂O): 1.5 g NH₄Cl, 0.2 g MgSO₄·7H₂O, 0.02 g CaCl·2H₂O, 3.55 g Na₂HPO₄, 1.5 g KH₂PO₄, 1 mg ZnSO₄·7H₂O, 0.035 mg MnCl₂·4H₂O, 0.35 mg H₃BO₃, 0.2 mg CoCl₂·6H₂O, 0.01 mg CuCl₂·2H₂O, 0.02 mg NiCl₂·6H₂O, 0.9 mg Na₂MoO₄·2H₂O, 0.02 mg Na₂SeO₄, 1.2 mg ferric ammonium citrate, 0.5 g peptone, 1 g pyruvate, and 0.25 g yeast extract (pH adjusted to 6.5). CO (100 ppm) was added to the headspace of each bottle and the slurries were incubated with shaking at 60 °C for 2 h. Slurries were serially diluted to 10⁻⁸ in 30 cm³ test tubes containing 2.7 ml CoxPPYE. Three subsamples were taken from each Puhimau biofilm and Kilauea Iki biofilm replicate for a total of 9 dilution series for both biofilm sites. The triplicate Puhimau soil slurries were serially diluted individually for a total of 3
dilution series for each soil site. Each test tube was spiked with 100 ppm CO and incubated at 60 °C at a 45° angle with shaking (100 rpm) to maximize gas exchange. CO headspace concentrations were measured for 10 randomly selected tubes to confirm initial headspace concentration. CO concentrations were determined after 5, 12, and 18 d. Growth was also visually scored at these timepoints.

Results

Site Characteristics

Puhimau biofilms were acidic (pH 3.9 ± 0.4), while the remaining site materials had near-neutral pH values (Table 3.1). The Puhimau soils were the hottest of the sites and typically experienced temperatures > 70 °C (Table 3.1). Both the Puhimau and Kilauea Iki biofilms had lower average temperatures near 50 °C (Table 3.1). All material had high water content. The temperature of a Kilauea Iki biofilm was fairly stable over time with small fluctuations < 6 °C (Table 3.2). Temperatures for Puhimau soil at the base of tree stumps were relatively stable over both short (5 d) and long-term (September 2011 versus February 2012) observations, typically fluctuating < 5 °C (Figure 3.2; Table 3.2). However, two soils experienced substantial diurnal fluctuations (replicates 1 and 2; Table 3.2). Puhimau microbial biofilms experienced elevated temperatures (average 42.0 °C ± 0.2) with broad fluctuations that occasionally dipped into mesophilic temperatures (Figure 3.2; Table 3.2).

Net CO consumption was observed at thermophilic temperatures for freshly collected intact material from all sites. Maximum potential CO uptake rates were ~5-fold higher for Kilauea Iki biofilms than the Puhimau biofilms and soils (Table 3.1). Puhimau biofilms and unvegetated soil had comparable rates that were 2-fold greater than the Puhimau stump soil
Puhimau unvegetated material had no net CO uptake at 80 °C and activity was not recovered at 60 °C.

**Table 3.1.** Selected properties of thermal site material. Values in parentheses represent ± 1 s.e.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Kilauea Iki biofilms</th>
<th>Puhimau biofilms</th>
<th>Puhimau stump soil</th>
<th>Puhimau unvegetated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)*</td>
<td>49 (1)</td>
<td>48 (1)</td>
<td>70 (2)</td>
<td>75 (2)</td>
</tr>
<tr>
<td>pH</td>
<td>7.6 (0.4)</td>
<td>3.9 (0.4)</td>
<td>6.43 (0.08)</td>
<td>6.75 (0.06)</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>90.0 (1.0)</td>
<td>92.0 (0.2)</td>
<td>45.0 (6.0)</td>
<td>36.0 (1.0)</td>
</tr>
<tr>
<td>Water potential (MPa)</td>
<td>-</td>
<td>-</td>
<td>-0.31 (0.01)</td>
<td>-0.32 (0.03)</td>
</tr>
<tr>
<td>Ex situ CO uptake (nmol gdw⁻¹ h⁻¹)**</td>
<td>45 (5)</td>
<td>10 (2)</td>
<td>5 (1)</td>
<td>11 (6)</td>
</tr>
<tr>
<td>Thermophile MPN (cells gfw⁻¹)</td>
<td>5.0×10⁸ (2.5×10⁸)</td>
<td>1.4×10⁶ (1.1×10⁶)</td>
<td>1.9×10⁵</td>
<td>8.6×10⁴</td>
</tr>
<tr>
<td>CO oxidizer MPN (cells gfw⁻¹)</td>
<td>4.0×10⁶ (3.6×10⁶)</td>
<td>239 (67)</td>
<td>4.2×10⁴</td>
<td>1.86×10⁴</td>
</tr>
</tbody>
</table>

*Temperatures represent means of instantaneous temperature recordings taken during sampling.  
**Ex situ CO uptake rates were determined from freshly collected material during March 2010 at 55 °C for Kilauea Iki biofilms, Puhimau biofilms, and Puhimau unvegetated soil and at 60 °C for Puhimau stump soil.

**Table 3.2.** Temperature regimes at sites recorded using HOBO loggers over 5 d. Puhimau soil and biofilm replicates 1, 2, and 3 were recorded during September 2011 while replicates 4, 5, and 6 were recorded during February 2012. The Kilauea Iki biofilm temperature profile was recorded in September 2011. Values in parentheses represent ± 1 s.e.

<table>
<thead>
<tr>
<th>Site</th>
<th>Average temperature (°C)</th>
<th>Temperature range (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Puhimau stump soil 1</td>
<td>61.6 (0.1)</td>
<td>57.6–71.4</td>
</tr>
<tr>
<td>Puhimau stump soil 2</td>
<td>46.2 (0.3)</td>
<td>32.0–67.2</td>
</tr>
<tr>
<td>Puhimau stump soil 3</td>
<td>76.55 (0.04)</td>
<td>74.2–79.0</td>
</tr>
<tr>
<td>Puhimau stump soil 4</td>
<td>81.06 (0.02)</td>
<td>79.9–81.8</td>
</tr>
<tr>
<td>Puhimau stump soil 5</td>
<td>75.718 (0.009)</td>
<td>75.0–76.2</td>
</tr>
<tr>
<td>Puhimau biofilm 1</td>
<td>43.1 (0.1)</td>
<td>30.5–49.0</td>
</tr>
<tr>
<td>Puhimau biofilm 2</td>
<td>30.7 (0.1)</td>
<td>21.0–40.3</td>
</tr>
<tr>
<td>Puhimau biofilm 3</td>
<td>47.8 (0.2)</td>
<td>30.5–58.6</td>
</tr>
<tr>
<td>Puhimau biofilm 4</td>
<td>42.1 (0.3)</td>
<td>22.6–51.8</td>
</tr>
<tr>
<td>Puhimau biofilm 5</td>
<td>40.0 (0.4)</td>
<td>19.1–59.2</td>
</tr>
<tr>
<td>Puhimau biofilm 6</td>
<td>48.1 (0.3)</td>
<td>31.1–65.1</td>
</tr>
<tr>
<td>Kilauea Iki biofilm</td>
<td>44.09 (0.04)</td>
<td>40.3–46.2</td>
</tr>
</tbody>
</table>
CO Oxidizer Activity and Abundance

Kilauea Iki biofilms had the greatest estimate of thermophilic CO oxidizers present. MPN estimates determined that Kilauea Iki biofilms had 130-fold more CO oxidizers present than at the Puhimau soil sites and 17,000-fold higher numbers than at the Puhimau biofilm site (Table 3.1). Based on MPN estimates, CO oxidizers represented 0.82% and 0.02% of the total estimated number of thermophiles at the Kilauea Iki and Puhimau biofilms, respectively (Table 3.1). CO oxidizers made up 22.6% and 21.6% of the total estimated number of thermophiles present at the Puhimau tree stump and unvegetated soils, respectively (Table 3.1).

![Figure 3.2](image)

**Figure 3.2.** Temperature profile of a representative microbial biofilm and soil associated with a Puhimau tree stump determined during September 2011. Data correspond to Puhimau stump soil 3 and biofilm 3 in Table 3.2.

Responses of CO Uptake to Varied Temperature

Kilauea Iki biofilm CO uptake rates were approximately 6-fold higher than Puhimau soil rates over the temperature range examined (Figure 3.3). Puhimau biofilm CO uptake rates across the temperature range assessed were 5-fold and 11-fold higher than Puhimau stump soil and
Puhimau unvegetated soil rates, respectively. Puhimau biofilms and Kilauea Iki biofilms had similar CO uptake rates over temperature (Figure 3.3, graphs a,b) with only 1.6-fold greater rates at Puhimau. The two Puhimau soils also had similar CO uptake rates over temperature (Figure 3.3, graphs c,d); although, tree soil rates were slightly higher (1.4-fold). While mean CO uptake rates did not significantly differ between sampling timepoints for Kilauea Iki biofilms, December 2010 uptake rates were on average 2 to 3-fold higher than May 2011 rates for the Puhimau sites.

Kilauea Iki biofilm uptake rates increased with temperature from 30 to 40 °C with a plateau from 40–60 °C followed by variable activity at 70 °C and net CO production at 80 °C (Figure 3.3, graph a). CO uptake by Puhimau biofilms and unvegetated soils increased with temperature from 30 to 60 °C followed by a sharp decline in activity at 70 °C (Figure 3.3, graphs b, c). Puhimau tree stump soils had variable CO uptake at 30 and 40 °C followed by an increase in CO uptake rates from 50 to 60 °C and variable activity at 70 and 80 °C (Figure 3.3, graph d). Notably, net CO consumption at 80 °C was observed for one replicate. During most assays, CO uptake continued to occur once CO concentrations approached atmospheric levels. An additional assay at 55 °C for Puhimau biofilms (Figure A.7) confirmed this observation.

CO uptake activation energies determined from Arrhenius analyses were similar for both sampling timepoints for Kilauea Iki, Puhimau mats, and Puhimau stump soil material (Figure 3.4, graphs a, b, d). The Puhimau unvegetated soil CO uptake activation energy from December 2010 (60.6 kJ mol⁻¹) was considerably lower than that from May 2011 (94.9 kJ mol⁻¹; Figure 3.4, graph c). CO uptake activation energy was lowest for Kilauea Iki biofilms (24.9 kJ mol⁻¹; Figure 3.4, graph a). Activation energies for CO uptake were lower for Puhimau stump soil (42.4 kJ mol⁻¹) than that for biofilms (62.8 kJ mol⁻¹; Figure 3.4, graphs b, d).
Figure 3.3. CO oxidation rates over temperature for (a) Kilauea Iki biofilms, (b) Puhimau biofilms, (c) Puhimau unvegetated soil and (d) Puhimau stump soil. Open symbols represent rates from the December 2010 assay; closed symbols represent rates from the May 2011 assay. All rates are means of triplicates ± 1 s.e.
Figure 3.4. Arrhenius plots for determining CO oxidation activation energies for (a) Kilauea Iki biofilms, (b) Puhimau biofilms, (c) Puhimau unvegetated soil, and (d) Puhimau tree stump soil. Open symbols denote rates from the December 2010 assay; trendline equation is listed first. Closed symbols denote rates from the May 2011 assay; equation is listed second.

Discussion

Net CO consumption was observed for freshly-collected geothermal soil and biofilm samples, indicating thermophilic CO oxidizers are active in situ in permanently hot environments. The observation of CO consumption at atmospheric concentrations (< 0.5 ppm) provides additional support that these sites are CO sinks rather than sources. Ex situ maximum
potential CO uptake rates, a proxy for CO oxidizer biomass (King and Weber, 2008), and MPN estimates show that thermophilic CO oxidizers were more abundant at Kilauea Iki biofilms than other sites examined. Oxygen profiling results demonstrated that Kilauea Iki biofilms have high rates of oxygenic photosynthesis (King and King, unpublished results) likely due to cyanobacterial populations in the green-pigmented outer layers of the biofilms. Increased organic carbon concentrations are correlated with greater abundance and diversity of CO oxidizers (Weber and King, 2010); therefore, increased organic carbon availability from primary production may be responsible for the higher CO uptake rates and MPN estimates for Kilauea Iki biofilms. Differences in site MPN estimates of CO oxidizers (130 to 17,000-fold) were more pronounced than changes in site maximum *ex situ* CO uptake rates (6-fold) perhaps due to the ability of CO oxidizers to proliferate under heterotrophic conditions over longer periods of time as opposed to short-term *ex situ* assays.

Puhimau stump soil had the lowest CO uptake rate. This might have been influenced by incubating these samples at 5 °C higher than other samples which may have unfavorably altered the activity of the CO-oxidizing community or increased CO production. The maximum potential CO uptake rates reported here are within the range of previously reported rates at cool volcanic sites (Weber and King, 2009) indicating that CO oxidizer community size at the thermal sites rivals that of temperate sites.

While average temperatures at both biofilm sites were similar (near 50 °C), Puhimau microbial biofilms experience substantial temperature fluctuations *in situ* while Kilauea Iki biofilm temperature remain relatively stable (Figure 3.2). Temperature oscillations at the Puhimau biofilm sites are not correlated to changes in ambient temperature (Figure 3.2) and instead likely reflect inconsistent heating due to wind disturbance of rising steam at the vent. In
general, soil temperatures at the Puhimau geothermal area were stable. Oscillations for Puhimau soil replicates 1 and 2 (Table 3.2) are correlated to changes in ambient temperature indicating these fluctuations were due to insolation. Despite experiencing the widest temperature range of all the sites, Puhimau biofilm CO uptake appeared to be more temperature sensitive than that of Kilauea Iki biofilms. The broader CO uptake temperature optima (40 to 60 °C; Figure 3.3, graph a) and lower activation energy (24.9 kJ mol\(^{-1}\); Figure 3.4, graph a) indicate that the CO-oxidizing community at the Kilauea Iki biofilms has the greatest thermal tolerance of the sites tested. These results are consistent with previous results from temperate volcanic deposits where temperature tolerance was unrelated to *in situ* temperature regimes experienced at sites (Chapter 2). Other factors such as organic carbon availability might be more important indicators of community stability in response to temperature.

Despite differences in material composition and *in situ* temperature regimes, all sites showed net CO consumption from 30 to 60 °C (Figure 3.3). Cardinal temperatures for CO activity did not correlate strongly to *in situ* temperatures at sites. These results are somewhat surprising since temperature is traditionally regarded as one of driving factors regulating microbial activity and diversity, and microbial communities from hotter environments are typically better adapted to higher temperature (McKinley and Vestal, 1984; Rannekleiv and Bååth, 2001; Petterson and Bååth, 2006). Nonetheless, results from thermal sites are consistent with previous results from temperate sites where temperature was not a primary factor structuring the CO-oxidizing members of the community (Chapter 2).

Activity in the mesophilic range is particularly surprising for the Puhimau soils which consistently exceed temperatures > 70 °C. Thermotolerant and spore-forming bacteria may become active during short-term low temperature incubation. Activity might also be attributed to
thermophilic species with broad temperature ranges of activity that include mesophilic temperatures. Net CO consumption at 80 °C for one Puhimau tree soil sample represents the highest recorded temperature for aerobic CO uptake to date for any material or culture. Further, it is possible that some CO oxidation occurred at higher temperatures for other samples but was masked when CO production exceeded CO consumption since only net CO concentration was measured. Collectively, this study shows that thermophilic CO oxidation occurs in natural environments and extends the known upper temperature limit for biological CO oxidation.
CHAPTER 4.
ISOLATION AND CHARACTERIZATION OF NOVEL THERMOPHILIC CO-
OXIDIZING BACTERIA

Introduction

All previous attempts to isolate aerobic CO oxidizers specifically have yielded Proteobacteria or Actinobacteria (King and Weber, 2007) with the exception of Bacillus *schegelii*, a Firmicute (Kruger and Meyer, 1984). Thermophilic CO-oxidizing isolates are limited to *B. schegelii*, *Pseudomonas thermocarboxydovorans* (Meyer and Schlegel, 1979), and four *Streptomyces* species (Gadkari *et al.*, 1990; Kim *et al.*, 1998; Kim and Goodfellow, 2002). All of these thermophiles were isolated as carboxydrotrophs, and *Streptomyces thermoautotrophicus* represents the only known obligately chemolithotrophic carboxydrotroph (Hugeldieck and Meyer, 1992). The first indication that CO oxidizers occupy more phyla followed the recent publication of several genomes which resulted in the discovery of *cox* operons in a surprising number of species, many of which were thermophiles (Chapter 1). *cox* genes, which encode CODH, are present in species from the phyla Chloroflexi, Bacteriodetes, Thermus-Deinococcus, and even the domain Archaea. Currently, thermophilic CO oxidizer diversity is phylum-rich, but species-poor.

CO uptake at elevated temperature has been documented for artificially- and naturally-heated material from volcanic sites (Chapters 2 and 3), but the microorganisms responsible for this activity are unknown. Previous culture-based approaches in temperate systems resulted in the isolation of several novel CO-oxidizing Proteobacteria and Actinobacteria and greatly expanded knowledge of CO-oxidizing communities (King, 2003). Therefore, similar approaches in thermal systems should expand our knowledge of CO uptake dynamics at these sites and increase the known diversity of thermophilic CO oxidizers.
This study sought to isolate and characterize thermophilic CO oxidizers responsible for CO consumption at geothermal sites (Chapter 3) and for artificially-heated material (Chapter 2). The capacity for CO oxidation by 31 newly isolated thermophilic CO-oxidizing strains in 8 genera is described. Two of these strains were also formally described as novel Chloroflexi isolates, *Thermomicrobium carboxidovorans* sp. nov. str. K13 and *Thermogemmatispora carboxidovorans* sp. nov. str. PM5. The isolation of three *Thermogemmatispora* strains in this study prompted analysis of potential CO uptake capacity of related Ktedonobacteria, since members of this group are widely distributed and might contain a multitude of new thermophilic CO oxidizers. The metabolic characterization of strain PM5 additionally provided insights into the functional role of *Thermogemmatispora* in Puhimau biofilms.

**Materials and Methods**

**Sites and Sampling**

Soils and microbial biofilms were aseptically collected from the Puhimau geothermal area and Kilauea Iki Crater (Chapter 3), transferred to Whirl-Pak bags, and shipped to LSU without temperature control. Samples were processed immediately upon arrival. Canopy soil and Bare cinders from the Pu’u Puai volcanic deposit described in Chapter 2 were aseptically collected, transferred to Whirl-Pak bags, and shipped to a laboratory at Louisiana State University (LSU). Canopy soil was collected from the upper 2 cm of soil beneath the leaf litter layer; fine roots and rocks were removed by sifting soil through a 2 mm sieve. Canopy soil was incubated at 55 °C for 30 d as described in Chapter 2. Bare cinders were incubated at 55 °C for 2 months. CO (100 ppm) was added to the headspace of Canopy and Bare samples after 1 and 2 months, respectively. CO uptake was measured by taking headspace subsamples periodically and determining CO concentrations using a gas chromatograph (RGA3; Trace
Analytical Laboratories, Muskegon, MI, USA) as described previously (King, 1999b). Samples that consumed CO were selected for enrichment of thermophilic CO oxidizers.

**CO oxidizer Enrichment and Isolation**

Initial slurries were created by adding 1 gfw sample material to 9 ml pre-warmed media in 60 cm³ serum bottles and vortexing. Samples were then serially diluted to 10⁻⁸ in the same media in 30 cm³ test tubes and incubated at 55 °C or 70 °C with shaking (100 rpm). CO (100 ppm) was added to the headspace of each dilution and CO uptake was monitored over time. The greatest dilution which consumed CO was plated onto appropriate media solidified with gellan gum (Phytagel; Sigma Aldrich, St. Louis, MO, USA) instead of agar based on the recommendations of Janssen et al., 2002 and Stott et al., 2008. Plates were wrapped with DuraSeal (Diversified Biotech, Dedham, MA, USA) and incubated at 55 °C or 70 °C. Individual colonies with distinct morphotypes were selected, inoculated into corresponding liquid media, and screened for CO uptake. Enrichments that oxidized CO were replated as necessary to obtain pure cultures.

Canopy soil enrichments were created using DSM medium 1033 (1033; DSMZ, 2012) and incubated at 55 °C. Bare cinder enrichments were created in DSM medium 592 (592; DSMZ, 2012) and incubated at 55 °C. Both DSM 1033 and DSM 592 are complex media containing yeast extract and tryptone in a mineral salts solution. Puhimau biofilms were enriched at 55 °C in Pyruvate Plate Count medium (PPC), Thermomicrobium Cellulose medium (TC), and medium VL55, a oligotrophic low pH (pH 5.5) medium containing an amino acid mixture described by Schoenborn et al. (2004). Puhimau tree stump soil enrichments were created at 55 °C and 70 °C using medium PPC and medium TC. Puhimau unvegetated soils were enriched in PPC, TC, 592, and Cox Succinate medium (CS) at 55 °C and 70 °C. Kilauea Iki
biofilms were enriched in PPC, TC, and CS at 55 °C and 70 °C. Medium PPC is an oligotrophic complex medium that has the following composition per L deionized H₂O: 0.1 g pyruvate, 0.5 g peptone, and 0.25 g yeast extract (pH 7.0). Medium TC had the same composition as medium 592 with tryptone omitted and 5 g/L microcrystalline cellulose added (pH 6.5). Medium CS is a mineral salts medium used previously in CO oxidizer isolations (Hardy and King, 2001) amended with succinate. Medium CS contained the following composition per L deionized H₂O: 1.5 g NH₄Cl, 0.2 g MgSO₄·7H₂O, 0.02 g CaCl·2H₂O, 3.55 g Na₂HPO₄, 1.5 g KH₂PO₄, 1 mg ZnSO₄·7H₂O, 0.035 mg MnCl₂·4H₂O, 0.35 mg H₃BO₃, 0.2 mg CoCl₂·6H₂O, 0.01 mg CuCl₂·2H₂O, 0.02 mg NiCl₂·6H₂O, 0.9 mg Na₂MoO₄·2H₂O, 0.02 mg Na₂SeO₄, 1.2 mg ferric ammonium citrate, 0.1 g yeast extract, and 25 mM succinate (pH 6.5).

**Determination of Maximum Potential CO Uptake Rates**

For CO uptake assays, strain CPP55 was grown in DSM medium 13 (13, pH 4.2; DSMZ, 2007) and strains PS3, KI7, and KI8 were grown in medium CS; all other strains were cultivated in medium 592. Turbid cultures were harvested by centrifugation and cell pellets were washed and resuspended in a minimal version of medium 592, except for isolate CPP55 which was resuspended in a minimal version of medium 13. To produce a minimal version of medium 592 (592-min), yeast extract and tryptone were omitted. To produce a minimal version of medium 13 (13-min), yeast extract and glucose were omitted. Aliquots (5 ml) of washed cells were transferred to triplicate 60 cm³ sterile test tubes and sealed with gastight neoprene stoppers. Cultures were incubated at 70 °C with shaking (100 rpm) for strains KI2 and PSS8 and at 65 °C for strain KI3. All other isolates were incubated at 55 °C. CO (100 ppm) was added to the headspace of all samples.
Headspace subsamples were taken at intervals using a sterile needle and syringe and analyzed by gas chromatography (Trace Analytical Laboratories) as described previously (King, 1999b). Data were recorded and areas of CO peaks were analyzed using PowerChrom Software (eDAQ, Inc., Colorado Springs, CO, USA). After completing assays, cells were harvested by centrifugation (25 °C, 15,000 x g), and cell pellets were lysed in a 5% SDS, 0.1 M NaOH solution with sonication (20 s) and incubation at 95 °C (15 min). Culture protein content was determined using a Pierce bicinchoninic acid protein assay kit (Thermo Scientific, Rockford, IL, USA) and CO uptake rates were expressed as units per mg protein.

Some species related to isolates, including Alicyclobacillus macrosporangiiidus DSM 17980, Thermomicrobium roseum DSM 5159, and Meiothermus ruber DSM 1279, were obtained from the German Resource Center for Biological Material DSMZ (Braunschweig, Germany) and assayed for CO uptake as described.

16S rRNA and coxL Gene Amplification and Sequencing

Cells were harvested from turbid cultures via centrifugation and the media was removed. DNA was extracted from cell pellets using either the QIAamp DNA Micro Kit (QIAGEN, Venlo, Netherlands) according to the user developed protocol for bacterial cells (QIAGEN, 2010) or the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA concentrations were measured on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The 16S rRNA gene was amplified using the PCR primers 27f and 1492r (Sambrook et al., 1989; Lane, 1991). PCR reactions were carried out in 25 µl reactions containing 12.5 µl GoTaq Green Master Mix (Promega, Madison, WI, USA) and 0.4 µM of each primer. Template concentrations were varied according to DNA concentrations. Amplification was carried out in an Eppendorf Mastercycler thermocycler.
(Brinkmann Inc., Westbury, NY, USA) using the following program: (1) initial denaturation at 94 °C for 3 min, (2) 27 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 1 min, elongation at 72 °C for 2 min, and (3) final elongation at 72 °C for 10 min. Amplicons were visualized and compared to a 100 bp DNA ladder (Promega) using gel electrophoresis in 1.2% (w/v) agarose gels stained with Gel Red (Biotium, Inc., Hayward CA, USA). The desired PCR product had a size of ~1500 bp.

The coxL gene was amplified using the PCR primers SOf (5′-GGCGGCTT[C/T]GG[C/G]AA[C/G]AAGGT-3′) and PSr (5′-[C/T]TCGA[T/C]GATCATCGG[A/G]TTGA-3′) (King, 2003). PCR reactions were carried out as described above using the following thermocycler program: 1) initial denaturation at 94 °C for 3 min, 2) 35 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 1 min, elongation at 72 °C for 2 min and (3) final elongation at 72 °C for 10 min. Amplicons were visualized as described above; the desired PCR product had a size of 1260 to 1290 bp.

PCR products of the proper size were purified using a MoBio UltraClean PCR Clean-up Kit (MoBio Laboratories), and sequenced bidirectionally using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the LSU Genomics Facility. Bidirectional 16S rRNA and coxL sequence reads were assembled and edited using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). coxL sequences were translated into predicted amino acid sequences using the ExPASy Translate Tool (Swiss Institute of Bioinformatics).

**Phylogenetic Analyses**

16S rRNA gene sequences of isolates were compared against the NCBI GenBank database to find related phylogenetic neighbors. 16S rRNA gene alignments were created using
ClustalW (Thompson et al., 1994) in MEGA5.1 (Tamura et al., 2011). Maximum-likelihood and neighbor-joining phylogenetic trees were created in MEGA5.1 using 100 and 1000 bootstrap replicates, respectively. 16S rRNA gene sequences of isolates were also aligned to the SILVA 16S rRNA database and taxonomically classified using SINA v1.2.11 (Pruesse et al., 2012).

coxL sequences were translated into predicted amino acid sequences and aligned to reference sequences using Muscle (Edgar, 2004) in MEGA5.1. Reference coxL sequences were obtained from the Integrated Microbial Genome and NCBI databases. Form II coxL sequences from *Rhodobacter sphaeroides* (YP_352939), *Mesorhizobium loti* (BAB48572), and *Ruegeria pomeroyi* DSS-3 (AAV94806) were used as an outgroup. Maximum-likelihood and neighbor-joining phylogenetic trees were created in MEGA5.1 using 100 and 1000 bootstrap replicates, respectively.

**Physiological and Biochemical Characterization of Strains PM5, KI3, and KI4**

Colony characteristics of strains were determined after growth for 4 d on medium 592 solidified with Phytagel at 55 °C for PM5 and 65 °C for strains KI3 and KI4. Cell morphology was examined with a Zeiss Axioscope fitted with a Neofluar 100X objective and an AxioCam MR digital camera. Gram staining was performed using standard methods (Smibert and Krieg, 1994).

Optimal pH for growth was determined by cultivating strains in medium 592 prepared with a pH range of 3.0–10.0 (55 °C PM5; 65 °C KI3 and KI4). Growth was assessed using medium 592 buffered at pH 3.1–6.5 with acetic acid and sodium acetate, at pH 7.0–9.0 with Na₂HPO₄ and HCl, and at pH 10.0 with Na₂HPO₄ and NaOH (0.1 M for all). Growth was also assessed using medium 592 buffered at pH 4.1–4.5 with pivalic acid, at pH 5.0–6.6 with 2-(N-morpholino)ethanesulfonic acid, at pH 7.1–8.5 with Tris, and at pH 9.1–9.5 with sodium
bicarbonate (0.1 M for all). Temperature optima were assessed using cultures grown at temperatures from 30–80 °C at pH 6.5. Optical density determined spectrophotometrically at 600 nm was used to measure growth of strains KI3 and KI4. Growth of strain PM5 was determined by measuring cell protein content of subsamples at over time. Cells were harvested by centrifugation (25 °C, 15,000 x g) and lysed as described previously. Protein content was determined using a Pierce bicinchoninic acid protein assay kit (ThermoScientific). All assays were conducted in duplicate.

Catalase activity was determined by pipetting a 3% H₂O₂ solution onto cells on a microscope slide and observing the formation of oxygen bubbles. API 20 NE test strips (BioMerieux Inc., France) were used to assess additional biochemical traits of strains according to manufacturer specifications.

Additionally, oxygen requirements of strain PM5 were assessed using sealed 60 cm³ serum bottles containing 5 ml of a basal salts media supplemented with 0.01% yeast extract and 25 mM glucose. Microaerophilic conditions were established by flushing the serum bottles with N₂ (10 min); subsequently, 7.85 cm³ headspace was removed and replaced with filtered ambient air to produce ~3% O₂. Anaerobic bottles were flushed with N₂ for 10 min. Nitrogen sources supporting growth were determined by monitoring growth as described on minimal media containing 25 mM glucose as a carbon source with the addition of 25 mM NH₄Cl, 25 mM KNO₃, or no added nitrogen. PM5 cells were washed in nitrogen-limited media prior to inoculating nitrogen assay bottles. All assays were conducted in duplicate and growth was measured as previously described.
Substrate Utilization by Strains PM5, KI3, and KI4

Utilization of various carbon sources for growth was assessed at 55 °C (PM5) or 65 °C (KI3 and KI4) with a basal salts medium supplemented with 0.01% yeast extract and 25 mM final concentrations of the following substrates: propionate, glycolate, malonate, tartrate, lactate, gluconate, acetate, fumarate, malate, pyruvate, succinate, citrate, glycerol, mannitol, inositol, glycine, alanine, valine, proline, glutamate, aspartate, serine, methylvamine, dimethylamine, trimethylamine, betaine, arabinose, fructose, glucose, galactose, mannose, ribose, lactose, sucrose, xylose, rhamnose, glucuronate, galacturonate, methanol, ethanol, isopropanol, and acetone. Controls contained 0.01% yeast extract only. Growth of PM5 was assessed after 4 d as described above. Growth of strains KI3 and KI4 was determined at intervals for 5 d as described above.

GENIII BioLog microplates (BioLog, Inc., Hayward, CA, USA) were used to compare carbon metabolism of strains to their closest phylogenetic neighbors. *Thermogemmatispora onikobensis* JCM 16817T was obtained from the Japan Collection of Microorganisms. *Thermomicrobium roseum* DSM 5159T was obtained from the German Resource Center for Biological Material DSMZ. BioLog plates were inoculated with cultures that were initially grown in medium 592, harvested by centrifugation, washed and resuspended in inoculating fluid A at an optical density of about 0.1 at 600 nm. Plates were wrapped with DuraSeal, placed in zip-top bags, and incubated at 55 °C (PM5 and *T. onikobensis* JCM 16817T) or 65 °C (KI3, KI4, and *T. roseum* DSM 5159T). Color development at 590 nm was determined at intervals using a Gen5 Microplate reader (Biotek Instruments, Winooski, VT, USA).

Polymer hydrolysis by strain PM5 was evaluated on a basal salts medium solidified with agar supplemented with 0.5% (w/v) of gelatin, casein, cellulose, starch, chitin, or xylan. Agar
was used in place of Phytagel since pitting around PM5 colonies was routinely observed on Phytagel media indicating that strain PM5 was capable of hydrolyzing gellan gum.

**Chemotaxonomic Analyses of Strain PM5**

DNA base composition (\% mol G+C) of strain PM5 was carried out by the Identification Service of the DSMZ (Braunschweig, Germany) following the procedure described by Mesbah et al. (1989). RNA-free genomic DNA was hydrolyzed and the nucleotides dephosphorylated to produce nucleosides which were analyzed by high pressure liquid chromatography (Tamaoka & Komagata, 1984). Fatty acid extraction and analyses were carried out by the DSMZ Identification Service using standard extraction methods (Miller, 1982; Kuykendall et al., 1988) and the standard protocol of the Sherlock Microbial Identification System (MIDI Inc., Newark, DE, USA). DNA-DNA hybridizations between strain PM5 and *T. onikobensis* JCM 16817\(^T\) were also carried out by DSMZ according to standard methods (De Ley et al., 1970; Cashion et al., 1977; Huss et al., 1983).

**CO Oxidation by Ktedonobacteria**

*Ktedonobacter racemifer* SOSP1-21 (= DSM 44963) was obtained from the German Resource Center for Biological Material DSMZ, and *Thermosporothrix hazakensis* SK20-1 (= JCM 16142) was obtained from the Japan Collection of Microorganisms. Strains T12, T26, T81, T104, P352, P359, P497, and OLθ16, isolated from geothermal soils in the Taupo Volcanic Zone of New Zealand (Stott et al., 2008), were provided by Dr. M. Stott (Wairakei Research Centre, Taupo, New Zealand). These isolates were selected for CO oxidation assays and *coxL* PCR screening based on their 16S rRNA gene sequence similarity to strain PM5. DNA was extracted from strains and the *coxL* gene amplified as described previously.
For CO uptake assays of Ktedonobacteria strains, isolates were initially grown in medium 592 (pH 6.5) in gastight serum bottles containing a 100 ppm CO headspace. All isolates were cultivated at 55 °C in a shaking incubator (100 rpm), except for *K. racemifer* DSM 44963 which was grown with shaking at 30 °C based on the reported temperature optimum (Cavaletti *et al.*, 2006). For CO uptake assays, 3 ml aliquots of washed cells resuspended in medium 592-min were transferred to triplicate 30 cm³ sterile test tubes and sealed with gastight neoprene stoppers. Test tubes were incubated with shaking (100 rpm) at a 45° angle to maximize gas exchange. CO (100 ppm) was added to the sample headspace, CO concentrations were determined at intervals, and culture protein content was determined as described above. *K. racemifer* DSM 44963 was also screened for its ability to oxidize CO in DSM medium 65 (DSMZ, 2007), medium 592, mineral salts media supplemented with 1% (w/v) Phytagel, and under microaerophilic conditions. Isolates T12, T104 and P352 were additionally screened for CO oxidation in medium 592 and mineral salts media supplemented with 1% (w/v) Phytagel.

To determine the capacity of isolates to grow with CO as a sole carbon and energy source, 160 cm³ serum bottles containing 9.5 ml of medium 592-min were inoculated with 0.5 ml of washed cells. Bottles were sealed with gas tight stoppers, and 30% CO and 5% CO₂ was added to the headspace. Controls did not receive CO or CO₂ additions to headspaces. All cultures were incubated with shaking (100 rpm) at 55 °C except for *K. racemifer* DSM 44963 which was incubated at 30 °C. Growth was monitored by periodically measuring absorbance at 600 nm. For strain PM5 and *T. onikobensis* JCM 16817ᵀ, 1 ml subsamples were harvested by centrifugation and protein concentrations were determined as described.
Results

Phylogenetic Analyses

A total of 31 thermophilic CO-oxidizing isolates were obtained; however several strains were redundant and only 13 isolates had unique 16S rRNA gene sequences (Table 4.1). All

Table 4.1. Bacteria isolated from artificially-heated volcanic deposits and geothermally-heated biofilms and soils with identification of the closest described neighbor and CO oxidation rates. When multiple isolates are given, strains have identical 16S rRNA sequences and a representative CO uptake rate is listed. CO uptake rates are expressed as CO nmol h\(^{-1}\) [mg protein\(^{-1}\)]. Values in parentheses represent ± 1 s.e.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Isolation Medium</th>
<th>Closest type species (% 16S rRNA identity)</th>
<th>CO rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pu‘u Puai Bare cinders</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BPP55</td>
<td>592</td>
<td>*Thermogemmatispora onikobensis ONI-1 (98.2)</td>
<td>24 (3)</td>
</tr>
<tr>
<td>Pu‘u Puai Canopy soil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPP55</td>
<td>1033</td>
<td>*Alicyclobacillus macrorosporangiidus 5A239-2O-A (98.8)</td>
<td>16 (2)</td>
</tr>
<tr>
<td>Puhimau biofilms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PM1</td>
<td>TC</td>
<td>*Brevibacillus thermoruber DSM 7064 (98.8)</td>
<td>7.42 (0.05)</td>
</tr>
<tr>
<td>PM2</td>
<td>VL55</td>
<td>*Geobacillus subterraneus 34 (98.4)</td>
<td>*</td>
</tr>
<tr>
<td>PM5</td>
<td>VL55</td>
<td>*Thermogemmatispora onikobensis ONI-1 (98.4)</td>
<td>10 (1)</td>
</tr>
<tr>
<td>PM6</td>
<td>VL55</td>
<td>*Thermogemmatispora onikobensis ONI-1 (97.9)</td>
<td>7 (1)</td>
</tr>
<tr>
<td>Puhimau tree stump soil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSS3</td>
<td>TC</td>
<td>*Alicyclobacillus contaminans NBRC 103102 (99.5)</td>
<td>11.9 (0.5)</td>
</tr>
<tr>
<td>PSS4</td>
<td>TC</td>
<td>*Brevibacillus thermoruber DSM 7064 (99.8)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>PSS2</td>
<td>TC</td>
<td>*Geobacillus subterraneus 34 (98.8)</td>
<td>21.5 (0.6)</td>
</tr>
<tr>
<td>PSS1, PSS9, PSS10, PSS11</td>
<td>PPC, TC</td>
<td>*Geobacillus vulcani 35-1 (100)</td>
<td>4.2 (0.1)</td>
</tr>
<tr>
<td>PSS6, PSS7</td>
<td>PPC, TC</td>
<td>*Meiothermus ruber DSM 1279 (100)</td>
<td>14.5 (0.6)</td>
</tr>
<tr>
<td>PSS8</td>
<td>TC</td>
<td>*Thermus scotoductus SA-01 (99.1)</td>
<td>6 (1)</td>
</tr>
<tr>
<td>Puhimau unvegetated soil</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS1</td>
<td>VL55</td>
<td>*Alicyclobacillus contaminans NBRC 103102 (99.6)</td>
<td>*</td>
</tr>
<tr>
<td>PS2</td>
<td>TC</td>
<td>*Anoxybacillus tepidamans GS5-97 (98.4)</td>
<td>3.5 (0.05)</td>
</tr>
<tr>
<td>PS3, PS5, PS6, PS7, PS8</td>
<td>592, CS</td>
<td>*Brevibacillus thermoruber DSM 7064 (99.8)</td>
<td>17 (2)</td>
</tr>
<tr>
<td>PS4, PS9</td>
<td>592, TC</td>
<td>*Meiothermus ruber DSM 1279 (100)</td>
<td>16.5 (0.6)</td>
</tr>
<tr>
<td>Kilauea Iki biofilms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KI5</td>
<td>TC</td>
<td>*Brevibacillus thermoruber DSM 7064 (99.8)</td>
<td>7.1 (0.6)</td>
</tr>
<tr>
<td>KI8, KI9</td>
<td>CS, TC</td>
<td>*Meiothermus ruber DSM 1279 (100)</td>
<td>5.9 (0.2)</td>
</tr>
<tr>
<td>KI2</td>
<td>CS</td>
<td>*Thermus scotoductus SA-01 (99.6)</td>
<td>1.9 (0.1)</td>
</tr>
<tr>
<td>KI7</td>
<td>TC</td>
<td>*Thermus scotoductus SA-01 (99.1)</td>
<td>*</td>
</tr>
<tr>
<td>KI3</td>
<td>TC</td>
<td>*Thermomicrobium roseum P2 (96.0)</td>
<td>6.7 (0.8)</td>
</tr>
</tbody>
</table>

*Strain consumes CO, but CO oxidation rate was not quantified since isolate shares > 99% 16S rRNA sequence identity to another listed strain.
isolates belonged to one of three phyla: Chloroflexi (Figure 4.1), Deinococcus-Thermus (Figure 4.2), or Firmicutes (Figure 4.3). The type of media used had no observable effect on cultivation of different organisms, and most isolates subsequently grew well in medium 592. All isolates were closely related to described species (> 98% 16S rRNA similarity) with the exception of strain KI3. A single spore-forming isolate was obtained from artificially-heated Bare cinders (Thermogemmatispora str. BPP55) and Canopy soil (Alicyclobacillus str. CPP55). Firmicutes, including Alicyclobacillus, Brevibacillus, Geobacillus, and Anoxybacillus sp., were isolated from all geothermal sites and dominated the isolates cultivated (Table 4.1; Figure 4.3); however, Brevibacillus was the only genus common to all geothermal sites. Thermogemmatispora were enriched from the Puhimau biofilms but not Puhimau soils. Meiothermus strains with identical 16S rRNA sequences were cultivated from both Puhimau soils and Kilauea Iki biofilms.

A novel organism (strain KI3) which shared 96% 16S rRNA similarity with Thermomicrobium roseum DSM 5159 was isolated from Kilauea Iki biofilms at 70 °C. An additional isolate (strain KI4) was enriched from the Kilauea Iki biofilms but did not oxidize CO. Strain KI4 shared 92% 16S rRNA similarity with Thermomicrobium roseum DSM 5159. 16S rRNA gene sequences from all isolates were deposited into GenBank under accession numbers KF193526 - KF193558 (pending approval). Representative isolates were deposited into the Leibniz-Institut DSMZ German Collection of Microorganisms and Cell Cultures (CPP55 = DSM 26552; PM1 = DSM 26550; PM5 = DSM 45816; PSS1 = DSM 26695; PSS2 = DSM 26696; PS2 = DSM 26645; KI2 = DSM 26573; KI3 = DSM 27067; KI4 = DSM 27169).
**Figure 4.1.** Taxonomy of isolates belonging to the phylum Chloroflexi based on 16S rRNA gene phylogeny generated using a neighbor-joining method with 1000 bootstrap replicates. *Bacillus subtilis* (AJ277905), *Streptomyces arenae* (AJ399485), *Thermus thermophilus* (X07998), *Thermotoga maritima* (M21774), and *Aquifex pyrophilus* (M83548) were used as outgroup sequences. Numbers at nodes indicate bootstrap support.
Figure 4.2. Taxonomy of isolates belonging to the phylum Thermus-Deinococcus based on 16S rRNA gene phylogeny generated using a neighbor-joining method with 1000 bootstrap replicates. *Bacillus subtilis* (AJ277905), *Streptomyces arenae* (AJ399485), *Thermotoga maritima* (M21774), and *Aquifex pyrophilus* (M83548) were used as outgroup sequences. Numbers at nodes indicate bootstrap support.
CO Oxidation

CO oxidation was documented for the first time in the genera *Alicyclobacillus*, *Anoxybacillus*, *Brevibacillus*, *Geobacillus*, *Meiothermus*, *Thermogemmatispora*, and *Thermus*. All isolates consumed CO at elevated temperatures (≥ 55 °C). CO uptake rates were generally similar among isolates (Table 4.1), with the exception of notably low rates for *Thermus* str. KI2 (1.9 CO nmol h⁻¹ [mg protein]⁻¹) and high rates for *Thermogemmatispora* str. BPP55 and *Geobacillus* str. PSS2 (24.0 and 21.5 CO nmol h⁻¹ [mg protein]⁻¹, respectively). CO oxidation was also confirmed for related previously described species including *A. macrosporangiidus* DSM 17980, *M. ruber* DSM 1279, and *T. roseum* DSM 5159.

The *coxL* gene was amplified and sequenced from *A. macrosporangiidus* DSM 17980, *Alicyclobacillus* str. CPP55, *Thermogemmatispora* strains BPP55, PM5 and PM6, and *Meiothermus* strains PSS6, PS4, and KI9 (Figure 4.4). Numerous attempts to amplify *coxL* from CO-oxidizing *Thermomicrobium*, *Thermus*, *Geobacillus*, *Brevibacillus*, and *Anoxybacillus* isolates were unsuccessful. All *coxL* sequences from isolates clustered within a polyphyletic clade containing predominantly thermophilic sequences (Figure 4.4). Within this clade, the *coxL* topology of isolates was mostly congruent with overall 16S rRNA gene phylogeny (Figure A.1). *coxL* gene sequences from isolates were deposited into GenBank under accession numbers KF193559 - KF193565 (pending approval).
Figure 4.3. Taxonomy of isolates belonging to the phylum Firmicutes based on 16S rRNA gene phylogeny generated using a neighbor-joining method with 1000 bootstrap replicates. *Thermus thermophilus* (X07998), *Thermotoga maritima* (M21774), and *Aquifex pyrophilus* (M83548) were used as outgroup sequences. Numbers at nodes indicate bootstrap support.
Figure 4.4. Inferred amino acid coxL phylogeny generated using a maximum-likelihood based method with 100 bootstrap replicates. Form II coxL sequences from *Rhodobacter sphaeroides* (YP_352939), *Mesorhizobium loti* (BAB48572), and *Ruegeria pomeroyi* DSS-3 (AAV94806) were used as outgroup sequences. Numbers at nodes indicate bootstrap support.
Morphology and Physiology of Strain PM5

Strain PM5 formed flat orange colonies embedded within the Phytagel. Cells stained Gram-positive, were non-motile, and formed mycelia of irregularly branched flexible filaments of indeterminate length (Figure 4.5, image a). Strain PM5 produced multiple exospores per cell via budding (Figure 4.5, image b). Strain PM5 formed orange irregular spherical aggregates in liquid culture. Detailed physiological characteristics of PM5 are given in Table 4.2. Strain PM5 grew at temperatures from 40–65 °C (optimum 55 °C) and pH 4.1–8.0 (optimum pH 6.0). Strain PM5 grew similarly well under aerobic and microaerophilic conditions, but anaerobic growth was not observed (Figure A.8).

Biochemical Characterization of Strain PM5

Strain PM5 grew better on ammonia than nitrate (Figure A.9), but also grew slowly under nitrogen-limited conditions (Figure A.10). *Thermogemmatispora* str. PM5 grows with gluconate, pyruvate, glycerol, alanine, glutamate, aspartate, glucose, mannose, lactose, sucrose, methanol, ethanol, and acetone. Strain PM5 does not grow with propionate, malonate, tartrate, lactate, acetate, fumarate, malate, succinate, mannitol, glycine, valine, proline, serine, methylamine,
dimethylamine, trimethylamine, betaine, galacturonate, isopropanol, glycolate, citrate, inositol, arabinose, fructose, galactose, ribose, xylose, rhamnose, or glucuronate. API test results revealed that strain PM5 respired nitrate and was β-glucosidase and β-galactosidase positive. Strain PM5 utilized all polymers tested as evidenced by colonies with clear zones of hydrolysis. Differences in GENIII substrate utilization of strain PM5 and T. onikobensis JCM 16817 are listed in Table 4.3. Notably, strain PM5 utilized more organic acids (N-acetyl-D-glucosamine, p-hydroxyphenyl acetic acid, methyl pyruvate, D-glucuronic acid, citric acid, D-malic acid, L-malic acid, and bromosuccinic acid) and amino acids (L-alanine, L-aspatic acid, and L-glutamic acid) than T.onikobensis JCM 16817.

**Chemotaxonomic and 16S rRNA Phylogenetic Analyses of PM5**

The major fatty acids of strain PM5 included iso-C17:0 (34.01%), iso-C19:0 (22.98%) and C20:0 (23.60%) (Table 4.4). The G+C content of strain PM5 was 59.0 mol% G+C. DNA-DNA relatedness between strain PM5 and T. onikobensis JCM 16817 was low at 46.4%. Based on 16S rRNA gene sequence phylogenetic analyses, strain PM5 clustered strongly with other members of the order Thermogemmatisporales (Figure 4.1) within the class Ktedonobacteria. When aligned to the SILVA database using SINA, which considers 16S rRNA secondary structure, strain PM5 was classified down to genus level, Thermogemmatispora, and the nearest neighbor was T. onikobensis ONI-1 (96.93% identity).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain PM5</th>
<th><em>T. onikobensis</em> ONI-1</th>
<th><em>T. foliorum</em> ONI-5</th>
<th><em>T. hazakensis</em> SK20-1</th>
<th><em>K. racemifer</em> SOSPI-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO uptake rate (CO nmol h⁻¹ [mg protein]⁻¹)</td>
<td>10 (2)</td>
<td>4.7 (0.8)</td>
<td>nd</td>
<td>6.3 (0.2)</td>
<td>nd</td>
</tr>
<tr>
<td>Colony color</td>
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<td>White</td>
<td>Orange</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>59</td>
<td>60.2</td>
<td>58.1</td>
<td>54</td>
<td>53.9</td>
</tr>
<tr>
<td>Temperature (°C):</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Optimum</td>
<td>55</td>
<td>60–65</td>
<td>60–65</td>
<td>50</td>
<td>28–33</td>
</tr>
<tr>
<td>Range</td>
<td>40–65</td>
<td>50–74</td>
<td>45–74</td>
<td>31–58</td>
<td>17–40</td>
</tr>
<tr>
<td>pH:</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>6</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>Range</td>
<td>4.1–8.0</td>
<td>4.6–8.0</td>
<td>4.6–8.0</td>
<td>5.4–8.7</td>
<td>4.2–7.2</td>
</tr>
<tr>
<td>Microaerophilic growth</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>+</td>
</tr>
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<td>Growth with:</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1% NaCl</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>2% NaCl</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>w</td>
</tr>
<tr>
<td>Hydrolysis of:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Gelatin</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Casein</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cellulose</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Chitin</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Xylan</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
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<td>Growth substrates:</td>
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<td></td>
</tr>
<tr>
<td>Mannitol</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Inositol</td>
<td>i</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Arabinose</td>
<td>i</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>Fructose</td>
<td>i</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>Glucose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Xylose</td>
<td>i</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>i</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>nd</td>
</tr>
<tr>
<td>Biochemical traits:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>-</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
**Table 4.3.** GENIII substrate utilization profiles of strain PM5 and *T. onikobensis* ONI-1. Substrates not metabolized by either strain are not listed. w = weakly positive reaction.

<table>
<thead>
<tr>
<th>GEN III substrates:</th>
<th>Strain PM5</th>
<th><em>T. onikobensis</em> ONI-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectin</td>
<td>w</td>
<td>-</td>
</tr>
<tr>
<td>tween 80</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>D-maltose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-melibiose</td>
<td>w</td>
<td>-</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-cellobiose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3-methyl glucose</td>
<td>-</td>
<td>w</td>
</tr>
<tr>
<td>D-glucose-6-PO₄</td>
<td>+</td>
<td>w</td>
</tr>
<tr>
<td>L-fucose</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-fructose-6-PO₄</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Glucuronamide</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-turanose</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>N-acetyl-D-glucosamine</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>p-hydroxyphenylacetic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>methyl pyruvate</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-glucuronic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>citric acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>α-keto-glutaric acid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-malic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-malic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>bromo-succinic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>α-Keto butyric acid</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>glycyl-L-proline</td>
<td>-</td>
<td>w</td>
</tr>
<tr>
<td>L-alanine</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-aspartic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-glutamic acid</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L-histidine</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inosine</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

**CO oxidation in Ktedonobacteria**

CO uptake rates were generally similar among Ktedonobacteria strains (Table 4.5), with the exception of particularly low rates for New Zealand strain P359 (2.7 CO nmol h⁻¹ [mg protein]⁻¹) and high rates for Hawaiian strain BPP55 (24.0 CO nmol h⁻¹ [mg protein]⁻¹).
Variability in rates among isolates was unrelated to phylogenetic relationships. For example, strain BPP55 and strain T81 have identical 16S rRNA gene sequences (Figure 4.2), yet CO uptake rates for strain BPP55 were 5-fold higher than those for strain T81 (Table 4.5). Strain OL016 and strain T12 share 99.7% 16S rRNA gene similarity (Figure 4.2), yet OL016 oxidized CO while strain T12 did not (Table 4.5). Thermogemmatispora strains were also capable of CO oxidation at atmospherically relevant concentrations (Figure A.11).


<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Strain PM5</th>
<th><em>T. onikobensis</em> ONI-1</th>
<th><em>T. foliorum</em> ONI-5</th>
<th><em>T. hazakensis</em> SK20-1</th>
<th><em>K. racemifer</em> SOSPI-21</th>
</tr>
</thead>
<tbody>
<tr>
<td>iso-C₁₅:₀</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.64</td>
<td>2.37</td>
</tr>
<tr>
<td>C₁₅:₀</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.76</td>
</tr>
<tr>
<td>C₁₆:₀</td>
<td>0.63</td>
<td>0.95</td>
<td>1.41</td>
<td>10.03</td>
<td>6.66</td>
</tr>
<tr>
<td>iso-C₁₆:₀</td>
<td>0.37</td>
<td>0.38</td>
<td>0.32</td>
<td>1.14</td>
<td>11.54</td>
</tr>
<tr>
<td>10-Methyl C₁₆:₀</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.26</td>
<td>7.79</td>
</tr>
<tr>
<td>C₁₆:₁</td>
<td>1.01</td>
<td>2.74</td>
<td>0.71</td>
<td>9.39</td>
<td>29.65</td>
</tr>
<tr>
<td>C₁₇:₀</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.76</td>
<td>-</td>
</tr>
<tr>
<td>iso-C₁₇:₀</td>
<td>34.01</td>
<td>44.13</td>
<td>44.87</td>
<td>52.79</td>
<td>25</td>
</tr>
<tr>
<td>anteiso-C₁₇:₀</td>
<td>0.64</td>
<td>0.49</td>
<td>0.72</td>
<td>10.3</td>
<td>9.61</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>5.06</td>
<td>2.55</td>
<td>6.62</td>
<td>7.28</td>
<td>2.01</td>
</tr>
<tr>
<td>iso-C₁₈:₀</td>
<td>8.31</td>
<td>2.19</td>
<td>2.22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C₁₈:₁₀ω₉c</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.71</td>
<td>1.21</td>
</tr>
<tr>
<td>iso-C₁₉:₀</td>
<td>22.98</td>
<td>19.34</td>
<td>27.27</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>anteiso-C₁₉:₀</td>
<td>1.41</td>
<td>0.64</td>
<td>1.29</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C₂₀:₀</td>
<td>23.60</td>
<td>26.55</td>
<td>14.56</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>unknown (ECL 17.08)</td>
<td>0.29</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>unknown (ECL 18.43)</td>
<td>1.69</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The coxL gene was readily amplified and sequenced for *T. onikobensis* JCM 16817, *K. racemifer* DSM 44963, PM5, PM6, BPP55, T26, T81, T104, P352, P359, and P497. *T. hazakensis* JCM 16142 and strain OL016 oxidized CO, but did not yield a coxL PCR product. Of
the 14 strains tested, only one strain (T12) lacked both CO uptake activity and evidence of \( \text{cox} \) genes. Strains P352 and T104 contained \( \text{coxL} \), but no measurable CO uptake was observed.

Notably, \( \text{K. racemifer} \) DSM 44963 also has not oxidized CO under multiple conditions tested, yet it contains all necessary \( \text{cox} \) operon genes. None of the Ktedonobacteria isolates were capable of carboxydotrophic growth.

Table 4.5. CO oxidation rates for strain PM5 and related strains. All rates are means of triplicate. Numbers in parentheses represent ± 1 std. dev.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Rate CO nmols h(^{-1}) [mg protein](^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{T. onikobensis} ) ONI-1</td>
<td>4.7 (0.8)</td>
</tr>
<tr>
<td>( \text{T. hazakensis} ) SK20-1</td>
<td>6.3 (0.2)</td>
</tr>
<tr>
<td>( \text{K. racemifer} ) SOSPI-21</td>
<td>-</td>
</tr>
<tr>
<td>PM5</td>
<td>10 (2)</td>
</tr>
<tr>
<td>PM1</td>
<td>7 (1)</td>
</tr>
<tr>
<td>BPP55</td>
<td>24 (5)</td>
</tr>
<tr>
<td>T26</td>
<td>6.9 (0.6)</td>
</tr>
<tr>
<td>T81</td>
<td>4.5 (0.4)</td>
</tr>
<tr>
<td>T12</td>
<td>-</td>
</tr>
<tr>
<td>T104</td>
<td>-</td>
</tr>
<tr>
<td>P359</td>
<td>2.7 (0.4)</td>
</tr>
<tr>
<td>P497</td>
<td>11 (2)</td>
</tr>
<tr>
<td>P352</td>
<td>-</td>
</tr>
<tr>
<td>OL( \theta )16</td>
<td>8.9 (0.7)</td>
</tr>
</tbody>
</table>

The \( \text{coxL} \) gene was readily amplified and sequenced for \( \text{T. onikobensis} \) JCM 16817, \( \text{K. racemifer} \) DSM 44963, PM5, PM6, BPP55, T26, T81, T104, P352, P359, and P497. \( \text{T. hazakensis} \) JCM 16142 and strain OL\( \theta \)16 oxidized CO, but did not yield a \( \text{coxL} \) PCR product. Of the 14 strains tested, only one strain (T12) lacked both CO uptake activity and evidence of \( \text{cox} \) genes. Strains P352 and T104 contained \( \text{coxL} \), but no measurable CO uptake was observed. Notably, \( \text{K. racemifer} \) DSM 44963 also has not oxidized CO under multiple conditions tested, yet it contains all necessary \( \text{cox} \) operon genes. None of the Ktedonobacteria isolates were capable of carboxydotrophic growth.
The *coxL* predicted amino acid sequences from strain PM5 and other *Thermogemmatispora*-like isolates strongly clustered together (99% bootstrap support) within a monophyletic Ktedonobacteria clade (100% bootstrap support) within a larger polyphyletic cluster of predominantly thermophilic *coxL* sequences (Figure 4.4). Within the *Thermogemmatispora* clade, the *coxL* topology was mostly congruent with 16S rRNA gene phylogeny. *coxL* amino acid sequences were all >95% similar when related to a species definition of 97% 16S rRNA gene similarity (Figure 4.6).

![Figure 4.6](image)

**Figure 4.6.** Relationship between Ktedonobacteria *coxL* inferred amino acid sequence similarities and 16S rRNA gene similarities.

**Morphological, Physiological, and Biochemical Characterization of Strains KI3 and KI4**

Strain KI3 formed small round off-white colonies, while strain KI4 formed tiny round semi-translucent pink colonies. Cells of both strain KI3 and KI4 were Gram-positive, non-sporeforming, non-motile, and rod-shaped (0.9 µM width, 2.0–2.5 µM length) with rods often found in pairs. Detailed physiological characteristics of KI3 and KI4 are given in Table 4.6.
Strain KI3 grew at temperatures from 50–70 °C (optimum 70 °C) and pH 6.5–9.5 (optimum pH 7.0). Strain KI4 grew at temperatures from 55–70 °C (optimum 65 °C) and pH 5.8–8.0 (optimum pH 6.7–7.1). Strain KI3 grew in the presence of up to 2% NaCl; strain KI4 did not grow with 1% NaCl.

Table 4.6. Characteristics of strain KI3 and KI4. Data for *T. roseum* P2 (= DSM 5159) derived from Jackson *et al.* 1973. nd = not determined. Numbers in parentheses represent ± 1 s.e.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Strain KI3</th>
<th>Strain KI4</th>
<th><em>T. roseum DSM 5159</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>CO uptake rate (CO nmol h⁻¹ [mg protein]⁻¹)</td>
<td>6.7 (0.8)</td>
<td>-</td>
<td>4.1 (0.3)</td>
</tr>
<tr>
<td>Colony color</td>
<td>offwhite</td>
<td>pink</td>
<td>Pink</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>nd</td>
<td>nd</td>
<td>64</td>
</tr>
<tr>
<td>Temperature (°C):</td>
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<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>70</td>
<td>65</td>
<td>70–75</td>
</tr>
<tr>
<td>Range</td>
<td>50–70</td>
<td>55–70</td>
<td>45–80</td>
</tr>
<tr>
<td>pH:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td>7.0</td>
<td>6.7–7.1</td>
<td>8.2–8.5</td>
</tr>
<tr>
<td>Range</td>
<td>6.5–9.0</td>
<td>5.8–8.0</td>
<td>7.5–8.7</td>
</tr>
<tr>
<td>Growth with:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% NaCl</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2% NaCl</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Growth on complex media:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2A</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>592</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>0.5% yeast extract</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Biochemical traits:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>-</td>
<td>nd</td>
</tr>
</tbody>
</table>

Biochemical Characterization of Strains KI3 and KI4

Neither strain KI3 or KI4 grew with any of the tested substrates as a sole carbon source. Differences in GENIII substrate utilization of Thermomicrobiales strains are listed in Table 4.7. Substrate utilization profiles were similar for strain KI3 and its closest phylogenetic neighbor *T. roseum* DSM 5159 (Table 4.7). Strain KI3 was differentiated from *T. roseum* DSM 5159 in its utilization of L-galactonic acid lactone and α-hydroxy-butyric acid, although the latter reaction was weak. *T. roseum* DSM 5159 was also weakly positive for a few aminosugars. Strain KI4
differed from other Thermomicrobiales strains in its utilization of D-galactose and D-salicin. CO oxidation rates for strain KI3 and *T. roseum* DSM 5159 were similar (Table 4.6); strain KI4 was unable to consume CO. The *coxL* gene could not be amplified from any of the 3 strains using the primer pair SOf and PSr.

**Table 4.7.** GENIII substrate utilization profiles of Thermomicrobiales isolates. Substrates not metabolized by any strain are not listed. w = weakly positive reaction.

<table>
<thead>
<tr>
<th>GEN III substrate</th>
<th>Strain KI3</th>
<th>Strain KI4</th>
<th>T. roseum DSM 5159†</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-D-glucose</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>D-glucose-6-PO4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-methyl glucose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-fructose</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-fructose-6-PO4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D-mannose</td>
<td>-</td>
<td>w</td>
<td>-</td>
</tr>
<tr>
<td>D-galactose</td>
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**Discussion**

Targeting thermophiles yielded several CO-oxidizing isolates in new phyla previously implicated in CO oxidation only from genome sequences. Thermophilic CO oxidizers were readily isolated from a variety of geothermal sites using traditional isolation techniques.
optimized for this functional group. Culture-based techniques were critical in identifying these organisms since \textit{coxL} genes could not be amplified from several isolates using existing primers. The lack of amplifiable \textit{coxL} from newly recognized CO-oxidizing thermophiles illustrates that less biased molecular methods, such as metagenome sequencing (von Mering \textit{et al}., 2007), should be implemented when possible to get a more accurate picture of CO oxidizer diversity, especially in thermal environments.

Surprisingly, no Proteobacteria or Actinobacteria were isolated despite thermophilic CO oxidizers existing in these phyla. None of the isolates tested were able to grow autotrophically. The isolation method may have enriched carboxydovores over carboxydotrophs due to the use of heterotrophic media and lower CO concentrations (100 ppm). CO oxidation was documented in 8 new genera. \textit{Alicyclobacillus} strain CPP55 grew and oxidized CO preferentially at pH 4 and represents the first documented acidophilic CO oxidizer. Analyses of the closely related strain \textit{A. macrosporangioides} DSM 17980 show that it also actively consumes CO at low pH. \textit{cox} genes were recently discovered in the genomes of two additional \textit{Alicyclobacillus} species (IMG Gene IDs 2523758565 and 2513792691), indicating that CO uptake may be a common trait in this genus. CO oxidation was also documented in isolates with 100\% 16S rRNA similarity to \textit{Meiothermus ruber} DSM 1279 which contains a \textit{cox} operon in its genome (Tindall \textit{et al}., 2010). CO uptake was confirmed for \textit{M. ruber} DSM 1279, and \textit{coxL} sequences from the \textit{Meiothermus} strains were also identical.

The presence of several \textit{Meiothermus} strains with identical 16S rRNA and \textit{coxL} gene sequences from distinct locations in this study and others (Chung \textit{et al}., 1997; Chen \textit{et al}., 2002; Zeng \textit{et al}., 2009), suggest that \textit{M. ruber} is a cosmopolitan species with a possibly high dispersal rate and a more conserved genome. In contrast, CO-oxidizing strains KI2, KI7, and PSS8 share
>99% 16S rRNA identity with *Thermus scotoductus* SA-01, yet the genome of *T. scotoductus* SA-01 does not contain a *cox* operon. The biogeography of *T. scotoductus* might be similar to that of *Sulfolobus islandicus*, a species with little gene flow between geographically isolated populations (Reno *et al.*, 2009). Indeed, *cox* operons were found in the genomes of *S. islandicus* strains from the Yellowstone National Park, but not strains from other locations. Further investigation, including genome sequencing, of these CO-oxidizing isolates might inform comparative analysis of congeneric isolates to help answer biogeographic questions about the global distribution of thermophiles and CO oxidizers.

Novel species related to *Thermomicrobium* and *Thermogemmatispora* were further characterized to aid in the development of a biogeography of thermophilic CO oxidizers on Kilauea volcano and elucidate the ecology of geothermal biofilm inhabitants. These strains not only add novel species to under-represented and poorly-studied groups of bacteria (*Thermomicrobia* and *Ktedonobacteria*), but also provided valuable insights into CO oxidation.

The deeply-branching “superphylum” Chloroflexi currently contains six classes that encompass vast phylogenetic and phenotypic diversity including thermophilic CO oxidizers (Wu *et al.*, 2009), anaerobes that reductively dechlorinate groundwater pollutants (Seshadari *et al.*, 2005), anoxygenic phototrophs lacking chlorosomes (Hanada *et al.*, 2002), nitrite oxidizers (Sorokin *et al.*, 2012), and filamentous spore-forming heterotrophs (Cavaletti *et al.*, 2007). However, recent extensive phylogenetic analyses have determined that the phylum Chloroflexi should only contain the classes Chloroflexi and Thermomicrobia, while the remaining divisions (*Ktedonobacteria, Anaerolinea, Caldilineae, and Dehalococcoidetes*) represent separate taxa distinct from Chloroflexi and require further investigation to establish their status (Gupta *et al.*, 2013).
The class Thermomicrobia currently contains 4 genera each represented by a single species: *Thermomicrobium roseum* DSM 5159 (Jackson et al., 1973), *Sphaerobacter thermophilus* DSM 20745 (Hugenholtz and Stackebrandt, 2004), *Nitrolancetus hollandicus* Lb (Sorokin et al., 2012), and ‘*Thermobaculum terrenum*’ YNP-1 (Botero et al., 2004; Kunisawa, 2011). Genome sequences are available for all four species and cox operons are present in *T. roseum* DSM 5159 and *S. thermophilus* DSM 20745 (Wu et al., 2009; Pati et al., 2010). While the CO-oxidizing capacity was revealed decades after its isolation (Jackson et al., 1973; Wu et al., 2009), *T. roseum* was the only thermophilic CO oxidizer obtained from a naturally hot environment prior to this study. A few environmental 16S rRNA clone sequences, most from hot springs, and a single geothermal soil isolate also cluster within Thermomicrobia.

Similarities in morphology, physiology, and 16S rRNA phylogenetic relatedness support inclusion of KI3 in the genus *Thermomicrobium*. Strain KI3 shares only 96.0% 16S rRNA gene identity with *T. roseum* DSM 5159. Therefore, based on the bacterial species definition of 97% 16S rRNA similarity (Stackebrandt and Goebel, 1994), strain KI3 represents a new species for which the name *Thermomicrobium carboxidovorans* sp. nov. is proposed. A more distant Thermomicrobiales isolate, strain KI4, was also isolated from the Kilauea Iki biofilms, but was unable to consume CO. Strain KI4 shares 92% and 94.5% 16S rRNA gene identity to *T. roseum* DSM 5159 and strain KI3, respectively. Strain KI4 is differentiated from *T. roseum* DSM 5159 and strain KI3 in its utilization of a broader range of sugars, inability to consume CO, and sensitivity to NaCl. Strain KI4 likely represents a new genus in the class Thermomicrobia, but further chemotaxonomic analyses are needed. Like *T. roseum* DSM 5159, strains KI3 and KI4 are fastidious species isolated from thermal microbial biofilms. The necessary growth
requirements for these strains are likely provided by the dense microbial community of the biofilms.

The class Ktedonobacteria includes a large number of uncultured environmental clone sequences, but only four formally described species in three genera: *Ktedonobacter racemifer* SOSP1-21 (Cavaletti *et al*., 2006), *Thermosporothrix hazakensis* SK20-1 (Yabe *et al*., 2010), *Thermogemmatispora onikobensis* ONI-1, and *Thermogemmatispora foliorum* ONI-5 (Yabe *et al*., 2011). Several other strains, some of which might represent novel species, have been isolated but not formally described (Cavaletti *et al*., 2006; Stott *et al*., 2008). Notably, several *Thermogemmatispora*-like strains were isolated from geothermal soils in New Zealand characterized by low pH and fluctuating temperature regimes (Stott *et al*., 2008).

The genome of *K. racemifer* SOSP1-21 has been sequenced and analyzed. It has the largest sequenced genome to date with a length of 13.7 Mbp (Chang *et al*., 2011). Genome annotations revealed greater than expected genetic redundancy due in part to an extremely high number of similar transposon-associated genes (7.5% of total protein-coding genes; Chang *et al*., 2011). Several xylose isomerase genes were also identified indicating a strong utilization of pentoses (Chang *et al*., 2011). The genome of *K. racemifer* SOSP1-21 also contains a form I *cox* operon encoding the potential for oxidizing CO; however, CO oxidation was not observed in this study.

Similarities in morphologies, fatty acid profiles, and G+C contents support inclusion of strain PM5 in the genus *Thermogemmatispora*. Strain PM5 shares 98.3% 16S rRNA gene identity with *T. onikobensis* ONI-1, but only 46.4% DNA-DNA relatedness; therefore, strain PM5 represents a new species for which the name *Thermogemmatispora carboxidovorans* sp. nov is proposed. Strain PM5 differed from *T. onikobensis* JCM 16817 in numerous respects,
particularly its ability to utilize organic acids and amino acids. Strain PM5 is further
differentiated from other *Thermogemmatispora* isolates by its lower temperature optima (55 °C),
hydrolysis of casein and starch, and inability to grow with mannitol, xylose, or rhamnose as sole
carbon sources.

Isolation of three *Thermogemmatispora* isolates from the Puhimau biofilms and Bare
cinders led to the exploration of the distribution of the CO oxidation trait in the class
Ktedonobacteria. CO oxidation was determined to be a common feature within the genus
*Thermogemmatispora* and a potentially useful diagnostic tool for differentiating closely related
strains (e.g. strain T12 and strain OL016). CO oxidation was also documented in *T. hazakensis*
JCM 16142, but not *K. racemifer* DSM 44963. Because *K. racemifer* DSM 44963 is mesophilic
and phylogenetically divergent from *Thermogemmatispora*, it may have different controls of *cox*
expression. Since none of the strains examined grew chemolithotrophically on CO, CO is likely
used as a supplementary energy source when heterotrophic substrates are limited. Further
isolation, characterization, and genome sequencing of strains will help resolve the taxonomy and
environmental role of Ktedonobacteria.

Several *coxL* clones from volcanic sites also clustered with Ktedonobacteria (Figure
A.12). Several Ktedonobacteria-related 16S rRNA and *coxL* environmental sequences grouped
together in clone clusters that currently have no cultured members. Results from isolates and
uncultured 16S rRNA and *coxL* clone sequences indicate that potentially CO-oxidizing,
phylogenetically diverse Ktedonobacteria are ubiquitous in volcanic and geothermal
environments. Indeed, *Thermogemmatispora* appear to be among the dominant members of
geothermal soil at Waikite Valley, New Zealand (Stott *et al.*, 2008) and Ktedonobacter-like *coxL*
sequences dominate the Bare site (Weber and King, 2010). In addition to Puhimau biofilms and
Bare cinders, *Thermogemmatispora*-related strains have been isolated from other sites characterized by low pH and fluctuating temperature regimes that reach thermophilic extremes (Waikite, New Zealand, Stott et al., 2008), indicating that *Thermogemmatispora* may be important CO oxidizers at sites that experience variable temperatures. The Ktedonobacteria-specific *coxL* evolutionary distance cutoff of 0.05, as opposed to the previously described 0.10 cutoff (King and Weber, 2008), might provide additional support in determining species boundaries among CO-oxidizing *Thermogemmatispora*.

Strain PM5 grows from 40 – 65 °C and produces exospores which may contribute to survival in the fluctuating temperature environment of the Puhimau biofilms. The ability of *Thermogemmatispora* str. PM5 to oxidize CO as a supplemental energy source and grow under limited nitrogen conditions may help overcome nutritional limitations in the biofilms. Since strain PM5 is able to hydrolyze cellulose and xylan, the tree stump upon which the biofilms grow may provide a carbon source. Tolerance of 1% NaCl may also be an important characteristic of PM5 since the Puhimau biofilms are subject to drying and rehydrating which can create osmotic stress. Overall, these characteristics make *Thermogemmatispora* str. PM5 particularly well suited to life in the Puhimau microbial biofilms.

**Description of Thermogemmatispora carboxidovorans sp. nov.**

*Thermogemmatispora carboxidovorans* (car.box.i.do.vo.rans, L. n. carbo, charcoal, carbon; Gr. Adj. oxys, sour, acid; L. v. voro, devour; M.L. part. Adj. carboxidovorans, carbon-acid devouring).

Cells are aerobic, Gram positive, non-motile, spore-forming filaments that form branching mycelia and produce exospores via budding. Grows at 40–65 °C (optimum 55 °C) and pH 4.1–8.0 (optimum pH 6.0). NaCl not required for growth; growth inhibited by 2% NaCl.
Grows under microaerophilic conditions. Catalase positive. Nitrate reduction, esculin hydrolysis, gelatinase, β-galactosidase positive. Indole production from tryptophan, arginine dihydrolase, fermentation, and urease negative. Principal fatty acids are iso-C_{17:0}, 12,17-dimethyloctadecanoic acid, and iso-C_{19:0}. G+C content 59%. Oxidizes carbon monoxide. Grows with gluconate, pyruvate, glycerol, alanine, glutamate, aspartate, glucose, mannose, lactose, sucrose, methanol, ethanol, and acetone. Does not grow with propionate, malonate, tartrate, lactate, acetate, fumarate, malate, succinate, mannitol, glycine, valine, proline, serine, methylamine, dimethylamine, trimethylamine, betaine, galacturonate, isopropanol, glycolate, citrate, inositol, arabinose, fructose, galactose, ribose, xylose, rhamnose, or glucuronate. Gelatin, casein, starch, cellulose, chitin, xylan, and gellan gum are hydrolyzed. The type strain PM5^T (= DSM 45816^T) was isolated from a geothermally-heated biofilm at the Puhimau thermal area (Hawaii, USA).

**Description of Thermomicrobium carboxidovorans sp. nov.**


Cells are aerobic, Gram positive, non-motile, non-sporeforming rods often found in pairs (0.9 µm width, 2.0–2.5 µm length). Grows at 50–70 °C (optimum 70 °C) and pH 6.5–9.0 (optimum pH 7.0). NaCl not required for growth; growth inhibited by 3% NaCl. Catalase positive. Urease, esculin hydrolysis, gelatinase, and β-galactosidase positive. Nitrate reduction, indole production, arginine dihydrolase, and fermentation negative. Oxidizes carbon monoxide. Grows on complex media. The type strain KI3^T (= DSM 27067^T) was isolated from a geothermally-heated biofilm at the Kilauea Iki crater (Hawaii, USA).
CHAPTER 5. 
COMPOSITION AND DIVERSITY OF CO-OXIDIZING BACTERIA IN 
GEOTHERMALLY-HEATED MICROBIAL BIOFILMS 

Introduction 

Activity assays, MPN analyses, and efforts to obtain isolates have shown that 
thermophilic CO oxidizers are active and abundant in a wide variety of geothermally-heated sites 
(Chapter 3, Chapter 4). However, other than insights obtained from isolates, little is known about 
the composition of thermophilic CO-oxidizing communities. Molecular ecological analyses of 
coxL gene sequences in temperate terrestrial volcanic environments on Miyake-Jima (Japan) and 
at Kilauea Volcano (Hawaii, USA) have found that Proteobacteria dominate vegetated deposits 
(> 75% of total coxL diversity; Dunfield and King, 2004; King et al., 2008; Weber and King, 
2010), while Chloroflexi, specifically Ktedonobacteria, dominate nutrient-poor unvegetated 
cinders (~70% of all coxL clones, Weber and King, 2010). Firmicute-like sequences appeared to 
dominate coxL libraries from sites on Miyake-Jima that consisted of a mosaic of unvegetated and 
recently-vegetated tephra (King et al., 2008), but at least some of these might actually belong to 
Ktedonobacteria (see Miyake-Jima volcanic clone sequence phylogenetic placement in Figure 
A.12). Most of the Kilauea and Miyake-Jima sites also harbored significant populations (5–25% 
total coxL diversity) of Actinobacterial CO oxidizers regardless of vegetation status (Dunfield 
and King, 2004; King et al., 2008; Weber and King, 2010). 

Although these same phyla harbor CO-oxidizing thermophiles, the extent to which the 
composition of CO-oxidizing communities in geothermal systems parallels that of temperate 
systems is unknown. Nonetheless, recent isolations of several novel CO-oxidizing species, 
including members of exclusively thermophilic genera (Chapter 4), suggest geothermal systems 
may harbor unique communities.
To assess the composition and diversity (richness and evenness) of thermophilic CO-oxidizing communities, cultivation-independent approaches were used to characterize geothermally-heated microbial biofilms colonizing dead trees at the Puhimau geothermal area (Kilauea Volcano, Hawai‘i). Clone libraries for the coxL gene were supplemented by results from a metagenomic analysis, which facilitated a comparison of communities in biofilms obtained from three distinct trees. Total microbial community composition was assessed in these same biofilms for additional context using a pyrosequencing-based analysis of 16S rRNA gene sequences. This study also investigated whether thermal sites harbor greater phylum-level CO oxidizer diversity than temperate sites and whether certain phylogenetic groups are ubiquitous in thermal biofilms.

Materials and Methods

Sample Collection

Microbial biofilms (described in Chapter 3) were scraped aseptically with ethanol-cleaned spoons from the bases of three separate tree stumps on February 15, 2012. Biofilm samples were transferred to Whirl-Pak bags, and frozen within 2 h of collection. Instantaneous in situ temperatures were recorded at the time of collection using a digital thermometer. Biofilm pH values were determined with a pH meter using slurries of biofilm material and deionized water (1:1 mass ratio). HOBO logger probes were embedded in each biofilm (< 1 cm) to record temperature regimes as described in Chapter 3. Frozen samples for DNA extraction were transported on dry ice to a laboratory at LSU and were stored at -80 °C until use.

DNA Extraction

DNA was extracted from biofilm samples using the MoBio PowerBiofilm DNA Isolation Kit (MoBio Laboratories) according to the manufacturer’s protocol. Ten extracts from each
biofilm sample were pooled to obtain >1 µg DNA per mat. DNA was quantified on a NanoDrop ND-3300 Fluorospectrometer (Thermo Fisher Scientific, Inc.) and using a Quant-iT PicoGreen dsDNA kit (Invitrogen).

**coxL Clone Library Construction**

**coxL** genes in the biofilm DNA extracts were amplified using the primers SOf (5’-GGCGGCTT[C/T]GG[C/G]AA[C/G]AAGGT-3’) and PSr (5’-[C/T]TCGA[C/T]GATCATCGG[A/G]TTGA-3’) (King, 2003). The PCR reaction mixture consisted of 0.2 µl Plantium Taq DNA Polymerase High Fidelity (Life Technologies Corp, La Jolla, CA, USA), 2.5 µl 10X High Fidelity PCR buffer, 0.2 µl 10 mM dNTPs, 1 µl 50 mM MgSO₄, 5 µl bovine serum albumin, 1.5 µl SOf primer, 1.5 µl PSr primer, 12.1 µl nuclease-free H₂O, 1 µl template. Thermocycler conditions included: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C 1 min, annealing at 58 °C 1 min, and elongation at 68 °C 2 min, followed by a final elongation at 68 °C for 10 min. DNA from biofilm 1 was diluted 100-fold in nuclease-free water prior to PCR amplification to facilitate successful amplification. PCR products were visualized using gel electrophoresis in a 1.2% agarose sodium borate gel and stained with Gel Red (Biotium, Hayward, CA, USA).

**coxL** PCR products (~1250 bp) were purified using the MoBio UltraClean PCR Cleanup Kit (MoBio Laboratories). DNA concentrations were determined using the Quant-iT PicoGreen dsDNA Kit (Invitrogen). PCR products were ligated into the pJet1.2/blunt cloning vector following the sticky-end cloning protocol of the CloneJet PCR Cloning Kit (Fermentas, Thermo Scientific). The vectors were transformed into OneShot TOP10 chemically competent *E. coli* cells (Invitrogen, Life Technologies). Cells were plated on Luria agar (Sigma-Aldrich) containing 100 µg ml⁻¹ ampicillin and incubated at 37 °C overnight. Transformed *E. coli*
colonies were resuspended in PCR tubes containing master mix for direct PCR amplification. The PCR reaction mixture consisted of 12.5 µl GoTaq Green, 1 µl 10 µM pJet1.2 forward (5’- CGACTCAGTATAGGGAGAGCGGC-3’), 1 µl 10 µM pJet1.2 reverse (5’- AAGAACATCGATTTTCCATGGCAG-3’), 9.5 µl nuclease-free H₂O, and a single clone colony. PCR products were purified using the Wizard SV 96 PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced bidirectionally using BigDye terminator chemistry (Applied Biosystems) on an ABI 3130XL Genetic Analyzer (Applied Biosystems) at the Louisiana State University Genomics Facility (Baton Rouge, LA).

**coxL Clone Library Analysis**

*coxL* forward and reverse reads were assembled, edited, and translated using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI, USA). Form II *coxL* sequences, containing GAGR at the active site instead of the diagnostic CSFR active site motif (King and Weber, 2007), were separated from authentic form I *coxL* sequences, but retained for further analysis. Translated form I *coxL* clone sequences were aligned against reference sequences in MEGA5.1 (Tamura et al., 2011) using Muscle (Edgar, 2004).

In order to define operational protein units (OPU), inferred amino acid *coxL* clone sequences were used to create a distance matrix using the program ProtDist within Phylib3.69 (Felsenstein, University of Washington). Numbers of OPUs and diversity indices for *coxL* libraries were estimated at an OPU evolutionary distance of 0.1 (King and Weber, 2008) and 0.05 (Chapter 4) using the program mothur v1.29.0 (Schloss et al., 2009). Phylogenetic trees were created using one representative *coxL* sequence per OPU. Maximum-likelihood and neighbor-joining phylogenetic trees were created in MEGA5.1 using 100 and 1000 bootstrap replicates, respectively. Form II *coxL* sequences from *Rhodobacter sphaeroides* (YP_352939),
*Mesorhizobium loti* (BAB48572), and *Ruegeria pomeroyi* DSS-3 (AAV94806) were used as outgroup sequences.

**High Throughput 16S rRNA Gene Sequencing**

The hyper-variable V4 region of the 16S rRNA gene was amplified from three biofilms using the primers 515f and 806r (Caporaso *et al.*, 2011) modified with barcodes and adaptor sequences (including 454 titanium primers A and B) required for pyrosequencing on the GS FLX System (454 LifeSciences, Branford, CT, USA). The same DNA extracts used to generate the Puhimau biofilm *coxL* clone libraries were used to generate the biofilm 16S rRNA pyrosequencing dataset. The PCR reaction mixture consisted of 0.2 µl Plantium Taq DNA Polymerase High Fidelity (Life Technologies Corp.), 2.5 µl 10X High Fidelity PCR buffer, 0.2 µl 10mM dNTPs, 1 µl 50 mM MgSO₄, 5 µl bovine serum albumin, 1.5 µl 515f primer, 1.5 µl 806r primer, 12.1 µl nuclease-free H₂O, and 1 µl template. Thermocycler conditions were as follows: 3 min at 94°C, followed by 28 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 1 min, and elongation at 68°C for 2 min, with a 10 min extension step at 68°C after the cycles were complete. PCR products were visualized using gel electrophoresis in a 1.2% agarose sodium borate gel and stained with Gel Red (Biotium, Hayward, CA, USA). Amplicons of the proper size (~350 bp) were purified using the MoBio UltraClean PCR Cleanup Kit (MoBio Laboratories). Six reactions for biofilm 1 and triplicate reactions for the other two biofilms were pooled separately. DNA concentrations were determined using the Quant-iT PicoGreen dsDNA Kit (Invitrogen) on a NanoDrop Fluorometer (Thermo Scientific). A final mixture was prepared for sequencing by adding amplicons from each sample in equal masses (~125 ng DNA each). Sequencing was conducted on a GS FLX instrument (454 Life Sciences) by the Environmental Genomics Core Facility at the University of South Carolina (Columbia, South Carolina).
16S rRNA Dataset Analysis

Sequences were processed using mothur v1.29.0 (Schloss et al., 2009). Barcodes and primers were removed and sequences were filtered based on quality using a moving window of 50 bp with an average quality score of 35 and minimum length of 100 bp. Trimmed sequences were aligned to the SILVA reference 16S rRNA dataset; chimeras were identified using UCHIME (Edgar et al., 2011) and removed. After uncorrected pairwise distances between aligned sequences were calculated, the average-neighbor method was used to cluster sequences into operational taxonomic units (OTU). OTUs were classified using the Ribosomal Database Project training set (version 7) for phylotype-based analyses. Representative sequences of unclassified OTUs which represented ≥ 1% of biofilm and soil datasets were analyzed by BLAST against the NCBI database to verify taxonomy. Abundant (≥ 1%) OTUs labeled as Cyanobacteria were also analyzed by BLAST to differentiate Cyanobacteria from chloroplast sequences.

Mothur was additionally used to generate diversity indices, rarefaction analyses, and OTUs shared among biofilms. Rarefaction analysis was performed to determine the number of observed OTUs (d = 0.03) as a function of the distance between sequences and the total number of sequences sampled. Venn diagrams were created to visualize species overlap, represented by shared phylotypes (d = 0.03), for the biofilm and soil communities. Genera known to harbor CO-oxidizing species were identified manually and curated to determine the relative contribution of potential CO oxidizers to the community.

Metagenome Sequencing

Puhimau biofilm 3 DNA was used to create a shotgun metagenome. DNA samples were processed and sequenced at the Argonne National Laboratory. Sample preparation included
sheering and size selecting DNA fragments (370 bp) and attaching barcodes and adaptors. Paired-end (2 x 100 bp) sequencing of fragments was performed on an Illumina HiSeq 2000 (Illumina Inc., San Diego, CA, USA).

**Metagenome coxL Fragment Analysis**

Raw reads were processed using a pipeline at CAMERA (California Institute for Telecommunications and Information Technology, San Deigo, CA). Open reading frames (ORF) were predicted from assembled contiguous sequences (contigs) and translated into amino acid sequences. The BLAST Command Line Application (National Center for Biotechnology Information) was used to create a database of all metagenomic ORFs and translated peptide sequences. These metagenome databases were analyzed by BLAST with full-length coxL nucleotide and amino acid query sequences from *Mycobacterium smegmatis* MC52 and *Bradyrhizobium* sp. BTAi to identify putative coxL gene fragments. Translated ORFs containing the form II active site motif in the results were identified and removed. All remaining BLAST results were then aligned to the NCBI GenBank database. Sequences were culled if they lacked similarity to form I coxL or were more similar to other genes. ORFs with ambiguous BLAST results were retained. Predicted amino acid sequences of these ORFs were used in subsequent analyses.

A reference alignment of full-length form I coxL amino acid sequences and several full-length form II sequences was created using ClustalW and manually edited as necessary in MEGA5.1. The trimmed metagenomic ORF dataset was aligned to the reference alignment using the profile alignment mode in Clustal X.2. A maximum-likelihood based reference tree and tree statistics file was created in PhyML v3.0 using an LG substitution model (Guindon *et al.*, 2010). Predicted coxL metagenomic fragments from the local BLAST results were analyzed using
pplacer which places query sequences on a fixed reference tree based on input alignments (Matsen et al., 2010). The software guppy (Matsen et al., 2010) and Archaeopteryx (Han and Zmasek, 2009) were used to visualize the phylogenetic placement output of pplacer.

**Results**

*coxL Clone Library Analyses*

A total of 154 form I *coxL* gene sequences were obtained from Puhimau mats: 19 from biofilm 1, 69 from biofilm 2, and 66 from biofilm 3. These sequences yielded five OPUs using an evolutionary distance of 0.1 (Figure 5.1). One OPU was shared among the mats, while the remaining 4 were represented in only one library. Each of the biofilms were dominated by an OPU comprised of Ktedonobacteria, which represented > 94% of the *coxL* sequences. The remaining OPUs clustered with α-Proteobacteria, but were not closely related to *coxL* sequences from known CO oxidizers (Figure 5.1).

In order to examine the Ktedonobacteria clone sequences in more detail, OPUs were also defined using a distance cutoff of 0.05 based on the relationship between *coxL* and 16S rRNA gene sequences determined for Ktedonobacteria in Chapter 4. This evolutionary distance resulted in 9 OPUs including 5 that clustered with Ktedonobacteria (Figure 5.2). Puhimau biofilm 1 resulted in 2 OPUs, but most (>94%) of the *coxL* sequences at this site belonged to one Ktedonobacteria OPU. Puhimau biofilm 2 was comprised of 4 OPUs, three of which likely represent different species within Ktedonobacteria (Figure 5.2). Puhimau biofilm 3 was dominated (76%) by the same Ktedonobacteria OPU as biofilm 1, but 19% of sequences at this site fell within a separate Ktedonobacteria OPU that also accounted for 38% of biofilm 2 sequences. Using the lower distance cutoff, the majority of *coxL* sequences were more closely
related to *Thermogemmatispora* isolates (< 7% sequence divergence) than *Ktedonobacter racemifer* (> 10% sequence divergence).

**Figure 5.1.** Phylogeny of inferred amino acid *coxL* OPU sequences created using a neighbor-joining method with 1000 bootstrap replicates. OPUs are defined at an evolutionary distance of 0.10. For OPUs comprised of sequences from multiple sites, a representative sequence is listed.
Figure 5.2. Phylogeny of inferred amino acid coxl OPU sequences created using a neighbor-joining method with 1000 bootstrap replicates. OPUs are defined at an evolutionary distance of 0.05. For OPUs comprised of sequences from multiple sites, a representative sequence is listed.
Overall, *coxL* diversity was low for the biofilms (Table 5.1) based on Shannon and inverse Simpson indices, which are diversity estimates that consider both species richness (the number of OPUs) and species eveness (the abundance of each OPU) (Magurran, 2004). The Shannon index quantifies the uncertainty in predicting the OPU for a randomly selected sequence from the dataset, while the inverse Simpson index is a measure of the likelihood that two randomly selected sequences belong to the same OPU (Magurran, 2004). Shannon index and inverse Simpson index values were highest at biofilm 2; although, values were relatively low across all biofilms (Table 5.1). Based on comparisons between observed and predicted OPU numbers from Chao1 estimates, all OPUs were recovered from the biofilms at a distance of 0.1, and all but one OTU was recovered at a distance of 0.05 (Table 5.1).

Table 5.1. Diversity estimates for *coxL* libraries at evolutionary cutoffs of 0.1 and 0.05. 95% confidence intervals are given in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Mat 1</th>
<th>Mat 2</th>
<th>Mat 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of clones</td>
<td>19</td>
<td>69</td>
<td>66</td>
</tr>
<tr>
<td>0.10 cutoff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of OPUs</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Chao1</td>
<td>2 (2.0)</td>
<td>3 (3.0)</td>
<td>2 (2.2)</td>
</tr>
<tr>
<td>ACE</td>
<td>0</td>
<td>4.1 (3.1, 18.4)</td>
<td>2 (2.2)</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td>0.21 (0, 0.52)</td>
<td>0.26 (0.06, 0.45)</td>
<td>0.139 (0, 0.288)</td>
</tr>
<tr>
<td>Simpson (1/D)</td>
<td>1.13 (0.93, 1.43)</td>
<td>1.13 (1.01, 1.27)</td>
<td>1.07 (0.98, 1.16)</td>
</tr>
<tr>
<td>Coverage (%)</td>
<td>94.4</td>
<td>98.5</td>
<td>100</td>
</tr>
<tr>
<td>0.05 cutoff</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of OPUs</td>
<td>2</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Chao1</td>
<td>2 (2.0)</td>
<td>7 (6, 20)</td>
<td>4 (4.0)</td>
</tr>
<tr>
<td>ACE</td>
<td>0</td>
<td>9.7 (6.4, 43.4)</td>
<td>5 (4.2, 9.1)</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td>0.21 (0, 0.52)</td>
<td>1.24 (1.05, 1.43)</td>
<td>0.69 (0.48, 0.91)</td>
</tr>
<tr>
<td>Simpson (1/D)</td>
<td>1.13 (0.93, 1.43)</td>
<td>2.99 (2.53, 3.65)</td>
<td>1.62 (1.34, 2.04)</td>
</tr>
<tr>
<td>Coverage (%)</td>
<td>94.4</td>
<td>97.1</td>
<td>98.4</td>
</tr>
</tbody>
</table>

Several (80) clones sequenced from biofilm 1 yielded form II *coxL* sequences. The primers also amplified a small number (≤ 5) of form II sequences from biofilms 2 and 3. BLAST
results determined most of these sequences were distantly related to an aldehyde dehydrogenase of *Nitrolancetus hollandicus* (63% amino acid sequence identity).

**16S rRNA Analyses**

A total of 70,536 raw reads with an average length of 294 bp was obtained from 16S rRNA pyrosequencing. After preprocessing, 39,164 sequences were used for downstream analyses. The three Puhimau biofilms were dominated by Acidobacteria, Chloroflexi, Armatimonadetes (formerly OP10), and Proteobacteria (Figure 5.3). Over 80% of the Chloroflexi reads belonged to the class Ktedonobacteria, specifically within the genus *Ktedonobacter* (Table 5.2). While overall patterns of diversity were similar, biofilms differed in the relative abundance of Firmicutes and Chloroflexi (Figure 5.3). Notably, *Alicyclobacillus* made up a significant fraction of biofilm 2 (14%), but were rare members (< 0.05%) of biofilms 1 and 3.

Figure 5.3. Phylogenetic composition of Puhimau biofilms based on phylum-level classification from mothur analysis. The category “Other” combines the reads assigned to the phyla Crenarcheota, Euryarchaeota, Aquificae, Bacteroidetes, Deinococcus-Thermus, Actinobacteria, Verrucomicrobia, Chlamydiae, Synergistetes, Archaea unclassified, and unknown unclassified.
Table 5.2. Phylogenetic composition (%) of Puhimau biofilm Chloroflexi OTUs.

<table>
<thead>
<tr>
<th>Site</th>
<th>Phylum Chloroflexi</th>
<th>Class Ktedonobacteria</th>
<th>Genus Ktedonobacter</th>
<th>Genus Thermogemmatispora</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mat 1</td>
<td>17.28</td>
<td>16.02</td>
<td>14.90</td>
<td>0.25</td>
</tr>
<tr>
<td>Mat 2</td>
<td>19.17</td>
<td>14.24</td>
<td>2.95</td>
<td>4.23</td>
</tr>
<tr>
<td>Mat 3</td>
<td>1.86</td>
<td>1.81</td>
<td>0.77</td>
<td>0.51</td>
</tr>
</tbody>
</table>

A large number of reads were unclassified by mothur and were therefore subjected to BLAST analysis. Most of the unclassified OTUs at the Puhimau biofilm sites were distantly related to Ktedonobacteria isolates (88–91% sequence identity), but a few unclassified OTUs appeared to be distantly related to members of the phylum Firmicutes (85–92% sequence identity) (Table 5.3).

Table 5.3. Phylogenetic composition of Puhimau biofilm unclassified OTUs (d = 0.03) based on closest neighbors from BLAST results. Only OTUs with ≥ 0.1% abundance at each site were analyzed.

<table>
<thead>
<tr>
<th>Closest 16S rRNA BLAST hit (% identity)</th>
<th>% Composition (± 1 s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterium SOSPI-165 (88%); Ktedonobacteria</td>
<td>5.3 (2.1)</td>
</tr>
<tr>
<td><em>Sulfobacillus acidophilus</em> DSM 10332 (86%); Firmicutes</td>
<td>2.3 (1.1)</td>
</tr>
<tr>
<td><em>Thermogemmatispora foliorum</em> (91%); Ktedonobacter</td>
<td>2.2 (2.1)</td>
</tr>
<tr>
<td>Bacterium Ellin 7237 (90%); Ktedonobacteria</td>
<td>2.2 (1.9)</td>
</tr>
<tr>
<td>Bacterium SOSP1-1 (91%); Ktedonobacteria</td>
<td>0.95 (0.5)</td>
</tr>
<tr>
<td><em>Ammonifex degensii</em> KC4 (90%); Firmicutes</td>
<td>1.2 (0.3)</td>
</tr>
<tr>
<td><em>Clostridium cellulosi</em> strain D3 (85%); Firmicutes</td>
<td>1.0 (0.6)</td>
</tr>
<tr>
<td><em>Oxalophagus oxalicus</em> (92%); Firmicutes</td>
<td>0.7 (0.5)</td>
</tr>
</tbody>
</table>

Phylum-level composition was similar among mats; however, comparisons across biofilms at an OTU definition of d = 0.03 show substantial differentiation at the species-level (Figure 5.4) mostly due to differences in rare OTUs. Species-level compositions showed that a few phylotypes dominated each Puhimau site; the top ten most abundant OTUs (d = 0.03) encompassed ~ 60% of the total sequence diversity. Notably, seven of the ten most abundant...
biofilm OTUs were Ktedonobacteria phylotypes. Interestingly, a *Chthonomonas* OTU (d = 0.03) was ubiquitous and abundant (4 – 11% of the total diversity) at the Puhimau biofilms.

![Venn diagram](image)

**Figure 5.4.** Venn diagram depicting shared OTUs among Puhimau biofilms at an evolutionary distance of 0.03.

Shannon index and inverse Simpson values indicated that total microbial diversity for the Puhimau biofilms was high (Table 5.4). Rarefaction curves did not approach saturation at species-level distance (d = 0.03) for any site (Figure 5.5). These results combined with Chao1 and ACE estimates of community richness compared to observed richness (Table 5.4) indicate that a substantial portion of the diversity at the sites was not captured despite the depth of sequencing. However, coverage values for sites were high (> 93%; Table 5.4) which indicates that the predicted un-sampled diversity consists of rare members of the communities.
Table 5.4. Diversity estimates for Puhimau biofilm and soil 16S rRNA libraries at an evolutionary distance of 0.03. 95% confidence intervals are given in parentheses.

<table>
<thead>
<tr>
<th>0.03 cutoff</th>
<th>Biofilm 1</th>
<th>Biofilm 2</th>
<th>Biofilm 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number OTUs</td>
<td>649</td>
<td>1211</td>
<td>907</td>
</tr>
<tr>
<td>Chao1</td>
<td>1994 (1638, 2477)</td>
<td>3852 (3334, 4496)</td>
<td>3322 (2764, 4047)</td>
</tr>
<tr>
<td>ACE</td>
<td>3448 (3136, 3799)</td>
<td>7861 (7326, 8443)</td>
<td>7365 (6818, 7962)</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td>4.29 (4.24, 4.34)</td>
<td>3.95 (3.91, 3.98)</td>
<td>3.7 (3.67, 3.73)</td>
</tr>
<tr>
<td>Simpson (1/D)</td>
<td>26.4 (25.2, 27.71)</td>
<td>18.02 (17.58, 18.48)</td>
<td>15.39</td>
</tr>
<tr>
<td>Coverage (%)</td>
<td>93.4</td>
<td>95</td>
<td>95.9</td>
</tr>
</tbody>
</table>

Figure 5.5. Rarefaction curves for pyrosequencing-derived 16S rRNA OTUs defined at an evolutionary distance of 0.03 for Puhimau biofilm sites.

Genera that harbor CO oxidizers occurred in each of the biofilm samples. *Ktedonobacter*, *Alicyclobacillus*, and *Thermogemmatispora* were the most abundant (Table 5.5); additional genera included *Thermomicrobium* and *Burkholderia*. Overall, CO-oxidizing genera made up 12.5% of the biofilm communities.
Table 5.5. Potential CO oxidizer composition of Puhimau biofilm communities. Values for each genus are percentages of the total sequences for each sample.

<table>
<thead>
<tr>
<th>Genus</th>
<th>Biofilm 1</th>
<th>Biofilm 2</th>
<th>Biofilm 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ktedonobacter</td>
<td>14.90</td>
<td>2.95</td>
<td>0.77</td>
</tr>
<tr>
<td>Alicyclobacillus</td>
<td>0</td>
<td>13.88</td>
<td>0</td>
</tr>
<tr>
<td>Thermogemmatispora</td>
<td>0.25</td>
<td>4.23</td>
<td>0.51</td>
</tr>
<tr>
<td>Thermomicrobium</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Burkholderia</td>
<td>0</td>
<td>0</td>
<td>0.26</td>
</tr>
</tbody>
</table>

Metagenomic Analyses

After removing redundant sequences that were present in output from the *Mycobacterium* and *Bradyrhizobium* queries, the BLAST results yielded 448 nucleotide ORF sequences and 213 translated amino acid ORF sequences; 93 of these sequences occurred in both datasets and 43 contained the GAGR active site motif for form II *coxL* (King and Weber, 2007). After removing redundant sequences and culling form II sequences, 525 unique sequences remained. The remaining nucleotide and amino acid sequences were aligned against the NCBI GenBank database, which led to a final trimmed dataset with 112 sequences varying in length from 33 to 997 amino acid residues (average 339). These sequences were placed on a reference tree of *coxL* sequences using pplacer. Of the 112 fragments analyzed, 33 sequences clustered with form I *coxL* sequences and 11 clustered with sequences from related molybdenum hydroxylases (Figure 5.7). Over half of the reads could not be placed confidently with either form I or form II branches of the reference tree. Subsequent BLAST analyses confirmed that the unclassified sequences were not form I *coxL*.

The single most abundant cluster of the metagenomic *coxL* reads analyzed were associated with the *Ktedonobacter racemifer* branch (Table 5.6; Figure 5.7). Actinobacteria, α-Proteobacteria, and Crenarchaeota *coxL* sequences were relatively abundant (>15 % for all) and diverse in the biofilm (Table 5.6; Figure 5.7). About 57% of the reads clustered closely to known thermophilic CO oxidizer groups, and 18% of reads appeared to be archaeal (Table 5.6).
Figure 5.7. Visualization of pplacer output depicting the phylogenetic placement of metagenome fragments from Puhimau biofilm 3 on a coxl reference tree. Branch thickness indicates the log-transformed number of placements on that particular branch. An additional visualization of the form I subtree is provided in Figure A.13.
Table 5.6. Phylogenetic composition (%) of form I coxL biofilm metagenome sequences.

<table>
<thead>
<tr>
<th>Phylum/Class</th>
<th>% Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ktedonobacteria</td>
<td>21.2</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>21.2</td>
</tr>
<tr>
<td>Crenarchaeota/Geoarchaeota</td>
<td>15.2</td>
</tr>
<tr>
<td>α-Proteobacteria</td>
<td>15.2</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>6.1</td>
</tr>
<tr>
<td>Deinococcus-Thermus</td>
<td>6.1</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>6.1</td>
</tr>
<tr>
<td>Euryarchaeota</td>
<td>3.0</td>
</tr>
<tr>
<td>β-Proteobacteria</td>
<td>3.0</td>
</tr>
<tr>
<td>δ-Proteobacteria</td>
<td>3.0</td>
</tr>
</tbody>
</table>

Discussion

Analyses of coxL clone libraries indicated that the Puhimau biofilm CO oxidizer community is less diverse than other CO-oxidizing communities (Weber and King, 2010), and was dominated by Ktedonobacter-like sequences. Most of the clone sequences were distantly related to known CO oxidizers and likely represent at least 5 novel species. The presence of novel α-proteobacterial coxL genes in the geothermally-heated biofilms is particularly interesting since ‘Pseudomonas thermocarboxydovorans’ is the only described thermophilic CO oxidizer in Proteobacteria (Meyer and Schlegel, 1978).

The total microbial communities at the Puhimau biofilms have lower diversity estimates than those of less extreme sites (e.g. temperate soils) and were dominated by just a few OTUs. This is consistent with previous studies which reported biofilms growing in extreme environments have low species diversity (Tyson et al., 2004; Brazelton et al., 2009; Liu et al., 2011). The community of a natural acidic biofilm (42 °C, pH 0.83) at an acid mine drainage runoff was dominated by Leptospirillum and Ferroplasma (Tyson et al., 2004). Biofilms growing on carbonate hydrothermal chimneys (> 90 °C, pH 9–11) consisted almost entirely of Thiomicronospira (Brazelson and Baross, 2009; Brazelton et al., 2009). A pilot study of
thermophilic, aerobic biofilms established at two wastewater reactors (~50 °C, pH 6.5–12.0) found that Proteobacteria and Bacteroidetes dominated the communities based on PCR banding patterns (Tiirola et al., 2003). Pyrosequencing of microbial biofilms at an alkaline hot spring at the Yellowstone National Park revealed that the biofilm community was dominated by phototrophic members of Cyanobacteria, Chloroflexi, Chlorobi, and Acidobacteria (Liu et al., 2011). Other geothermal biofilm diversity studies have focused on specific taxa, such as cyanobacterial populations in alkaline hot springs (Ferris et al., 2003) and Legionella occurrence in acidic springs (Sheehan et al., 2005), rather than overall community structure or specific functional groups such as CO oxidizers. Despite being an extreme environment, the Puhimau biofilms have much greater diversity than previous microbial biofilm studies and have an estimated number of microbial species > 3000 (Table 5.4).

While temperature does not appear to drive differences in CO uptake activity (Chapter 2, Chapter 3) or CO oxidizer abundance (Table 5.5), temperature may influence CO-oxidizing community composition. Consistent with coxL clone library results, Ktedonobacteria were dominant (~11 %) members of the microbial communities at the biofilms. Phylotypes in the phylum Armatimonadetes were also abundant at the Puhimau biofilm sites. Armatimonadetes currently only contains three isolates (Lee et al., 2011; Tamaki et al. 2011; Im et al. 2012), but is relatively common in molecular ecological analyses. Insights from the Puhimau biofilm metagenomes may assist in the enrichment of additional isolates in this under-represented phylum. CO-oxidizing genera made up significant portions (> 12%) of the biofilm communities. Actual numbers of CO oxidizers might be higher since several unclassified OTUs were distantly related to known CO-oxidizing species and coxL clone libraries indicated the presence of novel CO-oxidizing lineages.
Analysis of metagenomic coxL fragments showed that Ktedonobacter-like sequences dominated the CO oxidizers in the community. These results agree with previous results from coxL clone libraries and 16s rRNA gene sequence analyses. Several coxL fragments clustered with α-proteobacteria as observed in the clone library; however, several fragments also clustered with Actinobacteria, Archaea, Firmicutes, Chloroflexi and Deinococcus-Thermus. In silico analyses (Bikandi et al., 2004) of the coxL primers indicate that they do not amplify coxL from Sulfolobus, Thermomicrobium, Sphaerobacter, Meiothermus, Thermoaerobacter, or Sulfobacillus (King and King, unpublished results) which might explain their absence in the clone library. The relative abundance of Actinobacteria coxL fragments in the metagenome (> 20%) is surprising for two reasons. First, Actinobacteria coxL genes were undetected in the clone library even though they can be readily amplified (King, 2003). Second, Actinobacteria represented only 0.2% of 16S rRNA gene composition at biofilm 3. The primers used may have been biased against novel actinobacterial coxL genes or the depth of sequencing may have not been sufficient to pick up these sequences in the clone library.

Metagenomic analysis of another geothermally-heated microbial mat, the carbonate chimney biofilms at the Lost City Hydrothermal Field, revealed an unusually high number of transposases (> 8% of all reads) which suggests that horizontal gene transfer (HGT) is an important process in this system (Brazelton and Baross, 2009). Notably, Ktedonobacteria were abundant at the Puhimau mats, and Ktedonobacter racemifer also contains a very high number of transposases in its genome (Chang et al., 2011). Generally, HGT is suggested to play an important role in increasing phenotypic diversity in biofilm communities (Boles et al., 2004). Greater functional diversity, independent of phylogenetic diversity, increases community survival and stability over a broader range of environmental variables. So far the Puhimau
biofilm metagenome has only been analyzed in respect to CODH, but future analyses of the total metagenome may provide valuable insights including comparisons between the acidic terrestrial Puhimau biofilms and alkaliphilic aquatic Lost City biofilms. For example, several acidophilic phylotypes were present in the Puhimau biofilm community which is consistent with the lower pH at the sites.

The large number of unclassified reads from the Puhimau biofilm community demonstrates that the biofilms may be largely untapped sources of novel species. Further analyses of the biofilm metagenome may reveal adaptations to thermophilic and acidophilic lifestyles (e.g. biosynthesis of saturated fatty acids and production of pigments) and provide insights into phenotypes associated with life in these unusual thermal biofilms. Future analyses of 16S rRNA gene fragments from the metagenome would provide a useful complement to the community composition determined from the pyrosequencing dataset. The coxL clone libraries, 16S rRNA dataset, and coxL metagenome analysis, in conjunction with isolates obtained previously, all indicate that the Puhimau biofilms are dominated by Ktedonobacteria. This class of organisms appears to be an important CO-oxidizing group at sites that experience variable temperature regimes (e.g. Bare site, Puhimau biofilms and soils).
CHAPTER 6.
SURVIVAL AND ACTIVITY OF THERMOPHILIC CO-OXIDIZING BACTERIA
UNDER FLUCTUATING TEMPERATURE REGIMES

Introduction

Thermophilic proteins are uniquely adapted to function at high temperature (Kumara and Nussinov, 2001), yet thermophiles exist in a wide variety of mesophilic, cool and even frozen environments (Isaksen et al., 1994; Marchant et al., 2002; Rahman et al., 2004; Wu et al., 2006; Gorlach-Lira and Coutinho, 2007; Hubert et al., 2010; Mironov et al., 2013). Little is understood about the mechanisms that maintain thermophiles in these communities or their metabolic activity under sub-optimal conditions. Thermophilic populations may be maintained at temperate sites from exogenous sources (Hubert et al., 2009; Perfumo and Marchant, 2010). Alternatively, thermophiles in moderate habitats may be dormant (i.e. spores), have very low levels of activity, or become active only during brief periods of warming. Portillo et al. (2012) found that thermophilic Firmicutes made up 3.4% of the active community of a Spanish soil. This soil experiences a broad diurnal temperature regime, which includes thermophilic extremes (25–60 °C; > 45 °C, 7 h d⁻¹). The temporary exposure to elevated temperatures at this site may be enough to sustain a stable and metabolically-active thermophilic population.

CO oxidation at thermophilic temperatures has been documented for samples from several different temperate and geothermally-heated sites at the Hawai‘i National Volcanoes Park (Chapter 2, Chapter 3). Thermophilic CO oxidizers have been isolated from temperate sites with both small (15–25 °C) and large (15–55 °C) diurnal temperature fluctuations (Chapter 4). Differences in cultivable thermophilic CO oxidizers, CO uptake activity, and community composition at adjacent sites (i.e. Bare vs. Canopy and Puhimau biofilms vs. Puhimau soil) suggest that thermophilic populations are being actively selected rather than continuously added
by aeolian input (Chapters 2-5). This study examined whether temporary exposure to thermophilic temperatures allows for thermophilic growth and CO oxidation under otherwise suboptimal conditions.

**Materials and Methods**

**CO-oxidizing Strains**

Growth and CO uptake rates under different temperature regimes were assessed for three thermophilic and one mesophilic CO-oxidizing strain. *Meiothermus* strain PS4 was obtained from geothermal soil (78 °C) at the Puhimau geothermal area, Kilauea Volcano, Hawai’i (Chapter 4). *Alicyclobacillus* strain CPP55 was isolated from artificially heated (55 °C) soil from a temperate vegetated site (15–25 °C) at a recent volcanic deposit (Canopy site, Chapter 2). *Thermogemmatispora* strain PM5 was isolated from microbial biofilms at Puhimau (Chapter 4) that experience elevated but variable temperatures (average 42 °C; range 20 – 65°C; Chapter 3). The mesophilic *Burkholderia* strain WA was isolated at 25 °C from Canopy soil (Weber and King, 2012).

*Alicyclobacillus* strain CPP55 was cultivated in modified DSM medium 13 (medium 13 + 10 mg/L MnSO₄, pH 4.2; DSMZ, 2007), a low pH medium which favors growth of *Alicyclobacillus* (Darland and Brock, 1971). Strains PS4, PM5, and WA were grown in DSM medium 592 (medium 592, pH 6.5; DSMZ, 2012), a complex medium traditionally used to cultivate *Thermomicrobium* (Jackson, 1973). Initial cultures for each isolate were prepared by inoculating media (100 ml) in sealed flasks (500 ml) that were amended with CO (50 ppm) and incubated at 55 °C or 25 °C for thermophilic and mesophilic isolates, respectively. Stationary phase cultures actively oxidizing CO were used in the temperature response assays.
Oscillating Temperature Program

An EchoTherm Programmable Digital Chilling Incubator (Model IN45, Torrey Pines Scientific, San Marcos, CA, USA) was used to mimic in situ diurnal temperature fluctuations observed for an unvegetated volcanic deposit (Chapter 2). The temperature regime experienced by cultures in the incubator under (Figure 6.1) was recorded using a HOBO Data Logger (Onset Computer Corp., Pocasset, MA, USA) with a thermistor inserted into 5 ml water in a 75-cm³ serum bottle inside the incubator.

Figure 6.1. (a) In situ temperature profile of surface cinders at the unvegetated volcanic deposit at Pu‘u Puai, Kilauea Volcano, Hawai‘i and (b) oscillating temperature regime established in laboratory incubator.

Growth Assays

Growth assays were initiated by inoculating 9.5 ml media in 75-cm³ sealed serum bottles with 0.5 ml of stationary phase culture for which CO uptake had been established. Triplicate samples for each isolate and uninoculated media controls were incubated with shaking at 25 °C, 55 °C, and under an oscillating temperature regime (OTR). The OTR was initiated at 20 °C (time= 0 h). Sub-samples (0.75 ml) of strain PS4, CPP55, and WA cultures were collected with a sterile needle and 1 cm³ syringe at regular intervals, and growth was determined by optical density measurements (600 nm) using a spectrophotometer (Beckman DU 640
spectrophotometer, Beckman Coulter, USA) over 2 weeks. Growth rate constants were
determined from the slopes of curves of log turbidity plotted against time (h). Due to the
formation of small aggregates, growth of strain PM5 was determined using protein content.
Subsamples (1 ml) of PM5 cultures were obtained at intervals using needles and 1 cm$^3$ syringes.
Cells were harvested by centrifugation (25 °C, 15,000 x g) and the pellets were lysed in a 5%
SDS, 0.1 M NaOH solution with sonication (20 s) and incubation at 95 °C for 15 min. Culture
protein content was determined using a Pierce bicinchoninic acid protein assay kit (Thermo
Scientific).

**CO Uptake Assays**

Forty-five ml of the cultures used to initiate the growth assays were washed twice by
centrifugation and re-suspension in minimal media. Minimal medium 13 (13-min) for strain
CPP55 was prepared by omitting yeast extract and glucose. Minimal medium 592 (592-min) for
stains PS4, PM5, and WA was prepared by omitting yeast extract and tryptone. Volumes (5 ml)
of unwashed culture and washed cultures in minimal media were transferred to 75 cm$^3$ serum
bottles, which were sealed with gas tight stoppers and amended with CO (100 ppm). Triplicate
samples for both Unwashed and Min cultures of each isolate and uninoculated medium controls
were incubated with shaking at 25 °C, 55 °C, and OTR which was initiated at 20 °C. Headspace
CO concentrations were determined as described by King (1999b) using a gas chromatograph
(RGA3, Trace Analytical). At the termination of the uptake assays, cells were harvested by
centrifugation (25 °C, 15,000 x g) and protein content was determined as described above; CO
uptake rates were expressed as units per mg protein.
Results

Assays determined CO uptake (Table 6.1, Figure 6.2) and growth (Table 6.2, Figure 6.3) for four isolates under three temperature and two medium treatements. *Meiothermus* strain PS4 consumed CO under all incubation conditions (Figure 6.2, graph a). CO uptake rates were 5.5-fold and 16-fold greater under thermophilic conditions (55 °C) than with OTR and 25 °C, respectively (Table 6.1). Since strain PS4 depleted headspace CO prior to completion of a full OTR cycle, a second assay was initiated by re-establishing headspace CO levels and continuing the OTR program. CO uptake during the second assay (Figure 6.2, graph a, grey symbols) was nearly identical to the initial rate. Strain PS4 grew best at 55 °C, but it also grew under OTR (2-fold slower growth rate) and at 25 °C (5-fold slower) (Figure 6.3, graph a, Table 6.2). *Alicyclobacillus* strain CPP55 consumed CO and grew under both thermophilic and OTR conditions (Figure 6.2, graph b, Figure 6.3, graph b). CO uptake rates and growth rates under OTR were about 6.5-fold and 20-fold less than rates under thermophilic conditions, respectively (Table 6.1, Table 6.2). *Thermogemmatispora* strain PM5 consumed CO at 55 °C and during OTR (Figure 6.2, graph c), but only grew at 55 °C (Figure 6.3, graph c). CO uptake rates during OTR were about 9.5-fold less than at 55 °C (Table 6.1).

The mesophile, *Burkholderia* strain WA, grew at 25 °C but not 55 °C. Strain WA initially grew under OTR for the first 5 h of the program until temperatures reached 45 °C, after which no further growth was observed. The pattern for CO uptake for strain WA was similar to that for growth, with rapid CO uptake at 25 °C, no activity at 55 °C, and CO uptake during the first 4-6 h under OTR, after which uptake ceased. Even short-term exposure (3 h) to temperatures > 45 °C eliminated CO activity and growth without recovery after a return to lower temperatures. CO
uptake rates were slightly greater (1.5-fold) in Min media than for Unwashed cultures at all temperatures for all isolates examined (Table 6.1).

Figure 6.2. CO uptake by (a) *Meiothermus* strain PS4, (b) *Alicyclobacillus* strain CPP55, (c) *Thermogemmatispora* strain PM5, and (d) *Burkholderia* strain WA at 55 °C (■), during OTR (▲), and 25 °C (♦). Closed symbols denote unwashed cultures (see text); open symbols denote minimal media. Gray symbols indicate repeated OTR assay for strain PS4. Error bars represent ±1 s.e.

Table 6.1. Isolate CO uptake rates (CO nmol h\(^{-1}\) [mg protein\(^{-1}\)]) under different temperature regimes (±1 s.e.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Medium</th>
<th>55 °C</th>
<th>OTR</th>
<th>25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS4</td>
<td>rich</td>
<td>34 (1)</td>
<td>6.5 (0.1)</td>
<td>2.4 (0.1)</td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>53 (3)</td>
<td>9.5 (0.2)</td>
<td>3.1 (0.1)</td>
</tr>
<tr>
<td>CPP55</td>
<td>rich</td>
<td>10.1 (0.5)</td>
<td>1.5 (0.2)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>15 (4)</td>
<td>2.5 (0.6)</td>
<td>0</td>
</tr>
<tr>
<td>PM5</td>
<td>rich</td>
<td>3.3 (0.8)</td>
<td>0.31 (0.01)</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>4.3 (0.7)</td>
<td>0.48 (0.04)</td>
<td>0</td>
</tr>
<tr>
<td>WA</td>
<td>rich</td>
<td>0</td>
<td>0</td>
<td>2.56 (0.08)</td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>0</td>
<td>0</td>
<td>7.6 (0.5)</td>
</tr>
</tbody>
</table>
Figure 6.3. Growth of (a) *Meiothermus* strain PS4, (b) *Alicyclobacillus* strain CPP55, (c) *Thermogemmatispora* strain PM5, and (d) *Burkholderia* strain WA at 55 °C (■), under OTR (▲), and 25 °C (♦). Error bars represent ±1 s.e.

Table 6.2. Growth rate constants (k) for isolates under different temperature regimes (±1 s.e.)

<table>
<thead>
<tr>
<th>Strain</th>
<th>55 °C</th>
<th>OTR</th>
<th>25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS4</td>
<td>0.231 (0.001)</td>
<td>0.11 (0.01)</td>
<td>0.049 (0.001)</td>
</tr>
<tr>
<td>CPP55</td>
<td>0.7 (0.1)</td>
<td>0.345 (0.001)</td>
<td>0</td>
</tr>
<tr>
<td>PM5</td>
<td>0.41 (0.06)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>WA</td>
<td>0</td>
<td>0</td>
<td>0.3306 (0.0001)</td>
</tr>
</tbody>
</table>

Discussion

Three phylogenetically and phenotypically distinct thermophiles were all active under an oscillating temperature regime that only exceeded 45 °C for 3 h d⁻¹. In contrast, activity by a mesophile was completely inhibited after a short-term exposure (3 h) to temperatures between 45 – 55 °C. This suggests that at least some thermophiles might be better adapted to surviving low
temperature stresses than mesophiles are adapted to surviving high temperature stress. Environments with brief exposure to thermophilic extremes could select for thermophiles over mesophiles because the later may not survive short-term heating events. Nonetheless, mesophiles dominate soils, not thermophiles, even though the surface of many temperate soils and tropical soils experience temperatures that can exceed 50 °C when illuminated directly. Thus, the response of Burkholderia strain WA might be representative of temperature-sensitive mesophiles, and not mesophiles more generally.

Meiothermus strain PS4 had a wide range of activity including CO uptake and growth at 25 °C despite originating from a geothermally-heated soil at 78 °C. Strain PS4 shares 100% 16S rRNA gene sequence identity with the type strain Meiothermus ruber DSM 1279 (Chapter 4) which has a reported growth range of 35–70 °C (optimum 60 °C) (Longinova et al., 1984). Growth over a broad temperature range is a common characteristic of Meiothermus species (Chung et al., 1997; Chen et al., 2002; Zeng et al., 2009). The activity of strain PS4 at OTR and 25 °C suggests thermophiles have low levels of activity under suboptimal temperatures and is shared with other thermophiles, e.g. Kosmotoga olearia (20–80 °C, Dipippo et al., 2009) and Thermosynechococcus elongatus (30–60 °C, Onai et al., 2004). A wide growth range might facilitate transport and contribute to the ubiquity of some thermophiles, e.g., M. ruber, that have been isolated from geographically distant environments (Chung et al., 1997; Chen et al., 2002; Zeng et al., 2009; Chapter 4).

CO oxidation appeared less temperature sensitive than growth for Alicyclobacillus strain CPP55 and Thermogemmatispora strain PM5. Thermogemmatispora are prevalent in environments with fluctuating temperatures (Stott et al., 2008; Weber and King, 2010; Chapter 4), and thermophilic Firmicutes are commonly reported in temperate environments (Marchant et
The results presented here suggest that some spore-forming thermophiles might be active in situ rather than existing primarily in a dormant state. Brief periods of warming might maintain thermophilic populations in temperate habitats.

Some thermophiles are capable of activity under suboptimal temperature conditions; three hours per day above 45 °C is sufficient for growth and CO oxidation activity for some strains. CO oxidation at moderate temperatures could contribute to maintenance metabolism and survival of thermophiles under suboptimal conditions. Temperate sites with temporary exposure to elevated temperatures can potentially harbor diverse communities of thermophiles. Additionally, in periodically-heated environments, thermophiles might out-perform mesophiles due to their greater temperature tolerance. The temperature of the Earth is predicted to increase by 4 °C by the end of this century (USGCRP, 2009); therefore, the thermostolerance of CO oxidizers should be investigated further to help predict future fluxes in CO cycles as global temperature continue to increase.
CHAPTER 7.
CONCLUSIONS

As the role of CO in climate change expands, understanding the factors effecting microbial mitigation of CO emissions becomes increasingly important. Due in part to the discovery of novel cox genes in primarily thermophilic lineages, this research explored the role of temperature on CO uptake activity and the diversity of thermophilic CO oxidizers at geothermal sites. A temperate soil microbial community from a young (~ 50 y) volcanic deposit was capable of thermal adaptation despite not experiencing thermophilic temperatures in situ (Chapter 2). Differences in in situ temperature regimes did not coincide with differences in temperature optima or temperature sensitivities of CO oxidation activity for two adjacent temperate sites. Additionally, differences in CO oxidizer diversity (Weber and King, 2010) could not be attributed to differences in in situ temperatures despite the abundance of potential thermophilic CO oxidizers at the unvegetated site (Chapter 2). Other factors such as greater organic carbon availability may increase the CO oxidizer diversity of a system because the majority of reported CO oxidizers function preferentially as heterotrophs; this may also allow for the establishment of thermophilic populations in the community.

Chapter 3 provides the first documented CO uptake for thermal systems and microbial biofilms. CO-oxidizing communities are active in geothermal volcanic environments including unvegetated sites with low nutrient content and temperatures exceeding 75 °C. One geothermal soil was shown to have net CO consumption at temperatures as high as 80 °C, which represents the highest temperature under which aerobic CO oxidation has been documented. MPN estimates of CO oxidizers within Kilauea Iki biofilms rivaled those previously reported for Canopy soil (Weber and King, 2010) indicating that CO oxidizers are abundant in a variety of ecosystems.
Indeed, novel thermophilic CO-oxidizing bacteria were readily isolated from a variety of thermal materials (Chapter 4).

The enrichment of multiple *Thermogemmatispora* strains prompted an analysis of CO oxidation capacity among related Ktedonobacteria including *Ktedonobacter racemifer*, *Thermosporothrix hazakensis*, *Thermogemmatispora onikobensis*, and several undescribed isolates from a geothermal soil in New Zealand. CO oxidation was revealed to be common throughout Ktedonobacteria with possible diagnostic applications. Additionally, phylogenetic analyses of *coxL* genes from these organisms support the distinction of Ktedonobacteria as a separate phylum from other Chloroflexi (Cavaletti *et al.*, 2006; Gupta *et al.*, 2013; Chapter 4).

Molecular ecological analyses of 16S rRNA genes showed that Ktedonobacteria are dominant members at Puhimau representing 11% of the biofilm community (Chapter 5). The conservation of CO oxidation among Ktedonobacteria, isolation of CO-oxidizing *Thermogemmatispora* strains from the mats, and molecular analyses of *coxL* indicate that these organisms are the primary CO oxidizers present in the biofilms (Chapter 4, Chapter 5). The prevalence of this group of organisms in the Puhimau mats, unvegetated volcanic deposits (Weber and King, 2010), and geothermal soils in New Zealand suggests that Ktedonobacteria are important colonizers of organic-poor environments with temperature stress and emphasizes the need for further studies on these bacteria.

Thermophilic CO oxidizers were found to be active over broad temperature fluctuations which may aid in survival and maintenance metabolism under sub-optimal temperature conditions (Chapter 6). If a thermophilic population is able to remain active over a broad temperature range, relatively small differences in temperatures across sites might not act as a strong selection pressure. Additionally, results from assays suggest that thermophiles might be
better adapted than some mesophiles for life at sites with even brief exposure to extreme temperatures, although more assays are needed.

In general, results from these studies indicate that microbial communities are capable of adapting to high temperatures and that thermal environments should be included in future models of atmospheric CO dynamics. CO-oxidizing bacteria and archaea are extremely phylogenetically and physiologically diverse and appear to be ubiquitous in the environment.

The lack of amplifiable **coxL** genes encountered using the SOf and PSr primers on several new thermophilic isolates (Chapter 4) highlights the need for genome sequencing of novel CO oxidizers to obtain **cox** genes. Newly designed primers may also be suitable, although initial attempts utilizing alternative **coxL** primers were unsuccessful in amplification of **coxL** genes from most of the novel CO oxidizers presented in Chapter 4 (King and King, unpublished results). In particular, thermophiles and non-proteobacterial and actinobacterial lineages may have been missed in previous molecular ecological analyses of **coxL** due to primer limitations. Chapter 5 describes a process for analyzing **cox** fragments from a shotgun metagenome as an alternative approach to studying CO-oxidizing communities in the environment.

The observations presented here also highlight the need for future studies on aerobic CO oxidizers. While **cox** genes have been identified in numerous new lineages, CO uptake assays are needed to confirm the activity of potential CO oxidizers identified through genome analyses. Some species harbor complete **cox** operons, yet CO oxidation has not been observed under multiple conditions (e.g. *Ktedonobacter racemifer* DSM 44963 and *Natronorubrum tibetense* DSM 13204). Gene regulation of the **cox** operon is poorly understood and the conditions required for expression are currently unknown. Future studies should examine CO oxidizers in additional extreme environments since thermophiles, psychrophiles, halophiles, acidophiles, and
alkaliphiles have all been implicated in CO oxidation (Chapter 1, Chapter 4). Exploration of extremophilic CO oxidizers may help reveal the evolutionary history of $cox$, and elaboration on the role of these organisms in the environment will aid in our understanding of CO dynamics.


Gutpa, R. S., Pranay, C., & George, S. (2013). Phylogenetic framework and molecular signatures for the class Chloroflexi and its different clades; proposal for division of the class Chloroflexi class. nov. into the suborder Chloroflexineae subord. nov., consisting of the emended family Oscillochloridaceae and the family Chloroflexaceae fam. nov., and the suborder Roseiflexineae subord. nov., containing the family Roseiflexaceae fam. nov. *A Van Leeuw Microrob*, 103, 99–119.


APPENDIX A.
SUPPLEMENTARY FIGURES AND TABLES

Table A.1. List of known CO-oxidizers to date with coxl gene GenBank Accession number, IMG Gene ID, or reference cited. Numbers in parentheses indicate the number of strains listed in each phylum.

**Bacteria**

**Actinobacteria** (66)

- actinobacterium SCGC AAA027D23 2524326953
- Actinomycetospora chiangmaiensis DSM 45062 2515227915
- Actinoplanes globisporus DSM 43857 NZ_KB903358
- Actinoplanes missouriensis 431 AP012319
- Amycolatopsis methanolica 239 NZ_AQULO1000001
- Amycolatopsis orientalis DSM 46075 NZ_ASH010000104
- Amycolatopsis sp. ATCC 39116 75iv2 NZ_AFWY03000003
- Amycolatopsis taiwanensis DSM 45107 2515287142
- Amycolatopsis thermoflava N1165 DSM 44574 2512621063**
- Arthrobacter sp. 131MFC06 NZ_KB895541
- Arthrobacter sp. FB24 CP000454
- Conexibacter woesei DSM 14684 CP001854
- Cryptosporangium arvum YU 62921 DSM 44712 2510405120
- Gordonia rhizophera NBRC 16068 NZ_BAH010000197
- Ilumatobacter nonamiense YM16303 NZ_BAOL01000012
- Marmoricola sp. URHB0036 2523260516
- Micromonospora aurantiaca ATCC 27029 CP002162
- Micromonospora sp. L5 CP002399
- Mycobacterium africanum GM041182 339329439
- Mycobacterium bovis AF2122/97 (NP_854043)
- Mycobacterium bovis BCG str Pasteur 1173P2 NC_008769
- Mycobacterium bovis BCG str Tokyo 172 NC_012207
- Mycobacterium canettii CIPT 140010059 340003223
- Mycobacterium kansasii ATCC 12478 NZ_ACBV01000087
- Mycobacterium marinum M NC_010612
- Mycobacterium parascrofulaceum ATCC BAA614 NZ_ADNV01000025
- Mycobacterium smegmatis str MC2 155 NC_008596
- Mycobacterium sp. JLS NC_009077
- Mycobacterium sp. KMS NC_008705
- Mycobacterium sp. MCS NC_008146
- Mycobacterium thermoresistible ATCC 19527 AGVE01000046**
- Mycobacterium tuberculosis CDC1551 NC_002755
- Mycobacterium tuberculosis H37Ra NC_009525
Mycobacterium tuberculosis KZN V2475 CP001976
Mycobacterium ulcerans Agy99 NC_008611
Nakamuraella multipartita DSM 44233 CP001737
Nitriliruptor alkaliphilus DSM 45188 2513191069
Nocardiа araoenis NBRC 100135 NZ_BAFR01000351
Nocardiа jiangxensis NBRC 101359 NZ_BAGB01000170
Nocardioides sp. JS6141 CP000509
Nocardioopsis alkaliphila YIM 80379 NZ_ANBD01000132
Nocardioopsis ganjiahuensis DSM 45031 NZ_ANBA01000016
Nocardioopsis valliformis DSM 45023 NZ_ANAZ01000040
Nonomuraea coxensis DSM 45129 NZ_KB903944
Pseudonocardia asaccharolytica DSM 44247 2523187574
Pseudonocardia dioxanivorans CB1190 (651154792)
Rhodococcus equi 103S FN563149
Rhodococcus imteehensis RKJ300 NZ_AJJH01000129
Rhodococcus jostii RHA1 CP000431
Rhodococcus opacus B4 AP011115
Rhodococcus rhodochrous BKS646 NZ_AGVW02000037
Rhodococcus ruber Chol4 NZ_ANGC01000020
Rhodococcus sp. RHA1 NC_008268
Rhodococcus wratislaviensis IFP 2016 NZ_ANIU01000359
Saccharomonospora azurea NA128 NZ_CM001466
Saccharomonospora cyanea NA134 NZ_CM001440
Saccharomonospora glauca K62 NZ_CM001484
Saccharomonospora marina XMU15 NZ_CM001439
Saccharomonospora xinjiangensis XJ54 NZ_JH636049
Sciscionella marina DSM 45152 2515793467
Solirubrobacter soli DSM 22325 2524243496
Solirubrobacter sp. URHD0082 2523093192
Streptomyces sp. BoleA5 NZ_KB989214
Streptomyces sp. HGB0020 NZ_KB905814
Streptomyces sulphureus DSM 40104 NZ_KB905814
Streptomyces thermoautotrophicus (Gadkari, et al., 1990)*
Streptomyces thermocarboxydus (Kim et al., 1998)*
Streptomyces thermocarboxydovorans (Kim et al., 1998)*
Streptomyces thermospinisporus (Kim and Goodfellow, 2002)*
Thermocrispum agrestim DSM 44070 2513815143*
Thermocrispum municipale DSM 44069 2513802442*

α-Proteobacteria (43)
Afipia birgiae WP_019200216
Agromonas oligotrophica S58 AP012603
alpha proteobacterium SCGC AAA536B06 2236672425
Aminobacter sp. COX (King, 2003)
Bradyrhizobium elkanii WSM2783 2513671559
Bradyrhizobium japonicum USDA 124 2517104400
Bradyrhizobium japonicum USDA 38 2513942758
Bradyrhizobium japonicum USDA 6 AP012206
Bradyrhizobium sp. Ai1a2 2524464866
Bradyrhizobium sp. BTAi1 NC_009485
Bradyrhizobium sp. ORS278 NC_009445
Dinoroseobacter shibae DFL 12 CP000830
Jannaschia sp. CCS1 NC_007802
Labrenzia alexandrii DFL11 NZ_EQ973121
Mesorhizobium loti (King, 2003)
Mesorhizobium sp. NMB1 (King, 2003)
Mesorhizobium str. KP12W (Weber, 2009)
Methylferula stellata (WP_020174914)
Nisaea denitrificans DSM 18348 2525376734
Octadecabacter antarcticus 238 NZ_DS990628
Octadecabacter antarcticus 307 NZ_DS990575
Oligotropha carboxidovorans OM4 651235432
Oligotropha carboxidovorans OM5 650953878
Photobacterium sp. COX ABK91860
Polymorphum gilvum SL003B26A1 CP002568
Rhizobium sp. PD01-076 (2510841794)
Rhizobium sp. str NT26 FO082820
Rhodobacteraceae bacterium KLH11 NZ_DS999531
Roseobacter litoralis Och 149 CP002623
Roseobacter sp. GA1101 647648373
Roseobacter sp. MED193 NZ_AANB01000011
Roseobacter sp. SK20926 NZ_AAYC0100000
Roseovarius sp. 217 NZ_AAMV01000001
Roseovarius sp. TM1035 NZ_ABCL01000003
Ruegeria pomeroyi DSS3 NC_003911
Salinarimonas rosea DSM 21201 2523196126
Sediminimonas qiaohouensis DSM 21189 2523946139
Shinella zoogloeoides str. FG1M5 ACH99840
Stappia M4 (King, 2003)
Stappia aggregata IAM 12614 NZ_AAUW01000007
Stappia stellulata (King, 2003)
Sulfitobacter str. P10 (Weber, 2009)
Xanthobacter sp. COX (King, 2003)
β-Proteobacteria (13)
Burkholderia nodosa DSM 21604 2516018672
Burkholderia sp. LUP AEO14750
Burkholderia sp. DNBP22 AEO14747
Burkholderia sp. H160 NZ_ABYL01000003
Burkholderia sp. I2 AEO14746
Burkholderia sp. WA AEO14748
Burkholderia sp. YA AEO14749
Burkholderia xenovorans LB400 chromosome 1 CP000270
Herbaspirillum sp. (unpublished)
Hydrogenophaga pseudoflava U80806
Oxalobacteraceae bacterium IMCC9480 AEPR01000115
Pseudomonas thermocarboxydovorans' X77931*
Tepidimonas aquatica DSM 14833 unpublished*
Variovorax sp. (unpublished)

γ-Proteobacteria (5)
Alkalilimnicola ehrlichei MLHE1 NC_008340
Glaciecola pallidula DSM 14239 NZ_BAEQ01000016
Pseudomonas carboxydohydrogena AY463247
Saccharspirillum impatiens DSM 12546 2525350093
Serratia sp. (Weber, 2009)
Stenotrophomonas sp. LUPS (AAP75613)

δ-Proteobacteria (1)
Haliangiun ochraceum DSM 14365 CP001804

Firmicutes (9)
Alicyclobacillus herbarius DSM 13609 2523758565*
Alicyclobacillus pomorum DSM 14955 2513792691*
Bacillus methanolicus MG3 (ADWW01000002)**
Bacillus schlegelii’ (AAR26462)*
Planifilum sp. (unpublished)*
Sulfobacillus acidophilus DSM 10332 CP003179*
Sulfobacillus acidophilus TPY CP002901*
Sulfobacillus thermostulfidooxidans AT1 DSM 9293 2506241754*
Thermaerobacter marianensis DSM 12885 CP002344*
Thermaerobacter subterraneus DSM 13965 313902853*

Bacteroidetes (5)
Haliscomenobacter hydrossis DSM 1100 CP002691
Niastella koreensis GR2010 CP003178
Rhodothermus marinus DSM 4252 CP001807*
Rhodothermus marinus SG0 5JP17171 2506723904*
Rhodothermus marinus SG0 5JP17172 CP003029*
Chloroflexi (3)
  Ktedonobacter racemifer SOSP.121 DSM 44963 648603662
  Sphaerobacter thermophilus DSM 20745 CP001824*
  Thermomicrobium roseum DSM 5159 NC_011961*

Deinococcus/Thermus (2)
  Meiothermus ruber DSM 1279 (ZP_04039569)*
  Meiothermus rufus DSM 22234 2523182758*

Archaea
  Crenarchaeota (3)
    Sulfolobus islandicus Y G 57 14 NC_012622*
    Sulfolobus islandicus Y N 15 51 NC_012623*
    Sulfolobus solfataricus 98/2 WP_009990994*

  Euryarchaeota (3)
    Natronorubrum bangense JCM 10635 NZ_AOHY01000058**
    Natronorubrum sulfidificiens JCM 14089 NZ_AOHX01000041**
    Natronorubrum tibetense GA33 AOHW01000049**

  Geoarchaeota (4)
    Geoarchaeota archaeon OSP.B1 2504814364*
    crenarchaeote SCGC AAA261-C22 (WP_018031948)*
    crenarchaeote SCGC AAA261-F05 WP_018032234*
    crenarchaeote SCGC AAA261-N13 WP_018033649*

*Strain is thermophilic.
**Strain is thermotolerant.
Figure A.1. Maximum-likelihood based phylogeny (100 bootstrap replicates) of 16S rRNA gene sequences of selected CO-oxidizing taxa. Aligned 16S rRNA gene sequences were obtained from the GreenGenes 16S rRNA database (http://greengenes.lbl.gov). Numbers at nodes indicate bootstrap support.
**Figure A.2.** Neighbor-joining tree (1000 bootstrap replicates) depicting phylogeny of full length *coxL* predicted amino acid sequences with branches collapsed. Numbers at nodes indicate bootstrap support.
Figure A.3. Neighbor-joining (1000 bootstraps) tree of full length *coxL* nucleotide sequences with branches collapsed. Numbers at nodes indicate bootstrap support.
Figure A.4. Maximum-likelihood based tree (100 bootstrap replicates) depicting phylogeny of full length coxL predicted amino acid sequences. Same phylogeny depicted in Figure 1.3 with subtrees expanded. Numbers at nodes indicate bootstrap support.
Figure A.5. Mean maximum potential H$_2$ uptake rates for (a) Canopy and (b) Bare samples incubated at either 25 °C or 55 °C for 30 d. All data are means of triplicates ± 1 s.e.
Figure A.6. Mean maximum potential CO uptake rates for (a) Canopy and (b) Bare samples incubated at either 25 °C or 55 °C for 30 d. All data are means of triplicates ± 1 s.e. Values less than zero indicate net CO production.
**Figure A.7.** Puhimau biofilm CO uptake at 55 °C at low CO concentrations. All data are means of triplicates ± 1 s.e.

**Figure A.8.** Strain PM5 growth under different oxygen conditions. Error bars represent ± 1 s.e.
Figure A.9. Strain PM5 growth on different nitrogen sources. Error bars represent ± 1 s.e.

Figure A.10. Strain PM5 exhibits slow growth in nitrogen-free media. Error bars represent ± 1 s.e.
Figure A.11. CO uptake at 55 °C at low CO concentrations for strain PM5 (a) and PM6 (b). All data are means of triplicates ± 1 s.e.
Figure A.12. Detail of Ktedonobacteria 16S rRNA gene phylogeny. Tree generated using a neighbor-joining method with 1000 bootstrap replicates. Outgroup sequences not shown.
Figure A.13. Visualization of pplacer output depicting the phylogenetic placement of metagenome fragments from a Puhimau microbial biofilm (labeled as MAT3 in tree) on a coxl reference tree. Detail of form I coxl subtree of Figure 5.7.
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Caitlin Elizabeth King, a native of Baton Rouge, Louisiana, was born to Monica Moran King and Mark King in 1986. Caitlin graduated high school in 2004 from St. Frederick’s Catholic High School in Monroe, Louisiana. In May 2008, she graduated college with a Bachelor of Science degree in Microbiology from Louisiana State University (Baton Rouge, Louisiana). During Fall 2008, she began graduate study in biological sciences with a focus on microbial ecology at Louisiana State University in the laboratory of Dr. Gary M. King. Caitlin married Blake Wilson in September 2012. They have one daughter together.