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Distribution, diversity and ecology of aerobic carbon monoxide-oxidizing bacteria in Hawaiian volcanic deposits

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DISTRIBUTION, DIVERSITY AND ECOLOGY OF AEROBIC CARBON MONOXIDE-
OXIDIZING BACTERIA IN HAWAIIAN VOLCANIC DEPOSITS

A Dissertation

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By
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ABSTRACT

Carbon monoxide-oxidizing bacteria are among the first colonists of recently formed volcanic deposits despite environmental conditions that challenge their survival, such as oscillating water regimes and lack of endogenous sources of organic carbon and nitrogen. Activity assessments and molecular surveys of the coxL gene (large subunit carbon monoxide dehydrogenase) across a vegetation gradient on a 1959 volcanic deposit on Kilauea Volcano (Hawai‘i) indicated that CO-oxidizing communities continue to expand, diversify and remain competitive during ecosystem development. Distinct compositional shifts occurred across the gradient with Firmicutes-like coxL sequences dominating clone libraries from unvegetated sites (Bare) and Proteobacteria coxL sequences dominating libraries from vegetated sites (Canopy). Water regimes at the Bare and Canopy sites were distinct, with the former experiencing sometimes extreme diurnal oscillations in water potential (i.e. near 0 MPa to -60 MPa) and the Canopy remaining near 0 MPa. However, CO-oxidizing communities at these sites did not exhibit differential adaptations to water stress, which may not be an important factor controlling CO oxidizer distributions in these sites.

Of the major taxonomic groups represented in the coxL clone libraries, increased total richness across the transect was most highly correlated with the number of OTUs represented by β-Proteobacteria sequences, many of which were closely related to Burkholderia coxL. A qPCR approach developed to target Burkholderia coxL indicated that absolute gene copy numbers increased from undetectable quantities in the Bare site to an average of $8.6 \times 10^8$ copies gdw soil$^{-1}$ in the Canopy site. In addition, a 16S rRNA gene phylogenetic analysis of Burkholderia isolates obtained from these sites and their close relatives indicated that CO-oxidation may be a common trait among root-associated Burkholderia species. Their strong association with plants,
abundance and ability to consume atmospheric levels of CO, may explain their contribution to CO-oxidizer community expansion during ecosystem development and indicates that they may be an important constituent of active CO-oxidizing communities \textit{in situ}.
CHAPTER 1.
INTRODUCTION
Biogeochemistry of Carbon Monoxide (CO)

Despite relatively low atmospheric concentrations, 50-300 ppb (Novelli et al., 1994a), CO plays an important role in mediating the oxidative state of the atmosphere and concentrations of greenhouse gases (Crutzen and Gidel, 1983; Khalil, 1999). CO reacts with the hydroxyl radical (OH•) to form CO$_2$, which consumes about 75-85% of total annual CO emissions (Crutzen and Gidel, 1983; Khalil, 1999; Monson and Holland, 2001). The hydroxyl radical is also an important sink for other greenhouse gases, such as methane, which has a radiative forcing constant 20 times that of CO$_2$ and an atmospheric residence time of 8-9 years (Monson and Holland, 2001). Therefore, increased levels of CO can reduce the oxidative capacity of the atmosphere and indirectly enhance the greenhouse effect (Bates et al., 1995; Levine et al., 1985; Monson and Holland, 2001; Springer-Young et al., 1996; Zafiriou et al., 2003). The impacts of CO on atmospheric chemistry are reduced via microbial CO-oxidation in soils, which consumes 10-15% of CO emitted to the atmosphere annually (Conrad, 1996; Khalil, 1999; King, 1999b).

Abiological mechanisms of CO production are responsible for the largest inputs of atmospheric CO at present and across geological times scales. Concentrations of CO in Earth’s early atmosphere may have reached 100 ppm due to volcanic activity and may have played important roles in organic syntheses (Aylward and Bofinger, 2001; Miyakawa et al., 2002). A dramatic decrease in volcanic activity over geological time scales is likely responsible for the much lower atmospheric CO concentrations recorded today. Measurements of CO in Greenland ice cores indicate atmospheric concentrations of approximately 90 ppbv in 1800 A.D. (Monson and Holland, 2001). Fluctuations in the atmospheric CO concentrations since 1800 are likely attributable to anthropogenic sources such as fossil fuel combustion and biomass burning, which contribute an estimated 478 Tg CO y$^{-1}$ and 500-1500 Tg y$^{-1}$, respectively (Monson and Holland,
However, the largest source of atmospheric CO is the oxidation of methane, which occurs via reaction with the hydroxyl radical in the atmosphere and adds an estimated 744 to 924 Tg CO y\(^{-1}\). Methane is emitted from anthropogenic sources such as fossil fuel production, livestock management, biomass burning, landfills and rice cultivation (Monson and Holland, 2001).

Another abiological mechanism of CO production and the primary source of CO in marine environments is the photochemical degradation of dissolved organic matter (Bates et al., 1995; Haan et al., 2001; Kieber et al., 1989; Miller and Zepp, 1995; Valentine and Zepp, 1993; Zafiriou et al., 2003; Zuo and Jones, 1995). Photochemical degradation of organic matter quickly supersaturates marine surface waters with CO, making oceans a net source of CO to the atmosphere. However, CO-oxidizing bacteria reduce emissions from marine systems by about 86%, representing an important internal sink (Zafiriou et al., 2003). Photochemical degradation also occurs in soils and marine sediments along with chemical and thermodegradation that also produce CO (Conrad and Seiler, 1980; Conrad and Seiler, 1985; King, 2007; Moxley and Smith, 1998).

Biological sources of CO include production by microbes, leaves, roots and animals (Conrad et al., 1988; Hino and Tauchi, 1987; King, 2001; King and Crosby, 2002; Schade and Crutzen, 1999; Tarr et al., 1995; Wray and Abeles, 1993). CO has been reported as a byproduct or intermediate in several metabolic pathways. For instance, CO is an intermediate in acetyl-CoA production (Ragsdale, 2004), a product of bacterial aromatic amino acid metabolism, aldehyde decarboxylation in plants and heme degradation by heme oxygenase in mammals (Tenhunen et al., 1969; Wray and Abeles, 1993). In the soil environment, CO production by plant roots is of similar magnitude to diffusive inputs of CO from the atmosphere. Because most
soils are net sinks for atmospheric CO, the CO produced by plant roots must be consumed internally by CO-oxidizing bacteria (King and Crosby, 2002; King and Weber, 2007).

Microbiology, Physiology and Genetics of Aerobic CO-oxidizing Bacteria

Despite their widespread distribution and recognized importance in the biogeochemical cycling of CO, few aerobic CO oxidizers responsible for in situ CO oxidation have been identified. Prior to 2001, the only known CO oxidizers were a group called the carboxydotrophs, which can grow aerobically using CO as a sole carbon and energy source (King and Weber, 2007). These organisms are insensitive to high concentrations of CO and have been typically enriched and isolated in liquid culture under atmospheres containing 10% O₂, 5% CO₂ and 85% CO (vol/vol) (Meyer and Schlegel, 1978; Moersdorf et al., 1992). The first report of carboxydotrophy was published in 1903 (Beijerinck and van Delden, 1903) and additional reports of newly isolated carboxydotrophs were published in 1914 (Beijerinck, 1914) and 1922 (Lantzsch, 1922). However, these early reports did not provide firm evidence for CO oxidation and it wasn’t until 1953, that the first confirmed carboxydotroph, “Hydrogenomonas carboxydovorans”, was isolated from sewage sludge in the Netherlands (Kistner, 1953). Efforts to revive this culture in the late 1960s were unsuccessful (Mörsdorf et al., 1992), but a series of carboxydotrophs was isolated in the late 1970s and early 1980s from a variety of environmental sources (e.g. waste water, charcoal pile, compost) (Kalinowski, 1980; Kim and Hegeman, 1983; Kirkconnell and Hegeman, 1978; Meyer and Schlegel, 1978). However, the ecological niche for utilizing high concentrations of CO remains unknown and these isolates have not been considered important in in situ activities. The half-saturation constants determined for carboxydotrophs incubated under elevated concentrations of CO are greater than 500 nM (Conrad et al., 1981; Meyer and Schlegel, 1983), which is one to two orders of magnitude higher.
than those determined for samples from marine (2 to 5.4 nM; Tolli and Taylor, 2005), and soil and freshwater environments (5 to 50 nM; Conrad et al., 1981; Conrad and Seiler, 1982; Jones and Morita, 1984).

More recently developed enrichment strategies and CO oxidation assays that utilize relatively low CO concentrations (<1000 ppm) have aided in the isolation and identification of both novel and previously unrecognized CO-oxidizing isolates, which may represent organisms responsible for in situ activities (King, 2003a; Weber and King, 2007). This newly identified group of CO oxidizers, the carboxydovores, oxidizes CO at concentrations ranging from sub-ambient to about 1000 ppm; higher concentrations of CO inhibit CO oxidation (King, 2003a; Weber and King, 2007). Therefore, in the past, elevated CO concentrations (i.e., 85%) used to detect CO-oxidizing capabilities in cultures would have inhibited and masked the ability of carboxydovores to consume CO, necessitating the development of new enrichment strategies. One enrichment strategy involving the long-term incubation of forest soil under a constant flow of non inhibitory concentrations of CO (40-400 ppm) resulted in the isolation of Aminobacter sp. str. COX, the first isolate described to have a \(K_m\) similar to that of soil (Hardy and King, 2001).

Another recently employed strategy for enriching carboxydovores capitalizes on their mixotrophic capabilities. Carboxydotrophs and carboxydovores function preferentially as heterotrophs using a wide variety of organic substrates, but many will function mixotrophically using organics and CO simultaneously (Kiessling and Meyer, 1982; Weber and King, 2007). As a result, enrichment techniques that utilize low concentrations of CO and various organic substrates have resulted in the isolation of several novel CO oxidizers. These isolates include marine organisms in the genera Stappia (Weber and King, 2007), Ruegeria, Roseobacter (Tolli
et al., 2006), *Photobacterium*, and *Sulfitobacter* as well as a hotspring *Shinella* isolate and a *Serratia* strain from soil (Weber and King, unpublished; King, unpublished). CO oxidizers that have been obtained using similar methods from Hawaiian and Japanese volcanic deposits include members of the genera *Burkholderia, Pseudomonas, Mycobacterium* and *Mesorhizobium* (King, unpublished; Weber and King, unpublished).

Most of the physiology and genetics of aerobic CO oxidation has been learned from studies of carboxydrotrophs with an emphasis on *Oligotropha carboxidovorans*, an isolate from wastewater (Dobbek et al., 1999; Fuhrmann et al., 2003; Gnida et al., 2003; Kraut and Meyer, 1988; Meyer and Rajagopalan, 1984; Meyer et al., 1986; Meyer and Schlegel, 1983; Mörsdorf et al., 1992; Santiago et al., 1999; Schuebel et al., 1995). Carboxydrotrophs include members of the *Actinobacteria, Firmicutes* and *Proteobacteria* (King and Weber, 2007). All but one of these carboxydrotrophs, *Streptomyces thermoautotrophicus*, grow preferentially as heterotrophs using a variety of sugars, organic acids and amino acids as growth substrates (Gadkari et al., 1990; Meyer and Schlegel, 1983). As noted above, some carboxydrotrophs can also function mixotrophically and in at least one case (*Hydrogenophaga pseudoflava*), can utilize energy from CO oxidation to incorporate a surplus of organic carbon (Kiessling and Meyer, 1982). However, the group is most noted for its ability to function chemolithoautotrophically using CO as the sole source of carbon and energy (Futo and Meyer, 1986; Meyer et al., 1990; Meyer and Schlegel, 1978).

During chemolithoautotrophic growth, CO is oxidized by carbon monoxide dehydrogenase (CODH) and the CO$_2$ generated during the process can be fixed by ribulose 1,5-bisphosphate carboxylase oxygenase (rubisCO), the key enzyme in the Calvin-Bassham-Benson
Cycle (Futo and Meyer, 1986; Meyer and Schlegel, 1978). During the oxidation of CO by a membrane bound CODH, electrons are transferred to a CO-insensitive respiratory chain (Cypionka and Meyer, 1983; Mörsdorf et al., 1992) and a proton motive force is generated that can drive ATP synthesis (Cypionka et al., 1984). Reduced pyridine nucleotides are generated via reversed electron flow (Mörsdorf, 1992).

CODH is in the protein family of molybdenum hydroxylases (Bell et al., 1988; Hille, 2005; Meyer and Rajagopalan, 1984). Crystallographic structure determinations of the CODH from O. carboxidovorans have revealed that it is a dimer of heterotrimers, with the three subunit types: small (17.8 kDa), medium (30.2 kDa) and large (88.7 kDa) (Dobbek et al., 1999). The genes encoding these subunits are coxS, coxM and coxL, respectively. The large subunit is a molybdoprotein that contains the active site of the enzyme. The active site consists of a binuclear [CuSMoO₂] cluster (Gnida et al., 2003). The molybdenum ion at the center of the active site is coordinated by a sulfido ligand to a copper ion and the endithiolate group of a molybdopterin-cytosine dinucleotide (MCD). The copper is covalently linked to cysteine(S) (Gnida et al., 2003). In the active site, CO is oxidized at the molybdenum ion and the electrons are transferred via the conjugated bonds in the molybdopterin ring to the [2Fe-2S] clusters in the small subunit and the flavin adenine dinucleotide (FAD) in the medium subunit before reaching oxygen as the external electron acceptor (Dobbek et al., 1999). CO oxidation is also possible under anaerobic conditions if nitrate is supplied as the terminal electron acceptor (King and Weber, 2007).

In O. carboxidovorans, the cox genes are located on a 128 KB plasmid, pHCG3, along with genes that are necessary for hydrogenotrophy and CO₂ fixation via the Calvin Bassham
Benson Cycle (Fuhrmann et al., 2003; Santiago et al., 1999; Schuebel et al., 1995). The complete cox gene cluster consists of 12 genes in the following order: coxBCMSLDEFGHIK (Fuhrmann et al., 2003). As discussed above, coxMSL encode the three subunit types of CODH. Genes coxB, coxC, coxH and coxK encode putative transmembrane helicies. Genes coxC and coxH contain motifs of a two component signal transduction system and could be involved in CO-sensing. Gene coxI is a cytoplasmic protein and coxG plays a role in anchoring CODH to the cytoplasmic membrane. The gene cluster coxDEF is associated with the biosynthesis of the [CuSMoO₂] center, and mutation studies demonstrate the cluster’s essentiality in the synthesis of a fully functional enzyme (Santiago et al., 1999). In contrast, the genes cox BCHIK may not be essential and their presence in cox gene clusters varies among CO-oxidizing isolates. Although rubisCO is key to chemolithoautotrophic growth in O. carboxidovorans, and is located on the same plasmid as the cox genes, this seems to be an exception among CO oxidizers. Many recently discovered carboxydovores lack rubisCO altogether and in some, the absence of alternative CO₂ fixation pathways like the reverse tricarboxylic acid pathway, suggests that CO only serves as a supplementary energy source in these isolates.

Recently completed genome sequences have revealed the presence of more than one cox operon in some organisms (i.e. Burkholderia xenovorans LB400, Nocardioides sp. JS614). In the case of Nocardioides sp. JS614, the operons are in tandem while in all other cases the operons are at different locations on the chromosome. In all of the above cases, the sequences of the genes in the operons are different and in some of these cases, one of the genes is phylogenetically affiliated with cox genes in an organism from a different phylogenetic group, indicating possible horizontal gene transfer (HGT). For instance, Nocardioides sp. JS16 has one
coxl gene that is phylogenetically associated with those of other Actinobacteria coxl genes, but the second coxl gene is of unknown phylogenetic affiliation. Other possible cases of HGT have been detected in organisms in the genera Serratia, Photobacterium and Burkholderia, which have coxl genes that are phylogenetically affiliated with those of other \( \alpha \)-Proteobacteria. Because the coxl phylogeny largely parallels that of 16S rRNA genes, such possible HGT events can be detected. Collectively, these insights provide the basis for asking questions about the evolutionary history of the cox operon and how it is regulated, particularly in the scenarios when two operons are 1) in tandem or 2) located in different regions of the chromosome.

Sequences of cox genes from O. carboxidovorans and other carboxydotrophs have aided in identifying cox genes in several bacterial genomes as well as putative cox genes. Up until this point, all discussions of cox genes have referred to genes that contain all the essential motifs of the authentic aerobic CODH. These authentic or Form I cox sequences have helped to identify putative cox or Form II cox genes in a number of bacterial isolates and genome sequences (King and Weber, 2007). Form II genes encode a molybdenum hydroxylase that is more phylogenetically similar to the Form I CODH than to other molybdenum hydroxylases (King and Weber, 2007). The Form I and Form II coxl genes are 40-50% similar and contain several conserved amino acid motifs. However, the active site motif of form I coxl is AYXCSFR, while that of Form II is AYRGAGR. The active site motif encoded by Form II coxl is similar to that found in other molybdenum hydroxylases with broad substrate specificities. It is possible that the Form II genes encode a CO-oxidizing enzyme with broad substrate specificity, but this remains to be determined (King and Weber, 2007). Form II genes appear more commonly among completed bacterial genome sequences than Form I cox genes and have been identified as
important constituents in metagenomic studies (Mou et al., 2008) making their function and widespread distribution of interest.

The annotation of authentic *cox* operons in genome sequences have aided in identifying lineages not previously recognized to contain CO oxidizers and has thus expanded the currently known diversity of the functional group. As of March 2009, at least 16 CO oxidizers had been discovered out of about 700 finished bacterial genomes, of which 11 represent previously unrecognized CO oxidizers. However, some genome sequencing has been biased towards certain genera such as *E. coli*. As genome sequencing begins to encompass a greater diversity of organisms, more are likely to be discovered. Newly recognized *cox* operon containing organisms include *Arthrobacter* sp. FB24, *Nocardiooides* sp. JS614, *Mycobacterium* sp. MCS, *Mycobacterium* sp. KMS, *Mycobacterium* sp. JLS, *Mycobacterium* sp. CDC1551, *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. ORS278, *Rhodococcus* sp. RHA1, *Roseobacter* sp. MED193, *Roseovarius* sp. 217, *Haliangium ochraceum* DSM14365, *Thermomicrobium roseum*, and *Meiothermus ruber* DSM1279.

Affiliations of the *coxL* genes mined from environmental samples indicate that many CO oxidizers remain to be isolated, some of which belong to lineages not previously recognized to contain CO oxidizers. Dunfield and King (2004) found that 56% of Form I *coxL* sequences cloned and sequenced from a 1959 Hawaiian volcanic deposit were phylogenetically affiliated with *Mycobacterium* and *Bacillis* sequences, which suggests that species of the *Actinobacteria* and *Firmicutes* may be important *in situ*. Within the *Firmicutes*, the only known CO oxidizer is *Bacillus schlegelii*, but the sequence diversity from environmental samples indicates that many genera in this phylum contain CO oxidizers, but remain to be isolated and/or recognized. Cloning and sequencing of *coxL* genes from marine water column and sediment environments
revealed a similar diversity of CO oxidizers spanning the *Proteobacteria, Actinobacteria* and *Firmicutes* (King *et al.*, unpublished data).

**Ecology of CO-oxidation**

Although the ecological roles for CO oxidation have yet to be defined, the phylogenetic breadth and widespread distribution of CO-oxidizing bacteria as well as the ubiquity of CO may facilitate several different roles. Currently known CO-oxidizers occupy several ecological niches and include pathogens, plant symbionts and biogeochemically important taxa in both terrestrial and aquatic environments. In addition, two metabolic modes of metabolism for CO, either lithoautotrophically with CO as the sole carbon and energy source or mixotrophically with organics, may further diversify the ecological roles of CO and provide different advantages depending on carbon availability in the environment.

The genus *Mycobacterium* in phylum *Actinobacteria*, consists of both pathogenic and nonpathogenic species, of which several have been documented as CO oxidizers (Cho *et al.*, 1985; King, 2003b; Park *et al.*, 2003) or recently identified based on the presence of *cox* genes in their genome sequences. Two pathogenic, CO-oxidizing isolates are *M. tuberculosis* H37Ra and *M. bovis* BCG, which are causative agents of tuberculosis in humans and cattle, respectively. They along with *M. gordonae*, which can also infect lungs, are capable of oxidizing CO at concentrations found in ambient air and human respiratory gases (<1 to 5 ppm CO; King, 2003b). In the presence of nitrate, these organisms oxidize CO under suboxic and anaerobic conditions, which prevail in lung granulomas (King, 2003b). In addition, several *Mycobacterial* species were found to grow as carboxydrotrophs (Cho *et al.*, 1985; Park *et al.*, 2003). These
observations indicate that CO may be able to serve as a substrate for maintenance metabolism during infection.

In addition, recent findings that CO produced by host macrophages during infection plays a role in upregulating the dormancy operon of *M. tuberculosis* H37Ra (Shiloh et al., 2008). Similar interactions may occur in protists such as *Acanthamoeba*, which behave similarly to macrophages and are known to harbor several species of CO-oxidizing *Mycobacteria* (Greub and Raoult, 2004; Marques da Silva et al., 2002; Primm et al., 2004).

Both pathogenic and nonpathogenic CO-oxidizing members of the genus *Mycobacterium* as well as other members of the *Actinobacteria*, are commonly found in a number of other habitats in which CO oxidation may be important to their ecology. *Mycobacteria* are commonly found in nutrient poor environments such as municipal water sources, endolithic environments, dust and volcanic deposits (Dunfield and King, 2004; King and Weber, 2007; Primm et al., 2004; Walker et al., 2005). In young unvegetated volcanic deposits, CO has been found to contribute as much as 10% to total energy flow, indicating that CO may be an important substrate in nutrient-poor terrestrial environments, as it may be in mammalian lungs (King, 2003c). Furthermore, CO oxidation may continue even as intermittent pulses of organics become available from sources like rainwater or detritus allowing CO oxidizers to maximize carbon and energy acquisition. Similar roles may be played by CO-oxidizing members of the genera *Bacillus*, which appear to be common in nutrient poor, stressful environments (Dunfield and King, 2004; King et al., 2008).

Both carboxydotrophs and carboxydoxovores have been isolated from terrestrial and aquatic plant roots and have endophytic, epiphytic or symbiotic relationships or associations which may involve CO (King, 2003a; King and Crosby, 2002; King and Weber, 2007; Rich and
King, 1998). Terrestrial and aquatic plant roots, as sources of both CO and labile organics, may tap the mixotrophic capabilities of CO oxidizers and foster endophytic and epiphytic associations. Many mycobacteria and other members of the phylum Actinobacteria are involved in such interactions with plants. Three genomically sequenced Mycobacterium species, JLS, KMC and KMS, were recently recognized to contain cox operons and are known to be associated with barley roots (Child et al., 2005). Other mycobacteria comprise a significant portion of the endophytic community in wheat roots (Conn and Franco, 2004). Members of the phylum Actinobacteria, which include many CO oxidizers other than mycobacteria, was found to be associated with plant development on Mt. Pinatubo mud flows (Ohta et al., 2003).

Several CO-oxidizing species of Bradyrhizobium, Mesorhizobium, Sinorhizobium and Rhizobium are nitrogen-fixing symbionts in legume nodules (King, 2003a; King and Weber, 2007). Although it has yet to be specifically demonstrated, the association of CO-oxidizing bacteria with legume nodules may impact the efficiency of nitrogen-fixation by symbionts. CO produced in the nodules inhibits nitrogenase and leghaemoglobin, an oxygen transport protein that indirectly impacts nitrogenase by controlling oxygen delivery inside the nodule. It is possible that CO-oxidizing bacteria associated with the nodules may reduce inhibitory effects of CO produced inside the nodules and improve nitrogen-fixation efficiencies (King and Weber, 2007).

**CO-oxidizing Bacteria on Volcanic Deposits and Their Role in Biological Succession**

Recent studies document CO-oxidizing bacteria as early colonists of volcanic deposits where their metabolic and ecological versatility may enable them to play a role in ecosystem development. Despite the fact that CO oxidizers, along with a diversity of other microbes, are among the primary colonists of volcanic deposits, only a limited number of studies have focused
on various aspects of microbial succession on volcanic deposits (e.g., Bardgett and Walker, 2004; Bardgett et al., 2007; Ibekwe et al., 2007; Sigler and Zeyer, 2004; Tscherko et al., 2003), while most of the studies have focused on vegetational development (e.g., Del Moral and Clampitt, 1985; Del Moral and Wood; Grishin et al., 1996; Halpern et al., 1990; Kroh et al., 2000; Paijmans, 1973; Tsuyuzaki, 1995; Wood and Del Moral, 1988). The latter, as well as classical ecological studies of macroorganismal succession (Connell and Slayter, 1977; Glasser, 1982; Odum, 1969; Whitaker, 1975), have provided a firm foundation on which to build and refine ecological theories describing changes that occur during succession including functional shifts in energy flow, community composition, diversity, resistance, resilience and factors governing the progression of seral stages (Odum and Barrett, 2005). It is not clear to what extent such theories and principles apply to microbial systems, but has recently been argued by Prosser et al. (2007) that their application to microbial systems is vital to organizing data and observations in way that develops our ability to understand and predict behavior of microbial communities. The CO-oxidizing bacteria provide an intriguing functional group to examine in the context of ecosystem development because their presence and activity has been documented in both early and late succession and they are a phylogenetically and physiologically diverse group, which could potentially be important throughout the process. However, it is not clear how distribution, function and diversity of this functional group may change or is governed throughout succession.

Recent activity surveys of CO oxidizers on volcanic deposits have revealed that CO-oxidizers colonize deposits shortly after deposition where CO-oxidation contributes significantly to total energy flow (King, 2003c). Activity measurements at long-term monitoring sites on Kilauea volcano (Hawai`i), found that on fresh deposits, substantial rates of CO oxidation could
be detected within 6 months of deposit deposition (King, 2007). CO oxidation rates measured at a variety of unvegetated deposits in Hawai‘i reveal that CO oxidation can contribute as much as 10% to total energy flow in these environments (King and Weber, 2008). Similar activity measurements on unvegetated deposits in Miyake-jima, Japan as well as the continental United States indicate that early colonization of volcanic deposits by CO-oxidizers is a widespread phenomenon (King, 2003c; King et al., 2008).

The earliest stage of deposit colonization by microbes is likely driven largely by stochastic factors associated with transport, as it is for the early stages of plant succession (Glasser, 1982; Whitaker, 1975; Whitaker and Woodwell, 1972). This is particularly true in the colonization of remote volcanic deposits that are distant from established vegetation, as microbes reach these deposits primarily via atmospheric transport. However, across 70 to 150 year-old barren volcanic deposits at 9,000 ft. on Mauna Loa, which is well above the tree line, CO-oxidation was consistently observed at scales < 1 m to > 10 m (King and Weber, unpublished data). Despite the stochastic nature of atmospheric transport, this widespread distribution of activity on remote deposits indicates the ubiquity of CO-oxidizers and the potential importance of CO oxidation as mechanism for acquiring carbon in organic carbon-poor substrates.

Upon deposition, volcanic deposits are sterile substrates that lack endogenous sources of carbon and nitrogen, and the ability of pioneering colonists to acquire exogenous sources of carbon, such as atmospheric CO is important to their success. In addition to atmospheric CO and other trace gases, other exogenous sources for pioneering organisms may include dry or wet deposition of ammonium as well as the deposition of windborne organisms and organic detritus (Hodkinson et al., 2002; King, 2003c). Of these potential sources, King (2003c) approximated that wet
deposition of carbon could contribute significantly to the organic carbon pools on Hawaiian deposits that receive substantial rainfall even if only 10% of the organic matter is retained. Dust inputs, although important in some ecosystems, are only 250 mg m$^{-2}$ yr$^{-1}$ in Hawaii and are approximated to account for $<<10\%$ of organic inputs to these systems (King, 2003c). Inputs from microalgal photosynthesis may be significant in some deposits but are patchily distributed and requires further investigation (King, 2003c). In this regard, the advantage associated with being able to consume trace gases is their constant availability. In addition, many carboxydovores are able to functional mixotrophically (Kang and Kim, 1999; Kiessling and Meyer, 1982; Pearson et al., 1994; Ro and Kim, 1993; Weber and King, 2007) and may continue to utilize CO in conjunction with some of the more patchily distributed sources of carbon as they become available. Furthermore, a “mixotrophic advantage” or ability to incorporate more organics while growing with CO than without may exist in situ, but has yet to be demonstrated at environmentally relevant concentrations.

Classical ecological theory states that succession begins with autotrophic organisms, particularly on substrates that initially lack endogenous sources of organic carbon (Bardgett et al., 2005; Bardgett and Walker, 2004), but the early establishment of CO-oxidizing communities indicates pioneering colonist may be much more versatile in their metabolism. Some CO carboxydotrophs may function as chemolithoautotrophs, but many carboxydovores appear to lack suitable CO$_2$-fixation pathways and may use CO as an energy source in conjunction with an intermittent supply of organics. However, the ability of many CO oxidizers to function as preferential heterotrophs raises some interesting questions, as an increasing number of studies report the importance of heterotrophs as early colonists (Bardgett et al., 2007; Hodkinson et al., 2002; Sigler and Zeyer, 2004). For instance, in a study of succession on recently deglaciated
foreland in the Austrian Alps, Bardgett et al. (2007) found that heterotrophs were present in recently exposed sites even though they lacked autotrophic carbon inputs and carbon content was less than 0.1 mg g$^{-1}$. This ancient carbon, as determined by radiocarbon dating, was nearly 60% recalcitrant to degradation based on thermal analyses but still served as a carbon source for heterotrophic microbes at the site. In addition, in remote terrestrial systems such as the subantarctic Macquarie and South Orkney islands, it is proposed that initial sources of carbon for heterotrophs include windblown insects, invertebrates, pollen and other organic debris blown in from Australiasia and South America (Hodkinson et al., 2002). It is important to recognize that heterotrophs reported in these studies may actually have mixotrophic and lithotrophic capabilities as well, but this information would not be detected in culture-based studies using heterotrophic enrichments. Nonetheless, evidence of heterotrophic metabolism in young ecosystems challenges the notions of classical theory and warrants further attention.

The survival of early colonists is not only challenged by the lack of endogenous carbon and nitrogen sources in young deposits, but also a variety of other environmental stresses. The coarse, porous nature combined with the lack of organic matter, reduces the buffering capacity of deposits against environmental changes, which can result in frequent and rapid changes in temperature and water availabilities (Eggler, 1941; Griggs, 1933; Vitousek, 1994; Walker and Del Moral, 2003). On fresh deposits, it is not uncommon to see temperatures oscillate from 10 °C to 65 °C or more over a period of a few hours (Weber and King, unpublished data). Likewise, water potentials have been observed to oscillate between near 0 MPa and -60 MPa or lower within a few hours (Weber and King, unpublished data). Such rapid drying or decreases in water potential allow little time for acclimation, and adversely impact cell viability (Potts, 1994), which can be exacerbated by extreme temperatures and low nutrient availabilities. Although
microbial adaptation on volcanic deposits has not been studied specifically, we can surmise that several adaptations would be advantageous based on the responses of microbes to stresses imposed in the laboratory. Examples include endospore formation (Nicholson et al., 2000), trehalose and other compatible solute accumulation (Elbein et al., 2003; Potts, 1994), exopolysaccharide accumulation (Tamaru et al., 2005), efficient DNA repair systems (Battista and Earl, 2004; White, 2000), pigmentation (Jacobs, 2005) superoxide dismutase activity (Merkamm and Guyonvarch, 2001) and manganese accumulation (Horsburgh et al., 2002).

Consistent with this notion, a previous culture-based study of a successional gradient on a deglaciated foreland demonstrated that higher tolerance to nutrient limitation and temperature changes, as well as resistance to antibiotics, were characteristic of fast growing opportunistic bacteria, which occupied a greater fraction of the bacterial colony types isolated from younger sites (Sigler and Zeyer, 2004). Collectively, tolerance to these stresses likely plays an important role in regulating the activity and distribution of pioneering microbial colonists, including CO-oxidizing bacteria. For the latter, little is known regarding their adaptations or responses to such stresses.

After the initial colonization stages succession becomes a more directional process rather than stochastic (Glasser, 1982; Whitaker, 1975; Whitaker and Woodwell, 1972) and is a product of physical modification of the environment by communities and competitive interactions among their members (Odum and Barrett, 2005). In the facilitation model of ecosystem succession, the growth of pioneering plants, particularly nitrogen-fixing species, are known to play roles in increasing nutrient availability and water retention of young substrates, promoting the colonization by more plant species (Connell and Slayter, 1977). Microbes also participate in this process. For example, microbes have well documented roles in rock weathering, which increases
nutrient release from substrates and water retention, and increases favorability for colonization by plants and other microbes (Crews et al., 2001; Del Moral and Wood, 1993a; Joergensen and Castillo, 2001). Mechanisms of microbial weathering include 1) physical disaggregation, 2) acid production, 3) excretion of exopolysaccharides, 4) nutrient absorption and 5) interactions with organic ligands (Barker et al., 1998). Organisms involved in weathering include Bacillus sp., species in the phylum Actinobacteria, low G+C Firmicutes, Pseudomonas sp. and some root-associated bacteria and fungi (Calvaruso et al., 2006; Goldstein et al., 1999; Joergensen and Castillo, 2001; McNamara et al., 2006; Puente et al., 2006; Styriakova et al., 2004). The importance of fungi in weathering processes was pointed out by Joergensen and Castillo (2001), who suggested that ash deposit infertility in Nacaragua may be the result of low soil organic matter and lack of fungal phosphatase activity. Many of the bacterial genera listed above have been detected using molecular approaches or isolated from volcanic deposits (Dunfield and King, 2004; Hudson and Daniel, 1988; Nishiyama et al., 1998; Nuesslein and Tiedje, 1998). Many of these organisms are known to oxidize CO (Dunfield and King, 2004), providing indirect evidence that early colonizing CO oxidizers participate in facilitative processes.

In addition, carbon and nitrogen inputs from either free-living or root symbionts and lichens make it possible for many pioneering plant species to colonize nitrogen-poor substrates, facilitating subsequent plant colonization (Akers and Magee, 1985; Banfield et al., 1999; Barker et al., 1998; Burleigh and Dawson, 1994; Crews et al., 2001; Kurina and Vitousek, 2001; Perry et al., 2003; Puente et al., 2004; Thompson and Vitousek, 1997; Vitousek and Walker, 1989; Yamanake and Okabe, 2006). Such symbionts have enabled pioneering lupines to colonize young deposits on Mount St. Helens in Washington, USA (Del Moral and Clampitt, 1985). Early colonizing nitrogen-fixing trees include species of Dryas and Alnus (Bardgett and Walker,
2004; Halvorsen and Smith, 1995; Kamijo and Hashiba, 2003; Kohls et al., 1994; Yamanake and Okabe, 2006), Acacia koa (Leary et al., 2004; Nakao and Kitayama, 1996), Falcataria molucanna (Hughes and Denslow, 2005) and Myrica faya (Burleigh and Dawson, 1994; Vitousek and Walker, 1989). Several nitrogen-fixing symbionts in the genera Mesorhizobium, Burkholderia and Bradyrhizobium are now known to oxidize CO (King, 2003a) and have been isolated from volcanic deposits (Dunfield et al., 2004; King, 2003a), providing further indirect evidence of facilitative interactions involving CO oxidizers.

Specific plant-microbe interactions, such as those discussed above, have been well-documented, but plant-microbe interactions overall are complex and poorly understood. An increasing number of plant-microbe interactions are being recognized as species-specific and have positive and negative feedbacks (Kardol et al., 2007; Wardle et al., 2004). For example, in a study of microbial communities in the rhizosphere of pioneering plant species in a recently deglaciated area in Alaska, Alnus and Rhacomitrium rhizospheres had increased fungal: bacteria PLFA ratios while the Equisetum rhizosphere did not (Bardgett and Walker, 2004). In addition, in all three rhizosphere types, Gram-positive but not Gram-negative bacteria were enriched in comparison to bare soil in surrounding areas (Bardgett and Walker, 2004). Other studies have also reported an increase in Gram-positive bacteria in grassland rhizospheres (Felske et al., 1998). In contrast, several other studies report enrichment of Gram-negative bacteria in the root rhizosphere (Dunbar et al., 2002; Dunfield and King, 2004; Gomez-Alvarez et al., 2007; Singh et al., 2007; Zul et al., 2007) and several studies have suggested or have demonstrated directly that increased concentrations of labile carbon from root exudates in the rhizosphere may be the cause (Dunfield and King, 2004; Grayston et al., 1998; Ibekwe et al., 2007; Lu et al., 2006; Marilley and Aragno, 1999; Olsson and Persson, 1999; Singh et al., 2007; Zul et al., 2007). This
may be the case for CO oxidizers, as previous culture-independent assessments of coxL diversity in volcanic deposits in Hawaii and Miyake-jima, Japan suggest that the Gram-negative fraction of the community is enriched in the rhizosphere (Dunfield and King, 2004; King et al., 2008). In contrast, in some situations, competition between microbes and plants for key nutrients like nitrogen may be an important factor controlling succession (Halvorsen and Smith, 1995). Other apparent associations between microbes and plants do not impact the success of either. This was shown to be the case for vesicular-arbuscular mycorrhizae and six different pioneering plant species on Mt. St. Helens (Titus and del Moral, 1998). The sum of these interactions produce plant-microbe associations that are not always clear or predictable and require further study, particularly in the context of understanding ecosystem development.

Environmental conditions and species-specific interactions not only impact microbial communities in the present, but those in the past leave significant legacies on the composition and function of microbial communities (Martiny et al., 2006; Ramette and Tiedje, 2007; Strickland et al., 2009). The functional redundancy within microbial communities and their ability to adapt relatively rapidly to environmental change has lead to the common assumption that microbial communities are functionally equivalent (Strickland et al., 2009). However, recent studies indicate that bacterial communities collected from different environments behave differently even when placed under identical conditions; This indicates that history plays a role in determining community function (Langenheder et al., 2006; Strickland et al., 2009). In a recent study examining leaf litter decomposition rates by native and foreign microbial communities, it was found that most rapid decomposition rates occurred by native microbial communities (Strickland et al., 2009). Similarly, studies of plant litter decomposition on Mt. Etna also demonstrated that the plant source mattered, as well as the soil development (Hopkins
et al., 2007). Schipper et al., (2001) noted that inconsistencies between predicted and observed trends in microbial quotients (qCO$_2$) along successional sequences, which may be due to past and current ongoing environmental stresses that are complex and difficult to separate. Together, these insights do not support the functional equivalence hypothesis for microbial communities and indicate the importance in considering historical factors on community function and development.

While controls on microbial succession may be more complex than previously recognized (Hopkins et al., 2007), some ecological theories developed on the basis of macroorganismal studies seem to generally describe trends observed in microbial succession, particularly with respect to bioenergetics. In early succession, primary production (P) generally exceeds respiration (R), as most energy is being partitioned into biomass accumulation rather than maintenance (Odum and Barrett, 2005). As succession proceeds, the P/R ratio approaches one at which point the energy devoted to production and respiration is balanced. As systems progress through the early growth stage to a pulsing steady state, the ratio of respiration to biomass (B) decreases (Odum and Barrett, 2005). Although this phenomenon was initially described for macroorganisms, microbial R/B (microbial quotient: qCO$_2$) measured across successional sequences or vegetation gradients exhibited similar decreasing trends in most cases (Insam and Domsch, 1988; Insam and Haselwandter, 1989; Ohtonen and Vaer, 1998).

Concomitant with such shifts in energy flow are shifts in organismal strategies in energy partitioning to optimize the cost to benefit ratio (Odum and Barrett, 2005). In early succession, when environments are less crowded, selection favors organisms that partition more energy into reproduction vs. maintenance, or R-strategists (Odum and Barrett, 2005). In contrast, in the
crowded environments associated with late successional or climax stages, fierce competition for resources selects organisms that partition more energy into maintenance and survival, or K-strategists (Odum and Barrett, 2005). Consistent with this concept, a study of opportunistic bacteria across a deglaciated soil transect, found that younger sites had greater proportions of rapidly colonizing bacteria (Sigler and Zeyer, 2004). Further analyses demonstrated that the opportunistic bacteria displayed greater tolerance to stressors such as nutrient limitation and temperature oscillations, which are characteristic of early successional sites (Sigler and Zeyer, 2004). Successional studies in laboratory microcosms showed that bacterial culturability decreased while diversity, as measured using an tRFLP approach, increased during succession indicating a switch from R to K selection (Garland et al., 2001).

As the switch from R to K-selection occurs, organism diversity increases as an increasing number of niches are filled, biogeochemical cycles become more closed and energy use within the community is more efficient (Odum and Barrett, 2005). Consistent with these notions, a number of studies of microbial communities report increased taxonomic and functional diversity in older and more mature sites (Gomez-Alvarez et al., 2007; Tarlera et al., 2008; Tscherko et al., 2003). However, the increase in diversity is a function of organisms surviving competitive exclusion by well-adapted community members. Such competitive abilities vary between taxonomic and functional groups (Odum and Barrett, 2005). The presence of CO-oxidizing bacteria in both early and late succession is intriguing in this regard and brings about questions regarding their ability to remain competitive throughout succession.

In summary, soil microbes are an integral part of ecosystem development on volcanic deposits playing a variety of ecological roles ranging from antagonistic to facilitative. Despite
this, relatively few studies have focused on understanding microbial succession. Ecological principles and theories regarding ecosystem development derived from macroorganismal studies may apply to microbial systems, but the extent to which this is true remains unknown and warrants further study. Furthermore, how well the principles will apply to specific microbial functional groups remains largely unknown. In this regard, the CO-oxidizing bacteria are an interesting group. Our understanding of CO oxidizer ecology in itself remains limited, much less their roles in ecosystem development. Their ability to rapidly colonize and establish active communities on young volcanic deposits as well as remain a seemingly important part of mature soil communities spawns a number of questions. How do trends in CO-oxidizer community composition and diversity change with ecosystem development? Can CO oxidizers remain a competitive and active part of mature sites? What environmental factors drive their activity and distribution? Answering these questions will provide insights not only into the roles of CO oxidizers in succession, but also the general ecology of this abundant and ubiquitous functional group.

**Objectives of This Study**

The 1959 Pu’u Puai flow on the flank of Kilauea Volcano (Hawai’i) encompasses a vegetation gradient of uniform age and climate regime, which provides an ideal setting in which to study the interactions between plant development and the distribution of CO-oxidizing bacteria and served as the primary field site for this study. **Objective One**, was to use a culture-independent approach based on the coxL gene as well as activity measurements to investigate the distribution, diversity, community structure and relative abundance of CO-oxidizing bacteria across the vegetation gradient. Along with the distinct vegetation gradient, this site has a distinct
gradient in water regimes providing an opportunity to investigate the impacts of water stress on CO oxidizer activity and their ability to adapt to different water regimes experienced during succession (Objective Two). Results of accomplishing Objective One as well as efforts to enrich and isolate novel CO oxidizers provided the foundation for Objectives Three and Four. Culture-independent and dependent approaches indicated the potential importance of members in the genus *Burkholderia* in CO-oxidizing communities across the transect sites. **Objective Three** was to develop a qPCR approach to enumerate *coxL* gene abundance of this subset of the CO oxidizers across the transect. **Objective Four** was to investigate the physiology of novel CO-oxidizing burkholderias isolated from the sites and use molecular and physiological approaches to screen a wide variety previously isolated and described *Burkholderia* species to determine how widespread this trait is within the genus.

Results of completing the above objectives expand the known diversity of CO-oxidizing bacteria and provide insights into CO oxidizer diversity, abundance, distribution and resilience to water stress as a function of ecosystem development.
CHAPTER 2.
DISTRIBUTION AND DIVERSITY OF CARBON MONOXIDE-OXIDIZING BACTERIA AND BULK BACTERIAL COMMUNITIES ACROSS A SUCCESSION GRADIENT ON A HAWAIIAN VOLCANIC DEPOSIT
Introduction

Carbon monoxide-oxidizing bacteria are among primary colonists on recent volcanic deposits (Dunfield and King, 2004; King, 2003c; King and Weber, 2008). Initially, these deposits lack endogenous organic carbon to support heterotrophic metabolism, which forces early colonizers to rely on exogenous carbon and energy sources. Exogenous sources include atmospheric trace gases, such as CO. Although present at low concentrations in the atmosphere, 60-300 ppb (Crutzen and Gidel, 1983), CO serves as an effective energy (or carbon) source contributing up to about 6-9% of reducing equivalent flow for unvegetated tephra (King and Weber, 2008). Molecular surveys of the large subunit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase (cbbL) indicate that facultative lithotrophs, which include CO oxidizers, form diverse communities on volcanic deposits (Nanba et al., 2004). The ability of some facultative lithotrophs to rely on atmospheric CO for metabolism may contribute to their colonization success and roles in early ecosystem development.

In addition, results from activity assays and cloning and sequencing efforts based on coxL (large subunit gene of carbon monoxide dehydrogenase) reveal that CO-oxidizers are present and active in later stages of biotic succession (Dunfield and King, 2004; King, 2003; King and Weber, 2008; King et al., 2008). This is likely attributable to their metabolic and ecological versatility. Most CO oxidizers function preferentially as heterotrophs in laboratory culture, and at least some are capable of functioning mixotrophically, consuming organics and CO simultaneously (Kiessling and Meyer, 1982; Ro and Kim, 1993; Weber and King, 2007). In addition, many CO-oxidizing members of the Proteobacteria form N2-fixing symbioses with legumes, which include pioneering Acacia koa trees on volcanic deposits (King, 2003a; King
and Weber, 2007). Some members of the *Actinobacteria*, many of which are CO oxidizers, have also recently been associated with plant development on Mt. Pinatubo mudflows (Ohta *et al.*, 2003).

Recent molecular surveys and characterization of novel CO-oxidizing isolates have expanded the known diversity and metabolic versatility of this functional group (King and Weber, 2007). Little is known, however, about the diversity and distribution of CO oxidizers *in situ*, the environmental factors that govern diversity and distribution and how diversity relates to CO-oxidizer activity. Dunfield and King (2004) demonstrated that distinct CO oxidizer communities occurred on four volcanic deposits of varying age, chemical, physical and vegetation regimes. Clone libraries from unvegetated sites were overwhelmingly dominated by sequences from poorly characterized lineages, while vegetated sites were dominated by sequences derived from *Proteobacteria*. A survey of CO-oxidizers on volcanic deposits in Miyake-jima, Japan has revealed results similar to those from Hawai‘i (King *et al.*, 2008). These results paralleled findings of Gomez-Alvarez *et al.* (2007), who surveyed 16S rRNA gene diversity at sites established by King and co-workers. Collectively, the results suggest that CO-oxidizer community structure varies significantly among sites, but the sources of variation are unclear.

In a recent study, King and Weber (2008) described selected bacterial activities and physical and chemical properties along a short (< 10 m) transect of uniform age and climate regimes on Kilauea Volcano (Hawai‘i). The transect supports a vegetation gradient that provides a model system for investigating and understanding the distribution and diversity of CO oxidizers. With increasing vegetation and organic matter, *in situ* CO uptake rates decreased, while maximum potential CO uptake rates increased. These results suggested that as organic
substrate availability increases, CO-oxidizing communities continue to expand, but rely less on CO as a carbon and energy source.

In the present study, we investigated CO-oxidizer activity, abundance and diversity across this transect, along with overall bacterial community composition and diversity as assessed by 16S rRNA gene libraries. CO-oxidizer and total bacterial community compositions were statistically distinct and exhibited parallel shifts among the three sites. Increased diversity of both CO-oxidizers and total bacterial communities was positively correlated with increased vegetation influence and organic carbon. Most notably, however, coxL:16S rRNA gene OTU ratios indicated that as vegetation increases, CO-oxidizers account for an increasing fraction of total bacterial diversity.

**Materials and Methods**

**Site Descriptions**

Transect sites were established on a cinder deposit adjacent to Pu‘u Puai (19° 24’ 22.5” N X 155° 15’ 18.2” W), located on Kilauea Volcano (Hawai‘i Volcanoes National Park, Hawai‘i) at an elevation of about 1220 m (Figure 2.1). The Pu‘u Puai (“Gushing Hill”) deposit was the product of a series of eruptions of cinder, spatter and pumice from the Kilauea Iki Crater in 1959 (Hazlett, 2002). At present, Pu‘u Puai is characterized by a patchy distribution of mixed *Morella faya* (fire tree), and *Metrosideros polymorpha* (O’hia lehua) stands scattered among unvegetated tephra several meters thick. These stands form islands tens of meters in diameter. Within them, a litter layer up to 10 cm thick overlies the original tephra deposit, which has accumulated an organic-rich, peat-like soil. Adjacent unvegetated patches are comprised of cm to sub-cm sized cinders supporting sparse lichen growth. Transect sites include: 1) unvegetated tephra deposits 5 m from the edge of tree canopies (“Bare”); 2) the border between tree islands and unvegetated
Figure 2.1. a) Sampling sites Bare, Edge and Canopy on the 1959 Pu’u Puai volcanic deposit (Kilauea volcano, Hawai’i Volcano National Park), and b) surface materials at the Bare and Canopy sites. Scale bar shown in figure is applicable for both materials shown.
patches ("Edge"), and 3) areas within tree islands, at least 1 m from the Edge ("Canopy") (Figure 2.1). These three sites provide a distinct gradient in root biomass, water content, organic carbon and nitrogen, phospholipid phosphate content and acetylene reduction rates as described previously (King and Weber, 2008) and listed in Table 2.1.

**In situ and ex situ CO Fluxes and Maximum Potential CO oxidation Rates**

For Bare, Edge and Canopy sites, CO fluxes were determined *in situ* with ambient CO concentrations (~170 ppb) in March 2005 (Transect 1) and June 2005 (Transect 2). At each site, 3 ethanol-cleaned aluminum core tubes (7.5 cm diameter) were inserted 10-15 cm into the substrate about 1 m apart in a triangular pattern. Core tubes were capped with gas-tight caps with rubber septa sampling ports. Immediately after caps were in place, 3 cm³ gas samples were withdrawn from the core tube headspaces using needle and syringe and analyzed for CO content on a Trace Analytical RGD gas chromatograph as described previously (King, 1999b; King, 2003c). Headspaces were sampled at 3-min intervals for a total of 15 min. CO fluxes were also examined similarly in *ex situ* assays with 15 cm deep intact cores removed from the each of the sites. Maximum potential CO uptake rates for surface material (0-2 cm) collected aseptically from each site were determined using initial CO concentrations that saturate uptake (100 ppm). Within a few hours of collection, triplicate 5 g samples for each site were placed into ethanol-cleaned 110 cm³ jars that were capped with neoprene stoppers during uptake assays. One cm³ samples were removed at intervals with a needle and syringe and analyzed on a Trace Analytical RGD gas chromatograph.

**Most Probable Number (MPN) Estimates of CO Oxidizers**

Triplicate surface samples (0-2 cm) were aseptically collected from the three transect sites and placed into sterile “ziptop” plastic bags. Samples were transported to the laboratory at
ambient conditions, and processed within four days of collection. Debris and major roots (if any) were removed from all samples, and cinders in the Edge and Bare site samples were crushed. Triplicate 5 g samples (Edge and Canopy) or 12 g samples (Bare) were placed into sterile tubes with 30 mL of PYE medium (Meyer and Schlegel, 1983) containing 50 µg ml⁻¹ cyclohexamide to inhibit fungal growth. All nine tubes were incubated at room temperature on a rotating shaker for 5.5 h. The tubes were vortexed at maximum speed for 1 min and then centrifuged at 4,000 rpm for 1 min to pellet soil and cinders. Each of the nine supernatants was used to create three 10-fold dilutions. A 300 µl subsample of each of the 27 dilutions was serially diluted 5 times into 60 cm³ serum bottles containing 2.7 cm³ PYE-cyclohexamide medium. Each bottle was spiked with CO to an initial headspace concentration of 100 ppm. CO headspace concentrations in 27 arbitrarily selected bottles for each site were determined using gas chromatography to confirm initial CO concentrations. Headspace concentrations in all bottles were determined after 3 d and after 5 and 8 d for bottles that showed no uptake initially.

**DNA Extraction**

Surface samples (0-2 cm depth interval) were collected from Bare, Edge and Canopy sites using an ethanol-cleaned trowel and transferred into sterile bags. Leaf litter was removed from the surface of Edge and Canopy sites prior to sampling. Samples were transported to a laboratory in Maine on dry ice. Prior to DNA extraction, cinders from the Bare site were crushed and subjected to three cycles of freezing and thawing at -80 °C and 65 °C, respectively. Triplicate 10 g samples were extracted using a MoBio Ultraclean Mega Soil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s protocol. Large roots and other debris were removed from Edge and Canopy soils prior to freezing and thawing as described above. Triplicate 0.25 g samples from Edge and Canopy sites were extracted using a
MoBio UltraClean Soil DNA Isolation Kit (MoBio Laboratories, Inc. Carlsbad, CA) according to the manufacturer’s protocol. After extraction, Bare site DNA was concentrated using 5 M NaCl and 100% ethanol.

**PCR Amplification of 16S rRNA and **coxl** Genes**

For each of the sites, **coxl** was amplified in triplicate using 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, 2003a). PCR reactions were carried out in 50 µl volumes containing 5 µl of 10X Eppendorf PCR buffer, 10 µl of Eppendorf Taqmaster buffer, 3.5 mM MgCl₂, 0.1 µM of each primer, 100 µM of each deoxynucleoside triphosphate and 1.25 U of MasterTaq Polymerase (Brinkmann Inc. Westbury, NY). Template concentrations were varied to maximize PCR product yield. Amplification was carried out in an Eppendorf Mastercycler thermocycler (Brinkmann Inc. Westbury, NY) using the following 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 110 s and 3) final elongation at 72 °C for 10 min. PCR products were visualized using gel electrophoresis in 1% agarose stained with Gel Red (Biotium, Inc., Hayward, CA).

For each site, triplicate 16S rRNA gene PCR products were obtained using PCR primers 27f and 1492r (Lane, 1991; Sambrook et al., 1989). Concentrations of all other PCR reaction components were the same as described above for **coxl** amplification. Amplification was carried out using an Eppendorf Mastercycler Thermocycler (Brinkmann Inc. Westbury, NY) with the following program: 1) initial denaturation at 94°C for 3 min, 2) 30 cycles of denaturation at 94°C for 1 min, annealing at 55 °C for 1 min, elongation at 72 °C for 2 min and 3) final elongation at 72 °C for 10 minutes. PCR products were visualized as described above.
**16S rRNA Gene and coxL Library Construction**

For each site, triplicate coxL or 16S rRNA PCR products of the proper size (1,260 to 1,290 bp and 1500 bp, respectively) were pooled and purified using the MoBio UltraClean PCR Clean-up Kit (MoBio Laboratories, Inc., Carlsbad, CA). Within 24 h, purified products were cloned using a TOPO TA cloning Kit (Invitrogen Corporation, Carlsbad, CA). Transformed *E. coli* were grown at 37 °C on Luria Broth agar containing 50 µg ml⁻¹ kanamycin. Colonies were manually selected and lysed in 50 µL of PCR lysis solution (Epicentre Biotechnologies, Madison, WI) according to the manufacturer’s instructions. Vector inserts were amplified from the cell lysates in 50 µL PCR reactions containing 0.1 µM of vector primers T3 and T7. Concentrations of other PCR reaction components were as described above for coxL and 16S rRNA gene amplification. Reactions were carried out in an Eppendorf Mastercycler thermocycler (Brinkmann Inc. Westbury, NY) using the following program: 1) initial denaturation at 94 °C for 2 min, 2) 25 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, elongation at 72 °C for 1 min and 3) final elongation at 72 °C for 7 min. Amplified inserts of the proper size were purified using the MoBio UltraClean PCR Clean-up Kit (MoBio Laboratories Inc., Carlsbad, CA) and sequenced bidirectionally using an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Louisiana State University Genomics Facility (Baton Rouge, LA).

**coxL Clone Library Analyses**

Bidirectional coxL sequence reads were assembled, edited and translated using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Sequences that were form II or putative coxL genes (King and Weber, 2007) were eliminated from further analysis. Translated authentic form I coxL sequences were aligned using ClustalX version 1.0.1 (Thompson *et al.*, 2002).
Phylogenetic relationships were determined by completing a neighbor-joining analysis with 1,000 bootstrap replicates in PAUP (version 4.0b, Sinauer Associates, Inc. Sunderland, MA). All phylogenetic trees are rooted using a form II coxL gene sequence (King, 2003a).

In order to define operational taxonomic units (OTU) for the coxL libraries, inferred amino acid coxL sequences and 16S rRNA sequences from CO-oxidizing isolates were used to produce distance matrices using Phylip 3.67 (Felsenstein, University of Washington). CoxL similarities were plotted against 16S rRNA gene similarities (Figure B.1). The resulting relationship supported an OTU definition based on a coxL evolutionary distance of 0.1.

Numbers of OTUs, diversity indices, rarefaction curves and rank abundance curves for all coxL libraries were estimated at this distance using the software program, DOTUR (Schloss and Handelsmann, 2005).

Statistical differences among libraries were determined by performing pairwise comparisons of the libraries in webLIBSHUFF (Singleton et al., 2001) using 1000 bootstrap replicates. For comparison of all three of the libraries, the libraries were considered significantly different at a p-value of 0.0085, which was determined using the Bonferroni correction for multiple comparisons.

Statistical differences in the libraries were also determined by performing an Analysis of Molecular Variance (AMOVA) with Arlequin Software (Schneider et al., 2000), which estimates fixation indices (Fst values). Fst values near one indicate that variation within the libraries is small relative to variation among libraries, while Fst values near zero indicate that most variation in comparisons among libraries is found within them. Statistical significance is calculated based on the Fst values. The software also calculates nucleotide diversity, which is based on the variability in characters of aligned sequences and performs a mismatch analysis. The mismatch
analysis determines the frequency at which a given number of mismatched nucleotides occurs in pairwise comparison of sequences. The frequency distribution of mismatches is a function of the evolutionary distances among sequences in a library.

**16S rRNA Gene Library Analysis**

Bidirectional reads were assembled and edited manually using Sequencher 4.8 (Gene Codes Corporation, Ann Arbor, MI). Sequences were aligned using the NAST alignment tool ([http://www.greengenes.lbl.gov](http://www.greengenes.lbl.gov); DeSantis et al. 2006). In the alignment process, sequences less than 800 bp in length or that did not have at least 80% similarity to other 16S rRNA gene sequences in the database were eliminated from analysis. All sequences were checked for chimeras using Mallard ([http://www.cf.ac.uk.biosi/research/biosoft](http://www.cf.ac.uk.biosi/research/biosoft); Ashelford et al., 2006). Chimeric sequences were eliminated from subsequent phylogenetic and statistical analyses.

Numbers of OTUs in each of the 16S rRNA gene libraries were determined using an evolutionary distance of 0.03 in DOTUR (Schloss and Handelsmann, 2005). Libraries were also analyzed using webLIBSUFF and Arlequin software as described above. Each non-chimeric clone was classified at the phylum level using the Classifier Tool in the Ribosomal Database Project II release 9.59 ([http://rdp.cme.msu.edu/index.jsp](http://rdp.cme.msu.edu/index.jsp); Wang et al. 2007). Because of their dominance in all three 16S rRNA gene libraries, *Acidobacteria* sequences were subjected to separate phylogenetic, diversity and statistical analyses and the results have been detailed in Appendix D (Tables D.1-D.3). Unclassified sequences from the Bare site were also subjected to further phylogenetic analysis, as detailed in Appendix D (Figure D.2).

**Nucleotide Accession Numbers**

The 16S rRNA gene and *coxL* sequences in this study have been deposited in GenBank under accession numbers FJ465965-FJ466449 and FJ465756-FJ465964, respectively.
Results

CO-oxidizer Activity and Abundance

Patterns of in situ and ex situ atmospheric CO fluxes for Transects 1 and 2 were similar (Table 2.1). Net atmospheric CO consumption was observed at the Bare site, and was at least 1.5-times greater than net consumption observed at the Edge and Canopy sites. Net consumption was observed for Edge and Canopy sites on Transect 1, while net production was observed at these sites on Transect 2. Similar results were obtained from ex situ CO assays with intact cores. Maximum potential CO uptake rates displayed the opposite trend, with the lowest rates of consumption observed in the Bare sites (30 ± 15 nmol CO gdw⁻¹d⁻¹), and increased rates observed in the Edge (650 ± 200 nmol CO gdw⁻¹d⁻¹) and Canopy (5400 ± 570 nmol CO gdw⁻¹d⁻¹) sites. This 180-fold increase in maximum uptake rates in the Canopy site was accompanied by as much as a 5,000-10,000-fold increase in numbers of CO-oxidizers as determined by MPN estimates (Table 2.1).

Phylogenetic Analyses of coxL and 16S rRNA Gene Libraries

A total of 209 coxL gene sequences derived from Bare (76), Edge (60) and Canopy (73) sites were successfully sequenced and translated. The primers also amplified a small number of form II putative coxL or other molybdenum hydroxylase sequences from the three sites. A total of 485 non-chimeric 16S rRNA gene fragments were successfully sequenced from Bare (131), Edge (178) and Canopy (176) sites. From the latter libraries 14, 2 and 5 sequences were chimeric and eliminated, respectively.

The phylogenetic composition of coxL libraries encompassed the currently known phylogenetic breadth of CO-oxidizers, which spans the Firmicutes, Actinobacteria and Proteobacteria (Figure 2.2, Table 2.2). Libraries from vegetated sites (Edge and Canopy) were
Table 2.1. All quantities are the average of three replicates with the standard error reported in parentheses. Maximum CO uptake rates were determined using saturating concentrations of CO (100 ppm). *In situ* CO fluxes were determined under ambient conditions. *Ex situ* CO fluxes were determined under ambient conditions with intact cores. Transect 1= data collected in March 2005. Transect 2= data collected at an adjacent site in June 2005. "*"= Physical and chemical characteristics determined for surface samples (0-2 cm) at each of the transect sites as reported in King and Weber (2008).

<table>
<thead>
<tr>
<th>Site Characteristic</th>
<th>Bare</th>
<th>Edge</th>
<th>Canopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>6.9 (0.1)</td>
<td>4.5 (0.2)</td>
<td>3.9 (0.2)</td>
</tr>
<tr>
<td>Root Biomass* (gdw m$^{-2}$)</td>
<td>0 (0)</td>
<td>177 (43)</td>
<td>206 (34)</td>
</tr>
<tr>
<td>Water content* (g gfw$^{-1}$)</td>
<td>0.01 (0.00)</td>
<td>0.11 (0.02)</td>
<td>0.39 (0.10)</td>
</tr>
<tr>
<td>Total N* (%)</td>
<td>0.021 (0.006)</td>
<td>0.121 (0.022)</td>
<td>1.683 (0.022)</td>
</tr>
<tr>
<td>Total C* (%)</td>
<td>0.329 (0.102)</td>
<td>2.771 (0.523)</td>
<td>45.723 (0.795)</td>
</tr>
<tr>
<td>Transect 1: <em>In situ</em> CO fluxes (mg m$^{-2}$ d$^{-1}$)</td>
<td>3.2 (0.9)</td>
<td>2.2 (0.5)</td>
<td>1.3 (0.8)</td>
</tr>
<tr>
<td>Transect 2: <em>In situ</em> CO fluxes (mg m$^{-2}$ d$^{-1}$)</td>
<td>2.4 (0.7)</td>
<td>-2.5 (0.2)</td>
<td>-1.6 (0.5)</td>
</tr>
<tr>
<td>Transect 1: <em>Ex situ</em> CO fluxes (mg m$^{-2}$ d$^{-1}$)</td>
<td>1.6 (0.3)</td>
<td>1.3 (0.3)</td>
<td>0.02 (1)</td>
</tr>
<tr>
<td>Transect 2: <em>Ex situ</em> CO fluxes (mg m$^{-2}$ d$^{-1}$)</td>
<td>7.1 (3.1)</td>
<td>-1.2 (0.4)</td>
<td>-1.1 (1.1)</td>
</tr>
<tr>
<td>Maximum CO uptake (nmol gdw$^{-1}$ d$^{-1}$)</td>
<td>30 (15)</td>
<td>650 (200)</td>
<td>5400 (570)</td>
</tr>
<tr>
<td>CO-oxidizer MPN (cells gdw$^{-1}$)</td>
<td>360 (170)</td>
<td>4.8X10$^6$ (2.5X10$^6$)</td>
<td>4.9X10$^6$ (4.5X10$^5$)</td>
</tr>
</tbody>
</table>

dominated by *Proteobacteria*, which comprised as much as 75.3% of the sequences (Table 2.2). *α-Proteobacteria* were particularly abundant in the Edge library (about 67%), but accounted for only 42% of the Canopy library; *β-Proteobacteria* varied from 8% to 26% in Edge and Canopy libraries, respectively. The overwhelming majority of *coxL* sequences in the Bare library (69.7%) were affiliated with a clade tentatively assigned to *Firmicutes* (Figure 2.2). Other Bare library...
sequences cluster phylogenetically with CO-oxidizing members of phylum Actinobacteria, Nocardioides sp. JS16, Rhodococcus sp. RHA1 and various species and strains in the genus Mycobacterium (Figure 2.2).

Classification of 16S rRNA gene sequences using the Classifier Tool in RDPII (Wang et al., 2007) revealed that the phyla Acidobacteria, Actinobacteria, Proteobacteria (α-subphylum), Cyanobacteria, Planctomycetes and Verrucomicrobia were represented in all libraries (Table 2.2). With increasing plant development, however, there were distinct compositional shifts. Representatives of the Chloroflexi, Thermomicrobia and OP10 phyla were only found in the Bare library, while β- and γ-Proteobacteria and Bacteroidetes were only found in the Edge and Canopy libraries (Table 2.2; Figure 2.3). Distinct compositional shifts also occurred within the phylum Acidobacteria (Table B.1).

Out of 485 16S rRNA gene sequences, more than half (261) was classified as Acidobacteria, which was the dominant phylum in each of the libraries (Table 2.2; Figure 2.3). This phylum comprised 43.5, 60.1 and 55.1 % of Bare, Edge and Canopy libraries, respectively; sub-group composition varied among the libraries (Table B.1). For both the Edge and Canopy libraries, Proteobacteria comprised the second most abundant phylum at 21.3 and 29%, respectively, and were distributed among α, β, γ and δ sub-groups (Table 2.2). The Bare library contrasted sharply with the Edge and Canopy libraries in that 13% of the sequences were comprised of Cyanobacteria, while this phylum comprised 1% or less of the latter libraries.

Much smaller fractions of each library were comprised of Planctomycetes (1.5-6%), Actinobacteria (0.6-4.5%) and Verrucomicrobia (0.8-3.4). A number of sequences in Bare and Canopy 16S rRNA gene libraries could not be confidently assigned to any phylum using the Classifier Tool in the Ribosomal Database Project II. In the Bare library, 32 sequences were
Table 2.2. Phylogenetic composition (%) of the 16S rRNA gene and *coxL* libraries. “ND”= not determined. For the 16S rRNA gene libraries some of the sequences could only be classified as “possible firmicutes” and these are included in the “unclassified” category with other unclassified sequences.

<table>
<thead>
<tr>
<th>Phylogenetic Group (%)</th>
<th>16S rRNA gene</th>
<th></th>
<th></th>
<th></th>
<th><em>coxL</em></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bare</td>
<td>Edge</td>
<td>Canopy</td>
<td>Bare</td>
<td>Edge</td>
<td>Canopy</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Acidobacteria</em></td>
<td>43.5</td>
<td>60.1</td>
<td>55.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Actinobacteria</em></td>
<td>2.3</td>
<td>4.5</td>
<td>0.6</td>
<td>27.6</td>
<td>31.7</td>
<td>24.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacteroidetes</em></td>
<td>0</td>
<td>3.9</td>
<td>4.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proteobacteria</em></td>
<td>5.3</td>
<td>21.3</td>
<td>29</td>
<td>2.6</td>
<td>68.4</td>
<td>75.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α</td>
<td>4.6</td>
<td>11.2</td>
<td>16.5</td>
<td>2.6</td>
<td>66.7</td>
<td>42.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β</td>
<td>0</td>
<td>2.2</td>
<td>4</td>
<td>0</td>
<td>1.7</td>
<td>32.9</td>
<td></td>
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<tr>
<td>γ</td>
<td>0</td>
<td>5.1</td>
<td>5.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ</td>
<td>0.8</td>
<td>2.8</td>
<td>3.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td><em>Chloroflexi</em></td>
<td>24.4</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Firmicutes</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>69.7</td>
<td>0</td>
<td>0</td>
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<tr>
<td><em>Clostridia</em></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cyanobacteria</em></td>
<td>13</td>
<td>0.6</td>
<td>1.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OP10</td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Planctomycetes</em></td>
<td>1.5</td>
<td>6.2</td>
<td>4.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Verrucomicrobia</em></td>
<td>0.8</td>
<td>3.4</td>
<td>3.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Thermomicrobium</em></td>
<td>0.8</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unclassified</td>
<td>7.6</td>
<td>0</td>
<td>2.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 2.2.** Neighbor-joining tree (1000 bootstrap replicates) of inferred amino acid *coxL* sequences. Each branch represents one OTU defined at an evolutionary distance of $\leq 0.10$. Where one OTU was comprised of sequences from more than one site, a representative clone sequence from each site is listed. Bootstrap values $\geq 70$ are shown. Bare, Edge and Canopy sequences are denoted by B, E and C, respectively. Accession numbers: *Bacillus schlegelii* (AY463246); *Bradyrhizobium japonicum* USDA 6 (AY307921); *Carphophilus carboxydus* (FJ466455); *Mesorhizobium* sp. KP12W (FJ152139); *Shinella zoogloeoides* str. FG1M5 (FJ152138); *Stappia aggregata* (AY307918); *Stenotrophomonas* str. LUP (AY307920); *Oligotropha carboxidovorans* (CAA57829); *Zavarzinia compransoris* (AY463247); *Silicibacter pomeroyi* DSS-3 (YP166760); *Ruegeria* sp. WHOI JT-08 (AAW88347); *Burkholderia xenovorans* LB400 (YP558874); *Burkholderia* str. PP51-3 (FJ466454); *Burkholderia* str. CP11 (FJ152141); *Burkholderia* str. PP52-1 (FJ152140); *Hydrogenophaga pseudoflava* (HPU80806); *Pseudomonas thermocarboxydovorans* (X77931); *Alkalilimnicola ehrlichii* MLHE1 (YP742401); *Arthrobacter* sp. FB24 (YP831519); *Mycobacterium tuberculosis* H37Ra (NP214887); *Mycobacterium marinum* (YP001848973); *Mycobacterium gordonae* (AY333109); *Rhodococcus* sp. RHA1 (YP705171); *Nocardiooides* sp. JS16 (YP921462 and YP921462); B1(FJ465756); B2 (FJ465757); C60 (FJ465890); E4 (FJ465908); C2 (FJ465833); E19 (FJ465922); C57 (FJ465887); E10 (FJ465913); E13 (FJ465916); C14 (FJ465845); E15 (FJ465918); E21 (FJ465924); B18 (FJ465773); B30 (FJ465785); E60 (FJ465962); C56 (FJ465886); E9 (FJ465912); C18 (FJ465849); E61 (FJ465963); C52 (FJ465882); C1 (FJ465832); C13 (FJ465844); C50 (FJ465880); C15 (FJ465846); C72 (FJ465902); E42 (FJ465944); C73 (FJ465903); E1 (FJ465905); C19 (FJ465850); C26 (FJ465857); C27 (FJ465858); E7 (FJ465910); C28 (FJ465859); B73 (FJ465826); C31 (FJ465862); B29 (FJ465784).
Rhodobacter sphaeroides Form II coxl

Bacillus schlegelii

Bradyrhizobium japonicum USDA 6
Carbophilus carboxydus
Mesorhizobium sp. KP12W
Shinella zoogloeoides str. FG1M5
E4
C2
Stappia aggregata
Stenotrophomonas str. LUP
E19, C57
E10
E13, C14
E15
E21
B18
B30, E60
C56, E9
Oligotropha carboxidivorans
Zavarzinia compransoris
Siletbacter pomeroyi DSS-3
Ruegeria sp. JT-08
Burkholderia xenovorans LB400
Burkholderia str. PP51-3
Burkholderia str. CP11
Burkholderia str. PP521
C18, E61
C52
C1
C13
C60
Hydrogenophaga pseudoflava
Pseudomonas thermocarboxydovorans
Alkalamincola ehrlichei MLHE1
Arthrobacter sp. FB24
C15
Mycobacterium tuberculosis H37Ra
Mycobacterium marinum
Mycobacterium gordonae
Rhodococcus sp. RHA1
Mycobacterium smegmatis
C72
Nocardoides sp. JS16
E42
C73
E1
C19
Nocardoides sp. JS16 (gene 2)
C26
C27, E7
C28
C29
B73, C31

0.05 changes
Figure 2.3. Neighbor-joining trees (1000 bootstrap replicates) of representative OTUs (evolutionary distance ≤ 0.03) from the Bare 16S rRNA (a), Edge 16S rRNA (b), and Canopy
16S rRNA (c) gene libraries. Methanococcus maripaludis (AF005049) was used as an outgroup in
all trees. Only bootstrap values ≥ 70 are displayed. a) Accession numbers: Thermosinus
carboxydivorans str. Norl ctg53 (AAWL01000002); Ktedobacter racemifer str. SOSP1-21
(AM180156); Chthoniobacter flavus Ellin428 (AY388649); Blastococcus sp. str. CNJ868 PL04
(DQ448697); Conexibacter woeni DSM 14684T (AJ440237); Methylcyctis sp. str. Ch22
(AJ458487); Acidiphilium sp. str. DY1-3 (EF585285); Acidisphaera sp. str. NO-15 (AF376024);
Rhodopila globiformis (M59066); Solibacter usitatus str. Ellin6076 (AAIA01000029);
Edaphobacter modestum str. Wbg-1 (DQ528761); Peat Bog Clone TM2 (X97098);
Acidobacterium capsulatum (D26171); Nostoc sp. “Pannaria isabellina cyanobiont” 2Ch
(EF174226); Petalonia sp. ANT.GENTNER2.8 (AY493624); Tolypothrix sp. IAM M-259
(AB093486); Oscillatoria sp. PCC7112 (AB074509); Physcomitrella patens subsp. patens
(AP005672); Chlorella saccarophila str. 211-1d (D13149); Nostocoida limicola III str. Ben225
(AF244752); Pirellula sp. Schlesner 382 (X81943); B64 (FJ466090); B55 (FJ465972); B205
(FJ465973); B400 (FJ466023); B158 (FJ465982); B126 (FJ466043); B165 (FJ466077); B18
(FJ466018); B54 (X81944); B401 (FJ465971); B119 (FJ466014); B402 (FJ466048); B403
(FJ465966); B404 (FJ465984); B65 (FJ466005); B405 (FJ466016); B25 (FJ466025); B406
(FJ466050); B66 (FJ466095); B150 (FJ465983); B16 (FJ465974); B174 (FJ466022); B407
(FJ466081); B408 (FJ466087); B171 (FJ465995 ); B50 (FJ465998); B409 (FJ465968); B11
(FJ466030); B410 (FJ466042); B31 (FJ466071); B134 (FJ466067); B17 (FJ466053); B186
(FJ465991); B195 (FJ466024); B162 (FJ466044); B142 (FJ466017); B115 (FJ466008); B45
(FJ466089); B411 (FJ466019); B67 (FJ465978); B197(FJ466034); B210 (FJ466011); B412
(FJ466062); B413 (FJ466088); B62 (FJ466092); B69 (FJ466094); B208 (FJ466076); B70
(FJ466056); B140 (FJ465975); B414 (FJ466072); B415 (FJ466054); B416 (FJ466007); B143
(FJ466068); B417 (FJ465986); B28 (FJ465994); B156 (FJ466033); B418 (FJ466052); B176
(FJ466020); B21 (FJ466084); B187 (FJ465993); B138 (FJ466006); B32 (FJ465997); B155
(FJ466066); B125 (FJ466021); B61 (FJ465977); B214 (FJ465977); B178 (FJ466001); B419
(FJ466032); B198 (FJ466073). b) Accession numbers: Sphingoterrabacterium koreensis str.
Gsoil 3017 (AB267721); Niastella jeongjuensis str. GR20-13 (DQ244076); Edaphobacter
aggregans str. Wbg-1 (DQ528761); Acidobacteria str. Ellin345 (NC_008009); Solibacter
usitatus str. Ellin6067 (AAIA01000029); Chthoniobacter flavus Ellin428 (AY388649); Cryocola
sp. KAR37 (EF451667); Actinomycetales str. Ellin143 (AF408985); Mycobacterium savonii
siae str. E533T (AJ748836); Conexibacter woeni (AJ440237); Haliagnum tepidum (AB062751);
Chondromyces pediculatus str. Cm p17 (AJ233940); Aquicella lusitana str. SGT-39T
(AY359282); Dyella sp. CHNCT5 (EF471222); Sideroxysdans paludicola str. BrT (DQ386858);
Burkholderia sp. 14 (AY238506); Azospirillum amazonense str. 21R (AY741146); Acidiphilium
sp. CCP3 (AY766000); Rhizobiales bacterium A48 (AB081581); Asticcacaulis sp. SA7
(AB093140); Bradyrhizobium elkanii str. CBBAU 61184 (EF061103); Methylosinus
tricosporium (AF150804); Isosphaera sp. str. Schlesner 666 (X81958); Gemmata obscuriglobus
str. Schlesner 633 (X81957); Pirellula staleyi (M34126); E33 (FJ466279); E124 (FJ466345);
E400 (FJ466384); E48 (FJ466415); E175 (FJ466296); E401 (FJ466318); E178 (FJ466378); E7
(FJ466434); E46 (FJ466406); E116 (FJ466285); E177 (FJ466354); E402 (FJ466324); E109
(FJ466295); E403 (FJ466341); E10 (FJ466306); E45 (FJ466409); E125 (FJ466313); E113
(FJ466289); E404 (FJ466360); E197 (FJ466418); E405 (FJ466327); E6 (FJ466430); E160
(FJ466371); E183 (FJ466348); E105 (FJ466303); E195 (FJ466401); E137 (FJ466367); E42
c) Accession numbers:
Prosthecobacter dejongeii (U60012); Cytophagales str. MBIC4147 (AB022889);
Flavosolibacter ginsengiterrae str. Gsoil 643 (AB267477);
Sphingoterrabacterium koreensis str. Gsoil3017 (AB267721); Flexibacter Canadensis str. IFO 15130 (AB078046);
Rhodopila globiformis (M59066); Phenylobacterium immobile str. ET (Y18216);
Chlamydiae sp. str. FWC38 (AJ227774); Methylosinus trichosporium str. KS21 (AJ431385);
Rhizobiales genosp. AB CsBT10/2 (AJ810382); Erwinia mallotivora str. DSM 4565 (AJ233414);
Bradyrhizobium elkanii str. CCBAU 61184 (AF061103); Stella humosa str. DSM5900 (AJ535710);
Nitrosospira sp. str. Nsp57 (AY123791); Beggiatoa alba str. ATCC 33555 (AF110274);
Neveskia soli str. GR15-1 (EF178286); Chondromyces pediculatus str. Cm p17 (AJ233940);
Haliangium tepidum (AB062751); Stigmatella koreensis str. KYC-1019 (EF112185); “Desulfuromonas acetexigens” (U23140);
Bdellovibrio str. HD100 DSM 50701 (BX842648); Fervorhabdus acidiphilum str. T23 (AF251436);
Pirellula sp. str. 139 (X81945); Gemmata obscuriglobus str. ACM 2246 (X85248);
Ecalyptus subsp. globulus (AY780259); Thermosinus carboxydivorans str. Nor1 ctg53 (AAW0100002);
Pirellula sp. str. 678 (X81957); Nostocoida lenticulare III str. Ben222 (AF244749);
C216 (FJ466129); C214 (FJ466128); C118 (FJ466222); C130 (FJ466232); C44 (FJ466196); C189 (FJ466158); C210 (FJ466125); C194 (FJ466116); C183 (FJ466109); C32 (FJ466189); C3 (FJ466147); C85 (FJ466099); C69 (FJ466135); C203 (FJ466121); C104 (FJ466248); C100 (FJ466246); C75 (FJ466139); C156 (FJ466262); C72 (FJ466212); C62 (FJ466209); C64 (FJ466168); C107 (FJ466250); C196 (FJ466117); C211 (FJ466126); C75 (FJ466103); C95 (FJ466153); C49 (FJ466198); C36 (FJ466191); C73 (FJ466138); C163 (FJ466268); C53 (FJ466203); C212 (FJ466127); C167 (FJ499096); C70 (FJ466136); C126 (FJ466229); C84 (FJ466146); C169 (FJ466098); C68 (FJ466134); C106 (FJ466249); C51 (FJ466201); C5 (FJ466202); C97 (FJ466155); C17 (FJ466176); C120 (FJ466224); C164 (FJ466269); C31 (FJ466188); C83 (FJ466145); C21 (FJ466180); C215 (FJ466163); C2 (FJ466178); C39 (FJ466165); C86 (FJ466148); C113 (FJ266218); C77 (FJ466141); C185 (FJ466111); C110 (FJ466252); C96 (FJ466154); C162 (FJ466267); C200
(FJ466118); C176 (FJ466104); C197 (FJ466160); C81 (FJ466144); C123 (FJ466227); C172
(FJ466100); C46 (FJ466197); C168 (FJ466097); C116 (FJ466221); C79 (FJ466142); C166
(FJ466271); C11 (FJ466171); C43 (FJ466195); C205 (FJ466122); C23 (FJ466182); C80
(FJ466143); C121 (FJ466225); C184 (FJ466110); C179 (FJ466106); C55 (FJ466204); C98
(FJ466156); C71 (FJ466137); C63 (FJ466132); C187 (FJ466113); C173 (FJ466101); C128
(FJ466253); C94 (FJ466152); C34 (FJ466190); C177 (FJ466105); C201 (FJ466119); C157
(FJ466263); C190 (FJ466114); C74 (FJ466213); C136 (FJ466237); C155 (FJ466261); C154
(FJ466260); C218 (FJ466130); C143 (FJ466244); C182 (FJ466108).
Figure 2.3a.
(Figure 2.3b.)
(Figure 2.3c.)
identified as “possible Chloroflexus”. Further phylogenetic analysis demonstrated that these sequences indeed represented members of the *Chloroflexus*, and comprised the second most abundant phylum (24.4%) in the Bare library (Table 2.2; Figure B.2). The remaining unclassified sequences were designated as possible members of the OP10, *Actinobacteria* and *Firmicutes*. Unclassified sequences in the Canopy site were designated as possible members of the *Chlorobi*, *Bacteroidetes* or *Firmicutes* (Table 2.2).

**Statistical Comparisons of Libraries**

Based on webLIBSHUFF analyses, all possible pairings of the *coxL* libraries were significantly different (p-value < 0.001). For all three libraries, homologous coverages at an evolutionary distance of 0.10 were 90% or greater (Table 2.3). Heterologous coverages were highest for reciprocal comparisons of Edge and Canopy libraries (57 and 45%), indicating these libraries were most similar of the possible pairings (Table 2.3). Heterologous coverages were lowest for reciprocal comparisons of Edge and Bare libraries (1.3 and 8.3%). All possible pairings of 16S rRNA gene libraries were also significantly different based on webLIBSHUFF analysis (p < 0.002) with the highest heterologous coverages between reciprocal comparisons of the Edge and Canopy libraries and lowest coverages were observed for Bare and Canopy comparisons (Table 2.3).

Pairwise F$_{st}$ values derived from AMOVA analyses were significant at a p-value of 0.05 level for the *coxL* and 16S rRNA gene libraries, indicating that the libraries from each site were distinct (Table 2.4). All pairwise F$_{st}$ values for the *coxL* library comparisons were higher than for the corresponding values for the 16S rRNA gene libraries. Average pairwise differences for the *coxL* and 16S rRNA gene libraries exhibited the same trends (Table 2.4). Due to their dominance in all of the 16S rRNA gene libraries, sequences assigned to *Acidobacteria* were
subjected to a separate set of analyses in webLIBSHUFF and Arlequin; results of these analyses demonstrated that the *Acidobacteria* fractions of each of the three libraries were distinct (Table B.2).

**Table 2.3.** Homologous and heterologous coverages as determined by webLIBSHUFF analyses of 16S rRNA gene libraries and *coxL* libraries. Coverages are listed as percentages. Comparisons of 16S rRNA gene libraries or *coxL* libraries were made at evolutionary distances of 0.03 and 0.10, respectively. All comparisons between homologous and heterologous coverages were determined to be statistically significant at the p-values listed.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>16Sr RNA gene</th>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C_x</td>
<td>C_xy</td>
<td>C_yx</td>
<td>P-value</td>
<td>C_x</td>
<td>C_xy</td>
<td>C_yx</td>
<td>P-value</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bare (x) vs. Edge (y)</td>
<td>67</td>
<td>12</td>
<td>12</td>
<td>0.001</td>
<td>97</td>
<td>1.3</td>
<td>9.8</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edge (x) vs. Canopy (y)</td>
<td>66</td>
<td>51</td>
<td>44</td>
<td>0.001</td>
<td>97</td>
<td>58</td>
<td>45</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Canopy (x) vs. Bare (y)</td>
<td>58</td>
<td>5.1</td>
<td>3.1</td>
<td>0.001</td>
<td>91</td>
<td>12</td>
<td>22</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4.** Pairwise fixation indices (F_{st} values) and corrected average pairwise differences (θ[π]) for 16S rRNA gene library comparisons (below the diagonal) and for *coxL* library comparisons (above the diagonal).

<table>
<thead>
<tr>
<th>Pairwise Fixation Indices (F_{st} values)</th>
<th>Bare</th>
<th>Edge</th>
<th>Canopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
<td>0.31268</td>
<td>0.26349</td>
<td></td>
</tr>
<tr>
<td>Edge</td>
<td>0.10054</td>
<td>0.09397</td>
<td></td>
</tr>
<tr>
<td>Canopy</td>
<td>0.07505</td>
<td>0.01981</td>
<td></td>
</tr>
<tr>
<td>Corrected Average Pairwise Differences (θ[π])</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bare</td>
<td>193.9</td>
<td>167.1</td>
<td></td>
</tr>
<tr>
<td>Edge</td>
<td>18.1</td>
<td>47.7</td>
<td></td>
</tr>
<tr>
<td>Canopy</td>
<td>14.2</td>
<td>3.2</td>
<td></td>
</tr>
</tbody>
</table>

**Diversity Indices**

The total genetic variation (θ) and nucleotide diversity differences for both *coxL* and 16S rRNA gene libraries were lowest in the Edge and highest in the Canopy libraries (Table 2.5). Similar trends were observed among the 16S rRNA gene libraries. Mismatch frequency
distributions for all *coxL* libraries revealed that most of the mismatches occurred between closely related taxa, but distantly related taxa were distributed evenly within and among libraries (Figure 2.4). Bare and Canopy 16S rRNA gene libraries had flat mismatch frequency plots, indicating an even distribution of closely and distantly related taxa (Figure 2.4). In contrast, the Edge 16S rRNA gene library contained a much higher number of sequences than Bare and Canopy with mismatches in the 30 to 70-nucleotide range (Figure 2.4). Further analysis revealed that *Acidobacteria* sequences in the Edge library accounted for this unique trend in the mismatch frequency plot.

Using a definition based on an evolutionary distance of 0.1, 30 OTUs were identified across all *coxL* libraries. Rarefaction curves indicated that only 76 sequences representing 6 OTUs were necessary to observe a plateau in OTU discovery for the Bare site (Figure 2.5). In contrast, with similar sampling efforts, the Edge and Canopy rarefaction curves did not reach a plateau at all (Figure 2.5). The 73 Canopy sequences represented 19 OTUs, and the 60 Edge sequences represented 12 OTUs. Of the 30 OTUs identified among the libraries, 22 were represented in only one library: twelve, six and four OTUs occurred in Canopy, Edge and Bare, respectively. Six OTUs were shared between the Canopy and Edge libraries, one OTU was shared between the Canopy and Bare libraries, and one OTU was shared among all three.

ChaoI, a nonparametric index based on the probability of resampling an OTU in a particular library (Hughes and Bohannon, 2004), was calculated for each *coxL* library. Of the OTUs predicted by the ChaoI index, 85, 96 and 78 % were sampled in the Bare, Edge and Canopy libraries, respectively. Ace (*S_{ace}* ) and ChaoI indices were of similar magnitude (Table 2.5). The Shannon Index, which is more heavily weighted by the rare OTUs in a library, was
substantially lower in the Bare site than in the Canopy site; however, the 95% confidence interval overlapped for the Edge and Canopy libraries.

Table 2.5. Diversity estimates for the 16S rRNA gene and coxL libraries. The 95% confidence intervals for the $S_{ae}$, ChaoI and Shannon indices are in parentheses. The standard deviation for $\theta$ (genetic variation) and nucleotide diversity are in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>16S rRNA</th>
<th>coxL libraries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site</td>
<td>Bare</td>
</tr>
<tr>
<td>Clones</td>
<td></td>
<td>131</td>
</tr>
<tr>
<td>Diversity Estimates</td>
<td>No. of OTUs</td>
<td>69</td>
</tr>
<tr>
<td>$S_{ae}$</td>
<td></td>
<td>158</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(114,246)</td>
</tr>
<tr>
<td>ChaoI</td>
<td></td>
<td>155.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(107,262.7)</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td></td>
<td>3.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.81, 4.13)</td>
</tr>
<tr>
<td>Simpson (1/D)</td>
<td></td>
<td>57.1</td>
</tr>
<tr>
<td>$\theta$</td>
<td></td>
<td>204</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(88)</td>
</tr>
<tr>
<td>Nucleotide diversity</td>
<td></td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.11)</td>
</tr>
</tbody>
</table>

Although the Edge and Canopy coxL libraries had similar phylogenetic compositions at phylum levels, rank abundance curves showed that the OTUs were distributed quite differently (Figure 2.6). The most abundant OTU in the Edge library belonged to the phylum, *Actinobacteria*; this was followed by five members assigned to the $\alpha$-Proteobacteria with the remainder of the library consisting of less abundant $\alpha$-Proteobacteria and *Actinobacteria* sequences, and only one representative assigned to the $\beta$-Proteobacteria. In contrast, the most abundant OTU in the Canopy library was a representative of the $\beta$-Proteobacteria, with the remaining OTUs relatively evenly distributed among the *Actinobacteria*, $\alpha$- and $\beta$-Proteobacteria. The two most abundant OTUs in the Bare library were represented by
sequences assigned to *Firmicutes* (Figure 2.6), followed by sequences assigned to *Actinobacteria* and *α-Proteobacteria*. The increase in *coxL* OTU abundance across the transect was most highly correlated with the increased percentage of *β-Proteobacteria* OTUs at each site (*r* = 0.987). Correlations with the percentage of OTU *α-Proteobacterial* and *Actinobacterial* OTUs at each site were much weaker (*r* = 0.550 and 0.089, respectively).

**Figure 2.4.** Mismatch frequency plots for *coxL* libraries (a), 16S rRNA gene libraries (b), and cumulative mismatch plots for *coxL* (c) and 16S rRNA gene libraries (d).

The 16S rRNA gene sequences in the Bare, Edge and Canopy libraries represented 69, 91 and 100 OTUs, respectively, at an evolutionary distance of 0.03. None of the corresponding rarefaction curves reached a plateau. When the three libraries were pooled, 230 OTUs were
identified at an evolutionary distance of 0.03 of which 66, 59 and 70 OTUs were unique to Bare, Edge and Canopy libraries respectively. Thirty-one OTUs were shared between the Canopy and Edge libraries, two were shared between Edge and Bare libraries, one was shared between the Bare and Canopy libraries, and one was shared among all three. In addition, separate analyses of major *Acidobacteria* and *Proteobacteria* revealed that few of these OTUs were shared among libraries (Tables B.3 and B.4).

**Figure 2.5.** Rarefaction curves for *coxL* OTUs (evolutionary distance of ≤ 0.10) and 16S rRNA OTUs (evolutionary distance of ≤ 0.03) recovered from all sites, Bare site, Edge site and Canopy sites. Note the different scale on the y-axes.

The numbers of OTUs sampled by the 16S rRNA libraries were only 42-47 % of those predicted by the ChaoI index. The ChaoI index for the Bare library was lower than that of the Edge and Canopy libraries, but the 95% confidence intervals for all of the libraries overlapped and were not significantly different (Table 2.5). ChaoI indices did not plateau for the number of sequences sampled at any site. Ace indices were of similar magnitude as ChaoI, but consistently higher (Table 2.5). Shannon indices for the three 16S rRNA gene libraries were lowest for the Bare library followed by the Edge and the Canopy site (Table 2.2). The 95% confidence interval for Bare and Canopy did not overlap (Table 2.5). In contrast, the reciprocal Simpson Index
(1/D) indicated that the Canopy library was the richest followed by the Bare and Edge libraries, respectively.

Rank abundance analysis for the 16S rRNA gene libraries revealed that the Bare, Edge and Canopy libraries had 25, 27 and 33 OTUs, respectively, containing two or more individuals (Figure 2.6). Of these OTUs, Acidobacteria accounted for 12, 20 and 18 OTUs, respectively, including the most abundant OTU for each site. The remainder of the OTUs in the Bare library were distributed among Cyanobacteria, Chloroflexi, α-Proteobacteria, Thermomicrobia and unclassified clones. The remaining OTUs in the Edge library were accounted for by α- and γ-Proteobacteria, Bacteroidetes, Actinobacteria and Verrucomicrobia. In the Canopy, the OTUs were comprised of α-, β-, γ-, δ-Proteobacteria, Verrucomicrobia, Cyanobacteria, unclassified sequences (including possible Firmicutes), Bacteroidetes and Planctomycetes. Assuming that CO-oxidizers only belong to the Actinobacteria, Firmicutes and Proteobacteria, the number of OTUs containing more than two individuals that could contain CO-oxidizers was 2, 4 and 11 for the Bare, Edge and Canopy libraries, respectively.

The number of coxL OTUs sampled at each of the sites was strongly correlated with the number of 16S rRNA gene OTUs sampled ($r = 0.961$). However, the ratio of coxL OTUs: 16S rRNA OTUs increased with increasing vegetation as indicated by Bare, Edge and Canopy values (Figure 2.7). Because the ratio of coxL sampling effort to 16S rRNA sampling effort remained approximately constant across the three libraries, CO-oxidizers appeared to account for an increasing fraction of the total diversity as vegetation increased. The Pu‘u Puai transect was characterized by distinct gradients in pH, water content, root biomass and organic carbon and nitrogen (Table 2.1). Strong correlations existed among all of these variables, and there were strong correlations with the increased numbers of 16S rRNA gene and coxL OTU’s across
Figure 2.6. Rank-abundance plots for 16S rRNA gene and coxL libraries. A=Actinobacteria, F=Firmicutes, a=α-Proteobacteria and b=β-Proteobacteria.
the vegetational gradient (all r values $\geq 0.756$). Organic carbon and nitrogen and water content were more strongly correlated with the number of $coxL$ OTUs (r values 0.908, 0.911, 0.975, respectively) than with the number of 16S rRNA gene OTUs (r values 0.756, 0.760, 0.875, respectively). Site pH was negatively correlated with 16S rRNA gene OTUs and $coxL$ OTUs (r = 0.995 and 0.929). Maximum CO uptake potential was positively correlated with organic carbon (r = 0.998) and increasing $coxL$ richness (r = 0.931). However, in situ CO uptake rates decreased dramatically with increasing organic carbon (Table 2.1).

![Figure 2.7. Ratios of $coxL$ OTUs: 16S rRNA OTUs and $coxL$ ChaoI index: 16S rRNA ChaoI index observed in the Bare, Edge and Canopy clone libraries.](image)

**Discussion**

Previous molecular and activity assessments have documented the presence and potential importance of CO oxidizers for biotic succession on volcanic deposits (Dunfield and King, 2004; King, 2003c; King, 2007; King and Weber, 2007; King and Weber, 2008; King *et al.*, 2008). Little is known, however, about the relationship between diversity and composition of CO-oxidizing communities and plants as succession proceeds. Data presented here document
CO oxidizer activity, abundance, community composition and diversity across a successional gradient on a deposit of uniform age and climate that supports distinct gradients in vegetation, pH, water availability, organic C and N.

MPN estimates and activity assays show that CO oxidizer abundance and maximum potential CO uptake rates are highest where vegetation is greatest (Table 2.1). The difference between the magnitude of changes in MPN estimates (10^3- to 10^4-fold) and maximum potential CO uptake rates (180-fold) may reflect the fact that the MPN assay is based on the ability of CO oxidizers to proliferate under heterotrophic conditions during incubations of many days, while maximum CO uptake assays are based on CODH expression under approximately *in situ* conditions. Thus, MPN assays can detect both active and inactive populations that can grow under heterotrophic conditions.

MPN estimates for Canopy and Edge soil (4.9 x 10^6 and 4.8 x 10^6 CO oxidizers gdw⁻¹; Table 2.1) are comparable to previous estimates for carboxydotrophs in the A_h horizon (10 cm deep) of forest, rice paddy, cultivated and meadow cambisol soils (4.4 x 10^6 CO-oxidizers gdw⁻¹; Bender and Conrad, 1994). It should be noted, however, that Bender and Conrad (1994) used an atmosphere of 20% CO for six weeks to enumerate bacteria that use CO as the sole carbon and energy source. Thus, the MPN estimates in their study and this one may reflect different physiological groups of CO-oxidizers (e.g. carboxydotrophs vs. carboxydovores; King and Weber, 2007).

To date, all but one of the known CO oxidizers use organic substrates preferentially for growth (King and Weber, 2007). Thus, the increases in MPN and maximum uptake potentials with increasing vegetation likely reflect increased organic availability and its use by CO oxidizers. Organic carbon concentrations increase 140-fold with increasing vegetation and are
highly positively correlated with maximum uptake potential \( r = 0.998 \); Table 2.1). These results suggest that CO oxidizers are not only important during early succession, but that they can compete effectively for organic substrates during ecosystem development, and thus dramatically increase their population sizes. Similar increases in maximum potential CO uptake, a surrogate for community size, have been observed in other systems and studies (King, 2003c; King et al., 2008), but results presented here are the first indication that CO oxidizers remain competitive during ecosystem succession.

Changes in bacterial and CO oxidizer community composition and diversity accompany changes in abundance and maximum potential CO uptake across the successional gradient in this study. Statistically significant differences in heterologous coverages, \( F_{st} \) values and average pairwise comparisons (Tables 2.3 and 2.4), indicate the various communities differ significantly. Heterologous coverages highlight the distinction in community composition between the Bare and vegetated sites. Heterologous coverages between the Bare and Edge or Canopy sites range from 1.3-22%, while the heterologous coverages between the Edge and Canopy were more than twice as high (45-57%; Table 2.3). \( F_{st} \) and average pairwise differences support similar trends (Table 2.4). In addition, high numbers of unique OTUs in each of the libraries further confirm their distinctiveness.

Based on an OTU definition derived from the relationship between \( coxL \) and 16S rRNA gene identities for known CO-oxidizing isolates (King et al., 2008; Figure B.1), rarefaction analyses demonstrate that the Bare \( coxL \) library reaches a plateau in OTU discovery, while Edge and Canopy libraries do not (Figure 2.5). Comparisons of OTUs accumulated and 95% confidence intervals for the greatest common sampling effort (60 sequences) also reveal fewer OTU's for Bare than Edge or Canopy libraries. In addition, ACE and ChaoI indices for the
Canopy and Bare libraries differ statistically, providing further support for an increase in CO oxidizer richness with increasing vegetation (Table 2.5).

The congruence of 16S rRNA gene and coxL phylogenies for CO-oxidizing isolates provides a basis for inferring taxonomic assignments of coxL clone sequences (King and Weber, 2007). Results show that the composition of all three coxL libraries spans the phylogenetic breadth of known CO oxidizers, which include the phyla Firmicutes, Actinobacteria and Proteobacteria. Aerobic CO oxidation has also been recently confirmed for Thermomicrobium roseum in the phylum Chloroflexi (Wu et al., 2009). None of the Bare, Edge or Canopy coxL sequences cluster with the T. roseum sequence, however, indicating that representatives of this group of thermophiles may not be readily detectable in the Pu’u Puai cinder deposit.

At the phylum level, Canopy, Edge and Bare sites are compositionally distinct with Proteobacteria coxL sequences dominating the Edge and Canopy libraries, while sequences tentatively assigned to the Firmicutes dominate the Bare library (Table 2.2). Changes in the distribution of Proteobacteria sub-groups are especially intriguing. Analyses of the absolute and relative numbers of OTU’s in various phylogenetic groups show that only β-Proteobacteria OTU’s increase with increasing diversity as measured by ChaoI or Shannon indices. Two lines of evidence suggest that Burkholderia may be important members of the β-Proteobacteria CO oxidizers. First, most of the β-Proteobacteria coxL sequences cluster closely to or match exactly sequences from Burkholderia isolates obtained from the Canopy site (strains PP51-3, PP52-1, CP11; Figure 2.2). Second, all of the β-Proteobacteria 16S rRNA gene sequences obtained from the clone libraries are classified in the genus, Burkholderia. These results are consistent with the well-documented associations of Burkholderia with plants (Balandreau and Mavingui, 2007).
In contrast to *Proteobacteria* and *Firmicutes* clone sequences, *Actinobacteria* clone sequences are distributed evenly among the libraries, comprising 26-31% of the sequences. Many of these sequences cluster with *coxL* sequences from *Mycobacteria*, *Rhodobacter* and *Arthrobacter* species, but several cluster with a sequence from *Nocardioides* sp. JS16, which has two *coxL* operons in tandem (King and Weber, 2007). One of the *Nocardioides* sp. JS16 *coxL* gene sequences clusters with sequences from other *Actinobacteria*, while the second sequence appears to form a loosely related, but distinct clade. This latter clade may include novel or yet unrecognized *Actinobacteria* CO oxidizers, some of which colonize Kilauea volcanic deposits (Figure 2.2).

Increased organic carbon concentrations along the transect may not only explain increased abundance and diversity of CO-oxidizers as noted above, but may also account for the compositional shift from a *Firmicutes*-dominated community in the Bare site to a *Proteobacteria*-dominated community in the vegetated sites. These results are consistent with previous molecular surveys of CO-oxidizers on vegetated and non-vegetated volcanic deposits (Dunfield and King, 2004; King et al., 2008), as well as previous surveys of bacterial diversity in a variety of soil ecosystems (Dunbar et al., 2002; Singh et al., 2007; Zul et al., 2007). For example, numerous studies have documented plant roots as sources of labile carbon for rhizosphere bacteria (Grayston et al., 1998). Availability of organic carbon sources encourages the preferential colonization by fast-growing *Proteobacteria* (Lu et al., 2006; Marilley and Aragno, 1999; Olsson and Persson, 1999; Singh et al., 2007; Zul et al., 2007).

The distribution and activity of other functional groups, such as nitrogen-fixing bacteria, also varies with vegetation. Yeager et al. 2004, assayed nitrogen-fixation rates as well as abundance and diversity of *nifH* in pinyon pine, tarbush rhizospheres and unvegetated
interspaces. There was as much as a 7.5-fold increase in \textit{nifH} copy number in the vegetated areas compared to the interspaces, and a 2.6- and 10-fold increase in nitrogenase activity in the tarbush and pinyon rhizospheres, respectively. The composition of nitrogen-fixing communities did not change with increasing vegetation influence, however. In contrast, nitrate and nitrite reducer abundances decrease during succession, as determined by quantitative PCR of \textit{narG} and \textit{nirS}. Abundances of nitrite reducers and nitrous oxide reducers also either decreased or showed little change across a successional gradient as indicated by genes \textit{nirK} and \textit{nosZ} (Kandeler \textit{et al.}, 2006). Based on these observations, CO-oxidizers differ from denitrifiers and nitrogen-fixers in that they increase in diversity, remain important community members throughout succession, and respond differentially to environmental variables across vegetation gradients.

The composition and diversity of 16S rRNA gene libraries vary across the transect. Trends are less distinct than for \textit{coxL} libraries, but in general the Bare site bacterial community is less diverse than the Edge or Canopy communities (Table 2.5). Statistical distinctions among 16S rRNA gene libraries are supported by shifts in community structure with increasing vegetation that parallel some of the trends observed in the \textit{coxL} libraries. The most notable shift involves increases in the $\alpha$- and $\beta$- \textit{Proteobacteria} sequences in Edge and Canopy libraries. Of course, changes in the CO oxidizer communities appear more dramatic because they encompass only part of the total phylogenetic breadth represented in the 16S rRNA gene sequence libraries. Nonetheless, 16S rRNA gene sequences representing \textit{Proteobacteria} comprise only 5.3% of the Bare library, but account for 29% of the Canopy library. This trend is also consistent with previous reports of the associations between \textit{Proteobacteria} and plants (Grayston \textit{et al.} 1998; Lu \textit{et al.}, 2006; Zul \textit{et al.}, 2007).
The distributions of *Actinobacteria* among the 16S rRNA gene libraries also parallel the distributions observed among *coxL* libraries. *Actinobacteria* 16S rRNA gene sequences occupied small fractions (0.6 to 4.5%) of the three libraries, but were relatively evenly distributed among them. *Actinobacteria* were also evenly distributed among the three *coxL* libraries, but occupied much larger fractions (24.7 to 31.7%) of the total number of sequences (Table 2.2).

The distribution of *Firmicutes* among the 16S rRNA gene libraries, which appear to comprise most of the CO-oxidizers in the Bare site, is less clear. Only eight 16S rRNA gene clones have been classified as “possible *Firmicutes*”. These likely represent novel organisms within the phylum, but the lack of cultured relatives makes their phylogeny difficult to ascertain. In a separate study, Gomez-Alvarez et al. (2007) also examined 16S rRNA gene diversity at the Bare site and recovered few *Firmicutes* sequences. It is possible that even though *Firmicutes* dominate the CO-oxidizing communities, they occupy a very small portion of the overall bacterial community in the Bare site.

Other phyla not currently known to contain CO-oxidizers, such as *Acidobacteria*, exhibits patterns among Bare, Edge and Canopy sites that are similar to patterns that have been documented for other soil ecosystems (Dunbar et al., 2002; Gomez-Alvarez et al., 2007; Hansel et al., 2008; Janssen, 2006), including a survey across a successional gradient on dune soils (Tarlera et al., 2008). *Acidobacteria* compositions at the Bare, Edge and Canopy sites are statistically distinct and the distribution of *Acidobacteria* subgroups followed patterns noted previously (see Appendix B).

Although the 16S rRNA gene libraries under-sampled OTU richness, similar sampling efforts at each of the sites facilitate comparisons of relative diversity and relationships between
trends in 16S rRNA gene and coxL sequences. Assuming that total OTU richness is similarly underestimated at each site, the smaller numbers of coxL OTUs for the Bare site result in lower coxL:16S rRNA OTU ratios than for Edge and Canopy sites. This suggests that with increasing vegetation, CO-oxidizers account for an increasingly larger fraction of overall bacterial richness (Figure 2.7). This observation in turn implies that mixotrophs and facultative lithotrophs, as represented by at least some of the CO-oxidizing bacteria, do not appear to experience obvious competitive disadvantages that limit their ability to proliferate during succession.

Collectively then, results from this study provides the first clear demonstration of the relationship between plants and CO-oxidizer abundance, distribution and diversity. Although among the earliest colonists of volcanic deposits, CO-oxidizer communities continue to expand and diversify with the entire bacterial community as vegetation cover increases. Ratios of coxL OTUs: 16S rRNA gene OTUs also reveal that CO-oxidizers account a larger fraction of total bacterial diversity with increasing vegetation influence. These observations provide additional support for the notion that the metabolic versatility of CO oxidizers contributes to their ability to serve as both as pioneering colonists and active members of complex ecosystems in later stages of succession.
CHAPTER 3.
WATER STRESS IMPACTS ON BACTERIAL CARBON MONOXIDE OXIDATION ON RECENT VOLCANIC DEPOSITS
Introduction

Changes in water availability, measured as water potential, can substantially alter microbial communities and their activity. For example, T-RFLP patterns for 16S rRNA genes changed after drying oak soils, but not in grassland soils (Fierer et al., 2003). Since the latter experienced more frequent oscillations in water status than the former, the results indicated that water stress may play a role in the structure of the inherently different microbial communities in these two soils (Fierer et al., 2003). Other studies have also demonstrated that microbial communities in grassland soils are resistant to water stress (Griffiths et al., 2003).

Rapid drying or decreases in water potential allow little time for acclimation, and adversely impact cell viability (Potts, 1994). The effects of changing water potential can be exacerbated by extreme temperatures, low substrate availability and other physiological stresses (Kieft et al., 1987; Mary et al., 1985; Potts, 1994). Rapid water loss causes macromolecular and cellular destabilization, which inhibit enzyme activity and induce production of reactive oxygen species and DNA damage (Potts, 1994). Cellular defenses against desiccation-induced damage include accumulation of compatible solutes, exopolysaccharide production and enzyme synthesis to combat oxidative stress (Leblanc et al., 2008; Singh et al., 2005).

Likewise, rapid rehydration can adversely impact cell viability. In this case, cells must reduce compatible concentrations to avoid lysis due to elevated turgor pressure (Potts, 1994). Several studies have documented rehydration as a significant source of cell lysis and carbon turnover in soils (Bottner, 1985; Grierson et al., 1998; Magid et al., 1999; Schimel et al., 2007; Turner et al., 2003; Turner and Haygarth, 2001; Van Gestel et al., 1993; Wu and Brookes, 2005). Water availability can oscillate dramatically on unvegetated volcanic deposits, and likely plays an important role in regulating the activity and distribution of pioneering microbial colonists.
Regardless of age, unvegetated deposits are often coarse and porous with little buffering capacity against changes in water status. The lack of buffering capacity likely results from a combination of the texture and lack of organic matter (Rawls et al., 2003).

In spite of limitations imposed by water stress as well as limited substrate availability, a variety of bacteria colonize and are active on young unvegetated volcanic deposits. Carbon monoxide-oxidizing bacteria are among the earliest successful colonists. A field study by King and Weber (King, 2007) revealed that unvegetated, fresh lava chips supported measurable CO oxidation within six months. Additional studies have shown that atmospheric CO consumption accounts for 2-10% of reducing equivalent flow for Kilauea volcanic deposits (King, 2003c; King and Weber, 2008). Comparable rates of CO uptake have been observed on 23-year old volcanic deposits in Miyake-jima, Japan (King et al., 2008). Nonetheless, CO oxidizers, like other functional groups, must respond to local water regimes, but there are no published studies that document the response of CO oxidizers to in situ water regimes.

Plant colonization appears to play a major role in water regimes for Kilauea deposits. Plant colonization promotes weathering and organic matter accumulation, which increases water retention and results in more stable and less stressful water potentials. This may lead to a shift over time from water stress-tolerant to water stress-sensitive phenotypes. Little is known, however, about the impacts of water stress on microbial succession, and in particular how water stress impacts CO oxidizer community development and adaptation.

Very few studies have dealt with the impacts of soil water status on CO oxidizer activity (King, 1999a; Moxley and Smith, 1998; Spratt and Hubbard, 1981). Results from these studies suggest that CO oxidation is water sensitive, but that CO oxidizers may tolerate and even adapt to dry conditions. Spratt and Hubbard (1981) measured optimal CO consumption when soils
were incubated under relative humidities approaching 100%. Significant increases in CO consumption were observed, however, in soils that had been air-dried and subsequently rewwetted or equilibrated at relative humidities greater than 93%. Moxley and Smith (1998) found that the optimal CO oxidation rates of three Scottish soils occurred at water contents that were about field capacity. In the same study, two arable soils had optimal CO consumption rates at water contents that were lower than a woodland soil (10 to 15% vs. 25-30%). The authors suggested that the CO-oxidizing communities in each soil type may be adapted to these different water contents.

King (1999a) examined CO consumption by Maine forest “O”-horizon soils as they were dried under laboratory conditions and then gradually rewwetted by step-wise additions of deionized water. Soils produced CO when dried to water contents below 20%. Upon rewwetting, a hysteresis was observed for water contents of 20-80%. King (1999a), however, noted that limited drying and wetting cycles, which might better represent what occurs in situ, seemed to have no inhibitory effect on CO oxidation. This observation provides the first insights that CO oxidation may be somewhat resilient to in situ water dynamics in forest soils (King, 1999a).

In this study, we examined the impacts of water stress on CO oxidizer activity at two sites representing early and late successional stages on a 1959 deposit on Kilauea Volcano (Hawai’i). The two sites differ dramatically in water regimes with one site exhibiting diurnal oscillations in water potential (Bare site) and the other site remaining relatively moist (Canopy site). Previous molecular ecological surveys at these two sites have revealed distinct CO oxidizer communities (Chapter 2). The objectives of this study were: 1) determine the impact of decreased water potential on CO oxidation rates; 2) assess the ability for CO oxidation to recover
After drying and rewetting events, and 3) determine if the activity of distinct communities at the two sites demonstrate differential adaptations to local water regimes.

Materials and Methods

Site Descriptions

Volcanic material used in this study was collected from the Puʻu Puai deposit, which resulted from a 1959 eruption of Kilauea Iki (Kilauea Volcano, Hawaiʻi; GPS coordinates: 19° 24' 22.5" N X 155° 15' 18.2" W). A deposit several meters thick and comprised of cinders averaging about one cm in diameter, supported “tree islands”, which are irregularly shaped vegetated patches typically > 100 m² consisting largely of *Metrosideros polymorpha* (the Ohia lehua tree) and *Myrica faya* (fire tree). Sites designated “Bare” were comprised of unvegetated cinders and those designated “Canopy” were comprised of an organic-rich soil, which has accumulated on top of and within the original cinders deposit within tree islands. A 5-10 cm thick litter layer overlies the soil in Canopy sites and was removed prior to all sampling. Canopy and Bare sites were located within 10 m of each other. Physical and chemical characteristics of the materials at these sites as well as the composition of CO-oxidizing and bacterial communities have been described previously (King and Weber, 2008; Chapter 2).

Spatial and Temporal Variability: Relative Humidity, Water Potential and CO Uptake

Relative humidity (RH) and temperature profiles were obtained using U-series external channel HOBO Data Loggers (Onset Computer Corp.; Pocasset, MA) equipped with TMC50-HD soil temperature sensors. RH was measured at a height of about 1.5 m at each site. RH profiles were obtained during 6-7 October 2007, 28-30 January 2008 and 3-7 May 2008. During these periods, triplicate surface samples (upper 1 cm) were collected from the Bare and Canopy sites at regular intervals for water potential measurements. Samples were returned to the field
laboratory at ambient temperature and relative humidity in open containers to prevent alterations in water potential during transport. Samples were visually inspected to make sure that no condensation formed on the sides of the collection containers. Upon arriving at the lab, (no more than 20 min after field-collection), approximately 0.5-1 gram fresh weight samples were placed into 14 ml sample cups (Decagon Devices, Pullman, WA) in which water potentials were measured using a WP4-T dewpoint potentiometer (Decagon Devices, Pullman, WA) according to the manufacturer’s instructions. At each sampling, ambient relative humidity was measured using a Kestrel 4000 (Forestry Suppliers; Jackson, MS), a handheld instrument equipped with RH and temperature sensors. Surface temperatures (top 1 cm) at both sites were recorded at 10-minute intervals using a HOBO data logger.

To determine the distribution of water and maximum potential CO uptake activity in the upper 15 cm of the Bare sites, two sets of triplicate Bare site cores were collected in the morning (about 0800) and afternoon (about 1600) on 5 May 2008. Cores were collected using aluminum core tubes (7.2 cm dia) that had been ethanol-cleaned and baked dry in an oven. Cores were sectioned into the following depth intervals: 0-1 cm, 1-3 cm, 3-5 cm, 5-8 cm, 8-11 cm and 11-15 cm. Immediately after sectioning, approximately 5-gram fresh weight (gfw) samples of each fraction were transferred to 110 cm³ jars. Jar headspaces were spiked with CO to a final concentration of about 50 ppm. One cm³ volumes were removed at regular intervals and CO concentrations were determined using a Trace Analytical RGD Gas Chromatograph as described previously (King, 1999b). CO uptake rates were determined using curve fitting procedures as previously described (King, 1999b). Rates were normalized per gram dry weight (gdw). To determine the impacts of in situ drying on CO uptake at ambient concentrations by intact Bare cores and the water distributions in the upper 15 cm, triplicate intact cores were collected as
above in the morning and the afternoon of 7 May 2008. CO uptake assays were initiated no more than 30 min after collection. Core tubes were sealed with gas-tight plastic caps with rubber septa sampling ports. After sealing the core tubes, 18 cm$^3$ of ambient air were added to the headspaces to provide an overpressure that would accommodate multiple headspace samplings. At regular intervals, 3 cm$^3$ samples were removed and CO concentrations were determined as described previously (King, 1999b). Immediately following CO uptake rate analyses, cores were sectioned into the depth intervals described above for water potential and water content measurements. The water contents were determined after drying samples in the oven overnight at 176 °C.

**Response of CO-oxidation to Polyethylene Glycol 200-Amendment**

Maximum potential CO uptake rates were determined for triplicate 10 gfw samples of Canopy surface soil (0-1 cm) that were amended with 2 ml of sterile water or solutions of 50, 75 or 100% polyethylene glycol 200 (PEG 200), which resulted in water potentials of 0, -0.85, -1.50 and -2.37 MPa, respectively. Triplicate 20 to 21 gfw samples of Bare surface cinders (0-1 cm depth interval) were amended with 3 ml of water or solutions of 16, 32 or 50 % PEG 200, which resulted in water potentials of 0, -0.49, -1.25 and -2.77 MPa, respectively. After amending soil and cinders with water or PEG 200, samples were transferred into 500 cm$^3$ gas tight jars and allowed to equilibrate for 1 h. Jar headspaces were amended with CO to a final concentration of approximately 80 ppm with enough overpressure to accommodate multiple headspace samplings. Headspace concentrations were assayed immediately and then measured at appropriate intervals thereafter as described above. After completing the CO uptake assay, samples were removed from the jars to determine the water content and dry weight of the samples.
Response of CO-oxidation to Long-Term Desiccation and Rehydration

CO uptake by freshly collected Bare cinders (water content: 29%; 0 MPa) and Canopy soil (water content: 76%; 0 MPa) was measured as described previously (King, 1999b). Briefly, triplicate 6-6.5 gfw samples of canopy soil and triplicate 7-8 gfw samples of Bare cinders were placed into 110 cm³ gas-tight jars and amended with CO to a final concentration of 70-80 ppm with suitable overpressure to accommodate multiple headspace samplings. Headspace concentrations were determined immediately and at suitable intervals thereafter. From the same soil and cinder collections, 12 additional samples of cinders and Canopy soil were placed into WP4-T sample cups, and then placed into a desiccator for drying. Samples were desiccated to water potentials of -150 MPa, which were maintained for the duration of the experiment. At intervals of 14, 27, 38 and 63 d, triplicate samples were rehydrated by applying 2 ml of sterile deionized water and gently mixing with a sterile spatula. Immediately after rehydration, samples were placed into 110 cm³ gas-tight jars. Jar headspaces were amended with CO to concentrations of 70-80 ppm. Headspace concentrations were determined immediately and at suitable intervals thereafter. Seventy-five days after initiating the experiment, CO uptake assays were carried out as described above for triplicate Bare and Canopy samples that had been stored at field water content and potential in ziptop bags.

Response of CO-oxidation to Oscillating Water Regimes

Six samples of Bare cinders (13-15.5 gfw each) and Canopy soil (6-8.5 gfw each) were transferred into 110 cm³ jars and amended with CO to a final concentration of 60 ppm with suitable overpressure to accommodate multiple headspace samplings. After the CO uptake assay, samples were weighed and then dried at ambient room temperature and relative humidity (same as above) to a water potential of approximately -80 MPa. The dry masses of the samples
were measured to determine water contents. After 2 to 8 d, samples were rehydrated with 2 ml of sterile deionized water to a water potential near 0 MPa (water contents: 27% [Bare]; 66% [Canopy]) and placed into 110 cm³ jars to determine the CO uptake rates as before. After the CO uptake assay was completed, samples were re-weighed and dried again. Four additional cycles of drying and rewetting with CO uptake rate determinations were completed as above.

**Statistical Analyses**

For the experiments examining the response of polyethylene glycol amendment or long-term desiccation and rehydration on CO oxidation rates in Bare and Canopy samples, average rates of CO uptake for each treatment and control group were statistically analyzed by an ANOVA. Differences among treatments were assessed using a Tukey’s Honest Significant Difference test using KaleidaGraph Software (Synergy Software, Reading PA). Water potentials and water contents of depth intervals in Bare cores and whole core CO-oxidation rates were statistically examined in a similar fashion. The responses of CO oxidation in Bare and Canopy samples to multiple cycles of desiccation and rehydration were examined statistically using a repeated-measures ANOVA.

**Results**

**Spatial and Temporal Variability: Relative Humidity, Water Potential and CO Uptake**

Ambient temperature, cinder and soil surface temperatures and relative humidities (RH) varied diurnally at Bare and Canopy sites. Diurnal variations in ambient temperatures were always larger in the Bare site than in the Canopy site. The average low and high ambient temperatures observed during October 2007, January 2008 and May 2008 at the Bare site were 13.2 ± 1.6 °C and 25.2 ± 0.8 °C, respectively, and those observed at the Canopy site were 12.7 ± 1.5 °C and 19.4 ± 1.6 °C, respectively. Surface temperatures at the two sites also followed this
trend, but the difference in the average maximum surface temperatures for the two sites was much more dramatic. The average low and high surface temperatures observed at the Bare site were 13.8 ± 2.1 °C and 40.6 ± 2.6 °C, respectively, and those observed for the Canopy site were 13.1 ± 1.6 °C and 18.4 ± 1.8 °C, respectively. RH oscillated across a similar range for the two sites (Bare site: 50.8 ± 7.7 % to 88.8 ± 0.4 %; Canopy site: 55.1± 8.2 % to 89.2 ± 2.9 %) with lower values typically during mid-day (Figure 3.1).

During a relatively dry period (October 2007), water potentials at the Bare site decreased dramatically, dropping from near 0 MPa in the early morning to -60 ± 16 MPa by midday and increasing to -6.3 ± 1.1 MPa a few hours later (Figure 3.1). In contrast, during this interval average water potentials in the Canopy surface never dropped below 0 MPa. During wetter periods (January and May 2008), diurnal fluctuations in RH and water potential were smaller than in October 2007, but Bare site water potentials still decreased during the day to values as low as -3.7 ± 1.4 MPa (January 2008) to -7.4 ± 2.4 MPa (May 2008).

Water potentials and contents as well as maximum CO uptake rates did not differ significantly between the two sets of cores collected in the morning and afternoon on 5 May 2008. Water potentials in the upper 3 cm ranged from 0 to -0.33 MPa, while all water potentials at depths below 3 cm were near 0 MPa (Table 3.1). Water contents increased slightly with depth (Table 3.1). Maximum CO uptake rates were most variable, but highest for the 0-1 cm depth interval (morning: 62 ± 25 nmol gdw⁻¹ d⁻¹; afternoon: 190 ± 98 nmol gdw⁻¹ d⁻¹) and decreased at lower depths (Table 3.1).

Two additional sets of triplicate cores were collected at 1030 and 1400 on 7 May 2008. The first set of cores was moist from recent rainfall. Water content was lowest in the surface (12.5 ± 0.6%) and increased with depth to 36.9 ± 1.9 % in the 8-11 cm depth interval (Table
Average water potentials were near 0 MPa for all depth intervals assayed in the first set of cores (Table 3.2). The second set of cores was drier than the first. Average water contents for the 0-1 and 1-3 cm layers of the afternoon cores were significantly lower than those of morning cores (p = 0.0006, p = 0.027, respectively), with the water contents of the 0-1 cm and 1-3 cm fractions dropping to 2.0 ± 0.8 % and 11.0 ± 0.8%, respectively (Table 3.2). Likewise, water potentials for the 0-1 cm depth interval of the afternoon cores (-1.73 ± 0.6 MPa) were

**Figure 3.1.** Relative humidity (Canopy=●, Bare=▼) and water potentials (■) for Bare and Canopy sites for 6-7 October 2007 (beginning at 0700) and 3-7 May 2008 (beginning at 1600). Water potentials are averages of triplicate samples ± 1 S.E. Note the difference in the y-axis (water potential) for the October 2007 and May 2008 plots.
**Table 3.1.** Water content (%), water potential (MPa) and maximum potential CO uptake rates (nmol gdw$^{-1}$d$^{-1}$) for two sets of triplicate cores collected from the Bare site at 0800 (A) and 1600 (B) on 5 May 2008.

<table>
<thead>
<tr>
<th>Bare Site: Core Set</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Depth</td>
<td>Water content</td>
<td>Water potential</td>
<td>Maximum uptake</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0-1 cm</td>
<td>13.2 (1.7)</td>
<td>10.5 (2.3)</td>
<td>-0.02 (0.02)</td>
<td>-0.20 (0.02)</td>
<td>62 (25)</td>
<td>190 (98)</td>
</tr>
<tr>
<td>1-3 cm</td>
<td>17.1 (5.8)</td>
<td>20.5 (0.8)</td>
<td>-0.16 (0.10)</td>
<td>-0.03 (0.03)</td>
<td>71 (12)</td>
<td>68 (16)</td>
</tr>
<tr>
<td>3-5 cm</td>
<td>32.5 (1.3)</td>
<td>31.8 (1.4)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>43 (13)</td>
<td>46 (6)</td>
</tr>
<tr>
<td>5-8 cm</td>
<td>32.9 (1.7)</td>
<td>31.1 (0.5)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>22 (5)</td>
<td>22 (2)</td>
</tr>
<tr>
<td>8-11 cm</td>
<td>36.0 (1.9)</td>
<td>35.3 (1.2)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>11-15 cm</td>
<td>30.0 (2.6)</td>
<td>27.2 (0.9)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

significantly lower (p =0.0002) than water potentials for the morning cores (-0.04 ± 0.04 MPa). Average ambient atmospheric CO uptake rates for the afternoon cores (1.8 ± 0.8 mg m$^{-2}$ d$^{-1}$) were also lower than rates for the morning cores (5.0 ± 2.2 mg m$^{-2}$ d$^{-1}$), but the rates for the two sets were not statistically different.

**Table 3.2.** Average water potential (MPa), water content (%) and CO uptake rates (mg m$^{-2}$ d$^{-1}$) at ambient CO concentrations for triplicate cores collected from the Bare site in the morning and afternoon on 7 May 2008. Numbers in parentheses are standard errors.

<table>
<thead>
<tr>
<th>Collection Time</th>
<th>1030 7 May 2008</th>
<th>1400 7 May 2008</th>
</tr>
</thead>
<tbody>
<tr>
<td>Core section</td>
<td>Water potential</td>
<td>Water content</td>
</tr>
<tr>
<td>0-1 cm</td>
<td>-0.04 (0.04)</td>
<td>12.5 (0.6)</td>
</tr>
<tr>
<td>1-3 cm</td>
<td>0 (0)</td>
<td>18.2 (1.0)</td>
</tr>
<tr>
<td>3-5 cm</td>
<td>0 (0)</td>
<td>29.4 (1.5)</td>
</tr>
<tr>
<td>5-8 cm</td>
<td>0 (0)</td>
<td>36.1 (2.2)</td>
</tr>
<tr>
<td>8-11 cm</td>
<td>0 (0)</td>
<td>36.9 (1.9)</td>
</tr>
<tr>
<td>11-15 cm</td>
<td>0 (0)</td>
<td>32.0 (3.1)</td>
</tr>
<tr>
<td>Intact core CO uptake</td>
<td>5.0 (2.2)</td>
<td>1.8 (0.8)</td>
</tr>
</tbody>
</table>
**CO-oxidation Response to *in vitro* Water Potential Manipulation**

Maximum potential CO oxidation rates in Bare and Canopy samples were reduced substantially in response to lowered matric potentials adjusted by amendments with PEG 200. At water potentials ranging from -1.25 to -1.5 MPa, CO oxidation rates in the Bare and Canopy surface materials were reduced to 40% and 60% of water amended controls, respectively (Figure 3.2).

Rates of CO oxidation in Bare samples differed significantly from the water-amended controls at water potentials of -1.25 and -2.77 MPa (p=0.008 and 0.0209, Figure 3.2), but rates of the three PEG treatments did not differ significantly from one another (p> 0.35). Rates of CO oxidation in Canopy samples at water potentials of -0.92 to -2.03 MPa were significantly lower than the water-amended control (all p values ≤ 0.0267), but rates of the PEG 200-amended treatments did not differ significantly from one another (all p-values ≤ 0.339).

To determine the resilience of CO-oxidizing communities in Bare and Canopy surface materials to extended desiccation, maximum potential CO uptake was measured in samples that had been rehydrated after desiccation for 14, 27, 39 or 63 d. Maximum CO uptake rates for material stored at field water contents and potentials did not vary significantly over the duration of the incubation (Bare:  p = 0.624; Canopy:  p = 0.998).

Non-desiccated Canopy sample rates were significantly greater than rates of all desiccated and rehydrated samples (p< 0.0001; Figure 3.3). Rates of desiccated and rehydrated samples did not differ statistically from one another (p>0.05). Similar patterns were observed for Bare site samples (Figure 3.3).
Figure 3.2. Rates of CO oxidation (ppm h\(^{-1}\) gfw\(^{-1}\)) in PEG 200 amended and water amended (controls) Bare (a) and Canopy (b) samples. Rates are averages of triplicates ± 1 S.E.

Figure 3.3. CO oxidation response upon rehydration of (a) Bare and (b) Canopy materials after 14, 27, 39 or 63 d storage at -150 MPa. Rates of non-desiccated controls prior to the experiment (■) and after the experiment (◆). Data points are averages of triplicate samples ± 1 S.E. Note the scale differences in the y-axes.
To determine the impact of multiple oscillations in water status on CO oxidation, maximum potential CO uptake rates were measured in surface samples subjected to five cycles of desiccation to -80 MPa and rehydration to 0 MPa. Prior to desiccation, Bare and Canopy water potentials were 0 MPa with water contents of 33 % and 84 %, respectively; these Bare and Canopy samples oxidized CO at rates of $58 \pm 2.5 \text{ nmol gdw}^{-1} \text{d}^{-1}$ and $840 \pm 37.5 \text{ nmol gdw}^{-1} \text{d}^{-1}$, respectively (Figure 3.4). After one cycle of desiccation and rehydration, activity in Bare and Canopy samples was reduced to 55 and 50 % of the initial rates, respectively; these decreases were statistically significant ($p < 0.001$; Figure 3.4). For Bare samples CO oxidation rates increased through cycle 4 and decreased after cycle 5, but these changes were not statistically significant (Figure 3.4). Similar patterns were observed for Canopy samples.

**Figure 3.4.** CO oxidation response to multiple cycles of desiccation to -80 MPa and rehydration to 0 MPa in (a) Bare and (b) Canopy samples. Rates are averages of triplicate samples ± 1 S.E. plotted as a function of the number of desiccation/rehydration cycles. Note the scale differences in the y-axes on the two plots.
Discussion

*In situ* water dynamics in the Canopy and Bare sites differ markedly. Canopy surface water potentials remain at or very near 0 MPa; in contrast, Bare site surface cinders experience diurnal shifts in water potential that would inhibit activities of even some of the most stress tolerant bacteria (i.e. -5 MPa; (Griffin, 1981). Accordingly, CO uptake rates by intact Bare cores were higher for a morning sampling relative to an afternoon sampling during a period of moderate drying *in situ* (Table 3.2). This trend is consistent with observations of past studies that suggest that CO oxidizer activity is sensitive to water stress (King, 1999a, Moxley and Smith, 1998).

Maximum potential CO uptake by Canopy and Bare samples at various matric stresses adjusted using PEG 200 clearly demonstrated sensitivity to changes in water potential and content. Although the efficacy of using PEG as a proxy for matric stress imposed by drying has been questioned, recent transcriptomic work on *Bradyrhizobium japonicum* demonstrates that responses to matric stresses imposed by desiccation and PEG amendment are remarkably similar (Cytryn et al., 2007). Therefore, significant decreases in CO uptake rates in response to PEG amendment are likely representative of responses of CO oxidizers to matric stresses.

Even though CO oxidation is sensitive to water stress, results from additional experiments demonstrated that it recovers rapidly from extended periods of desiccation. Bare and Canopy surface samples that had been stored at a water potential of -150 MPa resumed rapid activity usually within 2 h after rehydration to 0 MPa, even after storage for 63 d. This indicates that despite the different water regimes at the two sites, both communities have core populations that withstand and recover from severe water stress. These findings are similar to those noted for
Maine forest soils, in which activity resumed after soils were dried to water contents lower than 20% (King, 1999a).

CO oxidizer activity in Bare and Canopy sites also responded similarly when exposed to multiple cycles of desiccation and rehydration. After one cycle of desiccation and rehydration, activity for both sites was significantly reduced, but subsequent cycles of drying and rewetting had little effect, and CO oxidation rates even increased slightly. Similar patterns were previously reported for soil nitrification rates (Fierer and Schmel, 2002). Reasons for recovery of some activity are unclear, but the results suggested that both Bare and Canopy CO-oxidizing communities have similar abilities to adapt to oscillations in water status.

The sensitivity of CO oxidation to water stress is similar to that of other soil processes, such as nitrification and aerobic methane oxidation. For instance, nitrification is inhibited by > 85% at water potentials < -3 MPa (Stark and Firestone, 1995). Methane oxidation is strongly inhibited by water potentials from -3 MPa to -4 MPa (Schnell and King, 1996). However, unlike methane oxidation, which does not recover from air-drying (Nesbit and Breitenbeck, 1992), CO oxidation resumes significant activity relatively quickly as documented here (Figures 3.3, 3.4). Nitrifiers also survive severe drought and resume activity within minutes after rehydration (Fierer and Schmel, 2002; Gleeson et al., 2008; Hastings et al., 2000; Steenwerth et al., 2005), although some nitrifiers appear more sensitive to water stress than others (Gleeson et al., 2008).

The similar responses of CO oxidation in Bare and Canopy sites to desiccation and rehydration seem remarkable considering the differences in water regimes they experience. Water potential at Canopy sites remains high, near 0 MPa, even during relatively dry periods (e.g., Figure 1), while in contrast, Bare site cinders experience diurnal variations with water potentials at times falling to values < -60 MPa (e.g., Figure 3.1).
Although Bare site water potentials regularly reach extreme values, they also frequently rise to near 0 MPa during periods of rainfall, and at night as temperatures decrease and dew forms. Thus, Bare site CO oxidizers experience favorable water potentials for a significant fraction of the day. This may reduce the selective pressure for specific adaptations that would maintain activity during periods of moderate water stress, and account for the similarity in responses observed for the Bare and Canopy sites.

Alternatively, exposure to water stress may select for stress tolerance by most, if not all, of the initial successful colonists of unvegetated, newly formed volcanic deposits. Over time, changes resulting from plant colonization or other variables may lead to shifts in community composition, but stress tolerance might be retained if new communities are largely drawn from members of the original assemblages. Thus, responses of CO oxidation by Canopy and Bare sites to imposed water stress would be similar.

Thus, dominance of Bare site CO-oxidizing communities by a putative Firmicutes clade may reflect water availability, while dominance of Canopy site CO oxidizers by Proteobacteria may reflect plant impacts (Chapter 2). This distribution is consistent with findings of previous studies, which indicate that Gram-positive bacteria (e.g., Firmicutes) dominate bacterial communities in arid environments, while Gram-negative bacteria dominate wetter, vegetated soils (Busse and Bottomley, 1989; Chanal et al., 2006; Chen and Alexander, 1973; Clark and Hirsch, 2008; Jawad et al., 1998; Nagy et al., 2005; Nesbit and Breitenbeck, 1992; Nicholson et al., 2000; Potts, 1994; Rainey et al., 2005; Rao and Venkateswarlu, 1983; Vriezen et al., 2007).

Although the distribution of specific CO-oxidizing taxa between Bare and Canopy sites could reflect impacts of the different water regimes, activity data indicate that those regimes do not elicit differential responses measured as CO uptake. Similar results have been reported
recently for analyses of microbial activity in a grassland soil that appeared to have dramatically oscillating water regimes (Pesaro et al., 2004). These observations suggest that short-term, frequent oscillations between extreme and moderate states do not select for physiological responses markedly different than those for systems that experience primarily moderate conditions.

Depth profiles for maximum potential CO oxidation rates at the Bare and Canopy sites provide support for the notion that exposure to periodic extreme water stresses may not strongly limit the distribution and abundance of active CO oxidizers. The Bare site community shows no specific adaptive responses in its activity relative to the Canopy site, which does not experience such extremes; Bare and Canopy site CO uptake depth profiles are also similar (King and Weber, 2008). If water potential regimes limited the distribution and abundance of Bare site CO oxidizers, CO oxidation should be higher in the in sub-surface, for which water potentials remain moderate. What has been consistently observed, however, is that the highest maximum potential CO oxidation rates occur in the upper 0-1 cm interval (Table 3.1; see also King and Weber, 2008).

Collectively, these observations show that CO oxidation is a water-sensitive process, but that CO oxidation recovers rapidly after extended periods of desiccation. The results here provide initial insights into the constraints of water potential on CO oxidation in unvegetated volcanic substrates, and show that CO oxidation in situ is a dynamic process that likely coincides closely with diurnal oscillations in water status. Striking similarities in CO oxidation responses to water stresses in Bare and Canopy sites indicate that the metabolic response of active CO-oxidizing communities at the two sites is not differentially adapted to water stress, despite contrasting water regimes. Nonetheless, the ability of at least some CO oxidizers to recover
from extended periods of desiccation provides evidence that they can persist in water-stressed environments, and offers insights into factors that contribute to their success as pioneering colonizers of unvegetated volcanic deposits.
CHAPTER 4.
QUANTIFICATION OF BURKHOLDERIA COXL GENES IN HAWAIIAN VOLCANIC DEPOSITS
Introduction

Recent studies of CO-oxidizing communities and activity in volcanic ecosystems have provided insights into the distribution and diversity of CO oxidizers in situ (Dunfield and King, 2004; King, 2003c; King; King and Weber, 2008). Significant rates of CO oxidation have been detected in deposits as little as 6 months after deposition, indicating that CO oxidizers are able to rapidly colonize recent deposits (King, 2007). On such young substrates, CO-oxidizing communities are dominated by Firmicutes (Chapter 2). As deposits become more vegetated and organic carbon increases during biological succession, CO-oxidizing communities remain active, increase in abundance and diversify, most likely as a result of increased organic substrate available for heterotrophic growth (Chapter 2). In carbon-rich soils characteristic of late succession, Proteobacteria dominate CO-oxidizing communities (Dunfield and King, 2004).

A recent molecular survey of CO-oxidizer communities across a vegetation gradient on a Hawaiian volcanic deposit demonstrated that the β-Proteobacterial coxL genes occupied an increasing fraction of coxL libraries generated from sites with increasing vegetation (Chapter 2). Total Proteobacteria comprised 2.6 % of a clone library generated for the unvegetated cinders (Bare site) and 75% of the library generated for the mature vegetated site (Canopy site). Although α-Proteobacteria comprised the majority of the Proteobacteria at each site, the highest percentage of α-Proteobacteria was actually in a site at the edge of the Canopy site (Edge site), a site representing an intermediate stage of vegetation development (Chapter 2). In contrast, β-Proteobacteria coxL was not detected in unvegetated cinders, but comprised 1.7 and 32.9 % of the Edge and Canopy site clone libraries, respectively. Several of these sequences were phylogenetically affiliated with Burkholderia xenovorans LB400 or PP52-1, a Burkholderia isolate previously isolated from the mature site (Chapter 2).
In addition, efforts to enrich novel CO-oxidizing bacteria from these sites resulted in more than 10 new β-Proteobacterial isolates, all members of the genus *Burkholderia*. Phylogenetic analyses of the 16S rRNA genes from these isolates revealed that the closest relatives (> 97% sequence identity) included *B. sacchari*, *B. unamae*, *B. nodosa*, *B. mimosarum*, *B. bryophila*, *B. ginsengesolis*, *B. bryophila* and *B. caledonica*. In addition, a *B. cepacia*-like isolate has also been identified as a CO-oxidizer (Chapter 2). Prior to these isolations, *Burkholderia xenovorans* LB400 was the only described CO-oxidizing member of the genus (King, 2003a). However, these observations indicate that CO-oxidizing capabilities could be widespread throughout the *Burkholderia*, which may be an important contributor to the expansion of CO-oxidizing communities during biological succession on volcanic deposits.

The isolation of CO-oxidizing relatives of root-associated *Burkholderias* supports the correlation between plants and CO-oxidizing β-Proteobacteria that was previously observed (Chapter 2). Close relatives, *B. sacchari* and *B. unamae*, are highly abundant in the rhizosphere of Maize and sugarcane (Braemer et al., 2001; Caballero-Mellado et al., 2004; Reis et al., 2004), where they fix-nitrogen that supports plant growth (Estrada-de los Santos et al., 2001; Perin et al., 2006). *B. mimosarum* and *B. nodosa*, two other close relatives of isolated CO-oxidizing *Burkholderias* were originally obtained from legume nodules and also fix nitrogen for their hosts (Chen et al., 2007; Chen et al., 2006). In addition, *B. byophila* has been demonstrated to promote plant growth and have antifungal properties (Vandamme et al., 2007). Other *Burkholderia* spp. such as *B. gladioli* and *B. caryophili* are associated with plants as pathogens causing grain rot and leaf-sheath browning in rice, slippery skin in onions and rot and wilt of carnations (Ura et al., 2006; Vandamme et al., 2007). Clearly, burkholderias are well-known associates of plants and may include a number of CO-oxidizers.
This study describes a QPCR approach to quantify *Burkholderia coxL* genes in soil, and represents the first molecular approach to enumerate any subgroup of CO-oxidizing bacteria. To date, the only estimates of CO oxidizer abundance *in situ* are from most probable number (MPN) assays (Bender and Conrad, 1994; Chapter 2) or relative abundances that have been inferred from maximum potential CO uptake rates (King and Weber, 2008). Using QPCR, *Burkholderia coxL* was enumerated in volcanic samples from a previously described vegetation gradient (King and Weber, 2008) and quantities were compared to parallel QPCR estimates of β- Proteobacterial 16S rRNA and total 16S rRNA gene copies. Results indicated that *Burkholderia coxL* gene copy numbers increased significantly with increasing vegetation. Ratios of *coxL*:16S rRNA gene copy numbers were similar to or greater than ratios reported for other functional genes (e.g. *nifH*, *narG*) in previous QPCR studies (Henry et al., 2006; Kandeler et al., 2006). This suggests that CO oxidizers are relatively abundant compared to other functional groups. Collectively, these results indicate that CO-oxidizing *Burkholderia* are common components of CO oxidizer communities on vegetated volcanic deposits, and that they are strongly associated with developing plant communities.

**Materials and Methods**

**Site Descriptions**

The three sampling sites used in this study, Bare, Edge and Canopy are located along a vegetation gradient on a 1959 volcanic deposit (Pu’u Puai) on Kilauea volcano in Hawai’i (19° 24’ 22.5” N X 155° 15’ 18.2” W). Chemical and physical characteristics of these sites as well as the diversity and structure of CO-oxidizing and total bacterial communities have been described previously (Dunfield and King, 2004; Gomez-Alvarez et al., 2007; King, 2003c; King and Weber, 2008; Nanba et al., 2004; Chapter 2). The Pu’u Puai deposit consists of cm-size basalt
cinders that support patches or islands of woody vegetation, including *Meterosideros polymorpha* and *Myrica faya*, which are surrounded by unvegetated patches. The Canopy site is located inside a vegetated patch about 2 m from the Edge site at the perimeter of the vegetation patch. The Bare site is located 5 m from the Edge site in unvegetated cinders. The Canopy and Edge sites were covered with a 5-10 cm layer of litter, while little or no litter occurs on the Bare site.

**Sample Collection and DNA Extraction**

In August 2008, triplicate surface samples (0-2 cm) were collected aseptically from the Bare, Edge and Canopy sites and placed into Whirlpak bags. Samples were transported to Louisiana State University on dry ice and stored at -80 °C until DNA was extracted. Major roots and debris were removed from all samples prior to extraction and cinders in Edge and Bare samples were crushed using a sterilized mortar and pestle. Prior to extraction, all materials were subjected to three cycles of freezing and thawing at -80 °C and 65 °C, respectively. DNA was extracted from triplicate Canopy samples (0.25-0.5 g fw) using a MoBio Power Soil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA). DNA was extracted from triplicate 5-7 g and 7-12 g subsamples of Edge and Bare materials, respectively, using a MoBio Power Max Soil DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA). Parallel subsamples were used for determining water content. DNA concentrations in the extracts were determined using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE).

**Primer Development and Testing**

*CoxL* sequences from *Burkholderia xenovorans* LB400 and three CO-oxidizing *Burkholderia* strains from Canopy soil (CP11, PP52-1, PP51-3), as well as *Burkholderia*-like *coxL* clone sequences previously obtained from the Edge and Canopy sites (Chapter 2) were
aligned using ClustalX version 1.0.1 (Thompson et al., 1997). Potential priming sites were identified using Primaclade (http://www.umsl.edu/services/kellogg/primaclade.html). Primers (F3 and R5; see Table 4.1) were chosen to amplify a 259 bp fragment of coxL, which includes the carbon monoxide dehydrogenase (CODH) active site motif; these primers exclude known coxL sequences from Actinobacteria, α- and γ-Proteobacteria, form II putative coxL genes, and known coxL sequences from β-Proteobacteria other than Burkholderia. Primer specificity was examined using PCR reactions with DNA extracts from various CO-oxidizing Actinobacteria and α- and β-Proteobacteria including members of the genera Mycobacterium (B8HB, M. neoaurum, M. parafortuitum, M. marinum), Stappia (S. aggregata, BrT7, MIO, S. stellulata, HI, M4), Shinella zoogloeoides str. FG1M5, Sulfitobacter str. P10 and Mesorhizobium str. KP12W. Burkholderia strains used in the primer design were PCR amplified as well as strains discovered subsequent to primer design (DNBP18, DNBP16, DNBP20, DNBP22, DNBP6-1, I7, PO-04-38-17, GA, WA, YA, B2of, PP51-2, PP52-1, CP11, I2, Rim).

To further confirm the specificity of Burkholderia coxL QPCR primers, Edge and Canopy site DNA was used in PCR amplifications as described below. PCR products were then cloned as described previously (Chapter 2). The PureYield Plasmid Miniprep System (Promega, Madison, WI) was used to isolate plasmids from individual clones grown in 0.6 ml of Luria Broth with kanamycin (50 µg ml⁻¹). Plasmids were screened for the presence of the insert by PCR amplification using primers F3 and R5. Inserts were bidirectionally sequenced from the plasmids using vector primers M13R and T7, and assembled as perviously described (Chapter 2). Sequences were analyzed by BLAST and then aligned and used in phylogenetic analyses to determine their affiliations. QPCR products were also cloned in a similar manner and clones
were screened for the proper sized insert using PCR amplification to confirm specificity of the QPCR reaction as well.

Table 4.1. Primers used for QPCR

<table>
<thead>
<tr>
<th>Target group</th>
<th>gene</th>
<th>Product length</th>
<th>Primer</th>
<th>Primer sequence (5'-3')</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Burkholderia</em></td>
<td><em>coxL</em></td>
<td>259 bp</td>
<td>F3</td>
<td>CGGCATGTCCAYGTCTG</td>
<td>This Study</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R5</td>
<td>ATCGTATTGAGKCCRAGC</td>
<td></td>
</tr>
<tr>
<td><em>ß-Proteobacteria</em></td>
<td>16S rRNA</td>
<td>360 bp</td>
<td>Eub338</td>
<td>ACTCCTACGGAGGCAGC</td>
<td>Lane, 1991; Fierer et al. 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Bet680</td>
<td>TCACTGCTACACGYG</td>
<td>Overmann et al. 1999, Fierer et al. 2005</td>
</tr>
<tr>
<td><em>All Bacteria</em></td>
<td>16S rRNA</td>
<td>200 bp</td>
<td>Eub338</td>
<td>ACTCCTACGGAGGCAGC</td>
<td>Lane, 1991; Fierer et al. 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Eub518</td>
<td>ATTACCGCGGCTGCTGG</td>
<td>Muyzer et al. 1993; Fierer et al. 2005</td>
</tr>
</tbody>
</table>

**QPCR Response Calibration**

The 16S rRNA and *coxL* gene copy numbers were quantified using standard curves generated from a series of 10-fold dilutions of *Burkholderia xenovorans* LB400 DNA extracted from 2-ml of culture grown overnight in pyruvate yeast extract (PYE) medium using a Microbial UltraClean DNA Isolation Kit according to the manufacturer’s protocol (MoBio Laboratories, Carlsbad, CA) (Weber and King, 2007). Possible DNA contamination in the kit reagents or processing was assessed with a culture-free extraction blank. Prior to extraction, culture and culture-free blanks were subjected to three cycles of freezing and thawing at -80 °C and 65 °C.
DNA concentrations in culture and culture-free extraction blanks were determined using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). The number of genome copies in the DNA extract was determined by assuming the average molecular mass of double-stranded DNA is 660 Da. Based on the known size of the \textit{B. xenovorans} LB400 genome, 9,731,138 bp (Chain \textit{et al.}, 2006), the average molecular weight of the genome is 10.7 fg. Genome copy number in the extract was then calculated using a formula adapted from previous QPCR studies (Joly \textit{et al.}, 2006): copy number = quantity of DNA (fg)/average mass of the \textit{B. xenovorans} LB400 genome. The 16S rRNA and \textit{coxL} gene copy numbers were then calculated based on the presence of six and two copies of these respective genes in the \textit{B. xenovorans} LB400 genome (Chain \textit{et al.}, 2006; Klappenbach \textit{et al.}, 2001; Lee \textit{et al.}, 2008).

Standard curves were generated using five 10-fold dilutions of \textit{B. xenovorans} LB400 DNA which ranged from 24 to \(2.4 \times 10^5\) genome copies. Triplicate sets of standards and no template controls (NTC) were run in parallel with all samples. The lower detection limit was determined from the highest DNA dilution that consistently amplified at a threshold cycle (Ct) lower than the NTC. Standard curves were plotted as Ct versus log of the calculated gene copy number.

\textbf{QPCR Reaction Conditions}\n
PCR conditions were optimized by amplifying genes from \textit{B. xenovorans} LB400 DNA and from soil extracts using a variety of primer concentrations and annealing temperatures. All QPCR reactions were carried out in 96-well ABI Prism MircoAmp optical plates using 25 \(\mu\)l reactions and the primers listed in Table 1. \textit{Burkholderia coxL} was amplified in reactions containing the following components: 12.5 \(\mu\)l Absolute QPCR SYBR Green Mix (Thermo Scientific, Epsom, Surrey, UK), 0.8 \(\mu\)l of each primer (0.32 \(\mu\)M final concentration each
primer), 1 µL ROX dye (80-fold dilution in sterile H2O; Thermo Scientific) and 0.5-4 ng template DNA. Amplification was carried out in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using the following program: Taq polymerase activation at 95 °C for 15 min, 35 cycles of denaturation at 95 °C (1 min), annealing at 60 °C (30 s) and extension at 72 °C (1 min). The β-Proteobacteria 16S rRNA genes and total 16S rRNA genes were amplified in the same manner using previously described primers and annealing temperatures of 60 °C and 53 ºC (Fierer et al., 2005) at final concentrations of 0.5 µM and 0.3 µM, respectively. Reactions were conducted in triplicate. Dissociation curve analysis and PCR product visualization using gel electrophoresis were carried out after each amplification to confirm reaction specificity. Gene copy numbers in soil DNA were determined based on the Ct values and by calculating the log of the gene copy number using the standard curves previously described.

In cases where amplification was detected in the no template control (NTC), the baseline was manually adjusted above the NTC to ensure that fluorescence signal did not contribute to standard or sample Ct values. For each gene quantified, all samples were run in a maximum of two separate QPCR assays to minimize error due to run-to-run variability; in these cases careful attention was paid to the behavior of the standard curves in each assay to ensure comparability of gene copy numbers. Samples were only quantified if they fell within the linear range of the standard curves. Gene copy numbers in cinder and soils extracts were expressed as copies ng DNA⁻¹ and copies gdw soil/cinders⁻¹, which was estimated from the total amount of DNA extracted from a known soil dry mass.

The presence of PCR inhibitors in cinder and soil DNA extracts was examined by amending extracts exhibiting gene copy numbers ranging from 1 x 10² to 1 x 10⁴ copies µl⁻¹ with
plasmid DNA containing either 16S rRNA or \textit{coxL} genes at final concentrations of $1 \times 10^6$ copies $\mu l^{-1}$. Gene copy numbers were also examined in 10-fold serial dilutions of DNA to determine if PCR inhibitors were present.

\textbf{Nucleotide Sequence Accession Numbers}

The \textit{Burkholderia coxL} clone sequences from Edge and Canopy libraries were deposited in GenBank under accession numbers FJ713673-FJ713692.

\textbf{Results}

\textbf{Primer Specificity}

A 259 bp \textit{coxL} fragment was successfully amplified from the \textit{Burkholderia} strains used for primer design (\textit{B. xenovorans} LB400, CP11, PP52-1, PP51-3) and from 14 other CO-oxidizing \textit{Burkholderia} strains isolated from Canopy soil (DNBP18, DNBP16, DNBP20, DNBP22, DNBP6-1, I7, I2 GA, WA, YA, B2of, PP51-2, PP52-1, CP11) and a strain isolated from soil from Pico de Orizaba, Mexico (PO-04-17-38). The \textit{coxL} genes from one CO-oxidizing \textit{Burkholderia} strain isolated from unvegetated volcanic deposits could not be amplified (strain Rim). No PCR products were amplified from any of the CO-oxidizing \textit{Actinobacteria} or \textit{α-Proteobacteria}. Dissociation curves from QPCR reactions containing soil DNA contained only one peak corresponding to the amplified fragment in the standards, confirming the specificity of the reaction (Figure 4.1). Likewise, visualization of PCR products by gel electrophoresis typically revealed the presence of a single band. Occasionally, a faint larger band (about 1000 bp) could be seen in some replicates, but this did not impact Ct values.

Twenty \textit{coxL} clones from libraries generated from Canopy and Edge DNA were chosen at random and sequenced. All 20 clone sequences were identified as form I \textit{coxL}; these sequences were most closely related to other \textit{Burkholderia coxL} genes by BLAST
Figure 4.1. Dissociation curves for *Burkholderia coxL* amplicons from environmental DNA. analysis, and clustered phylogenetically with *Burkholderia coxL* genes (Figure 4.2). Clones generated from the QPCR products had the same sized inserts as the form I *coxL*-containing clones, demonstrating the specificity of the QPCR.

**Sensitivity, Detection Limit and Amplification Efficiency**

Standard curves generated from serial dilutions of *Burkholderia xenovorans* LB400 DNA for the *coxL*, β*-Proteobacterial* 16S rRNA and 16S rRNA gene fragments were linear between $4.6 \times 10^3$ to $4.7 \times 10^6$, $1.42 \times 10^3$ to $1.43 \times 10^6$, and $1.42 \times 10^3$ to $1.42 \times 10^6$ gene copies, respectively (Figure 4.3). The lower detection limits for *coxL*, β*-Proteobacteria* 16S rRNA and 16S rRNA gene fragments assays were $4.7 \times 10^3$, $1.42 \times 10^3$ and $1.42 \times 10^3$ copies reaction$^{-1}$, respectively. Amplification of serial dilutions of environmental DNA yielded the expected corresponding decrease in copy numbers when 0.5 to 10 ng were loaded in a PCR reaction indicating that there was no inhibition. Amplification efficiencies for all genes were similar, ranging from 1.6 to 1.7, allowing for comparisons of gene copy numbers.
coxL, β-Proteobacteria 16S rRNA and Total 16S rRNA Gene Copy Numbers

Gene copy numbers for coxL, β-Proteobacterial 16S rRNA and total 16S rRNA increased across the vegetation gradient from the Bare to Canopy sites (Table 4.2). Numbers of Burkholderia coxL genes were below the detection limit in the Bare site (4.70 x 10^3 copies reaction^-1), while coxL copy numbers for the Canopy and Edge sites ranged from 7.20 x 10^3 to 1.48 x 10^4 copies per ng DNA and did not differ statistically. When expressed per gdw soil, however, Canopy and Edge coxL gene copy numbers differed significantly (p = 0.0007), and ranged from 7.10 x 10^8 to 9.52 x 10^8 and 7.58 x 10^7 to 1.47 x 10^8, respectively (Table 4.2).

Copy numbers of β-Proteobacteria 16S rRNA genes in Bare and Canopy sites ranged from 1.47 x 10^2 to 7.04 x 10^2 and 3.93 x 10^3 to 9.91 x 10^3 copies ng DNA^-1, respectively, and differed statistically (p = 0.01). The number of β-Proteobacteria 16S rRNA gene copies per ng DNA in the Edge site did not differ significantly from either Bare or Canopy numbers. Gene copy numbers per gdw soil for Bare and Edge were not statistically different, and ranged from 1.42 x 10^5 to 8.4 x 10^5 and 2.36 x 10^7 to 4.04 x 10^7, respectively (Table 4.2). Copy numbers per gdw soil in the Canopy were significantly higher than for Bare or Edge sites (p = 0.0099) ranging from 3.13 x 10^8 to 8 x 10^8 (Table 4.2).

Copies of 16S rRNA genes in the Bare, Edge and Canopy sites ranged from 3.4 x 10^4 to 1.97 x 10^6 copies ng DNA^-1 and 3.87 x 10^7 to 1.78 x 10^11 copies gdw soil^-1. Copy numbers corrected per ng DNA or per gdw soil did not differ statistically among sites. However, p-values were much lower for comparisons of Canopy site copy numbers per gdw soil with those of the Edge and Bare sites (p=0.0814 and p=0.0718, respectively) than for comparison of Edge and Bare sites (p = 0.9995).
**Figure 4.2.** Neighbor-joining tree (1000 bootstrap replicates) of *Burkholderia coxL* (259 bp) clones from Edge and Canopy DNA and culture reference sequences. Clone sequences are designated with “Clone” followed by the site from which they were obtained. Accession numbers: *Bradyrhizobium* sp. ORS278 (YP001207897); *Burkholderia* sp. I7 (pending); *Burkholderia* sp. I2 (pending); *Burkholderia* sp. DNBP6-1 (FJ466453); *Burkholderia* sp. EB-2 (pending); *Burkholderia* sp. DNBP16 (FJ466452); *Burkholderia* sp. DNBP18 (FJ466451); *Burkholderia* sp. DNBP20 (FJ466450); *Burkholderia* str. Rim (pending); *Burkholderia* sp. PP51-2 (FJ713671); *Carboxiphilus carboxydus* (FJ466455); *Mesorhizobium* str. KP12W (FJ152139); *Shinella zoogloeoides* str. FG1M5 (FJ152138); *Stenotrophomonas* str. LUP (AY307920); *Oligotropha carboxidovorans* (CAA57829); *Silicibacter pomeroyi* DSS-3 (YP166760); *Ruegeria* sp. WHOI JT-08 (AAW88347); *Burkholderia xenovorans* LB400 (YP558874); *Burkholderia* str. CP11 (FJ152141); *Burkholderia* str. PP521 (FJ152140); *Burkholderia* str. PO-04-17-38 (FJ713672); *Hydrogenophaga pseudoalvata* (HPU80806); *Alkalilimnicola ehrlichei* MLHE1 (YP742401); *Rhodobacter sphaeroides* (YP352939); Clone Canopy 8 (FJ13679); Clone Canopy 12 (FJ13683); Clone Canopy 9 (FJ13680); Clone Canopy 11 (FJ13682); Clone Edge 3 (FJ13688); Clone Edge 1 (FJ13686); Clone Edge 2 (FJ13687).
Figure 4.3. Standard curves utilized for quantifying *Burkholderia* coxL, β-Proteobacteria 16S rRNA, and total 16S rRNA gene copy numbers in QPCR reactions. Points represent averages of triplicate standards ± 1 S.E.; some error bars are smaller than the symbols.

The coxL copy numbers corrected per ng DNA or gdw soil for the Edge and Canopy sites did not differ significantly from the β-Proteobacterial 16S rRNA copy number (p=0.74-0.99, p =1). The coxL copy numbers for Edge and Canopy site differed significantly from total 16S rRNA genes at α =0.05 and 0.1, respectively (p=0.0001-0.029, p= 0.075-0.08). Average numbers of coxL genes expressed as a percentage of the total 16S rRNA genes for the Edge and Canopy sites were 6.2 and 0.7 %, respectively. Average numbers of β-Proteobacterial 16S rRNA genes expressed as a percentage of total 16S rRNA genes for the Bare, Edge and Canopy sites were 0.1, 2.0 and 0.5 %, respectively (Table 4.2).
**Table 4.2.** Gene copy numbers gdw soil$^{-1}$ or gdw cinders$^{-1}$ and ng DNA$^{-1}$. “detect limit” = 4.3 x 10$^3$ copies reaction$^{-1}$. “16S”=β-Proteobacteria 16S rRNA genes and “16S” =total 16S rRNA genes. Average copy numbers are reported for triplicate quantifications for each sample with the standard error in parentheses. “se”= standard error among samples.

<table>
<thead>
<tr>
<th>Site</th>
<th>Gene copy number per gram dry weight soil/ cinders</th>
<th>Ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coxL</td>
<td>se</td>
</tr>
<tr>
<td>Canopy</td>
<td>7.10x10$^6$</td>
<td>(1.45x10$^5$)</td>
</tr>
<tr>
<td>Canopy</td>
<td>9.52x10$^6$</td>
<td>(1.03x10$^5$)</td>
</tr>
<tr>
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<td>9.21x10$^6$</td>
<td>(4.51x10$^5$)</td>
</tr>
<tr>
<td>Edge</td>
<td>7.65x10$^6$</td>
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<tr>
<td>Bare</td>
<td>&lt; detect limit</td>
<td>NA</td>
</tr>
</tbody>
</table>

**Discussion**

This study describes a QPCR assay designed to enumerate coxL genes from the genus *Burkholderia*. This represents the first attempt to enumerate coxL from any subgroup of CO oxidizers in environmental samples using molecular techniques. Results show that numbers of
*Burkholderia coxL* genes increase with increasing vegetation cover on a recent volcanic deposit. These results are consistent with trends suggested by recent molecular and activity surveys across the same gradient (Chapter 2). Similarities between numbers of *Burkholderia coxL* genes and β-Proteobacterial 16S rRNA genes at the sites also suggest that CO-oxidizing *Burkholderia* dominates the β-Proteobacteria at these sites. Numbers of *Burkholderia coxL* genes expressed as a percentage of total 16S rRNA genes at the sites are similar to or greater than percentages previously reported for other functional genes (i.e. *nifH, narG, nosZ*) in soils (Henry et al., 2004; Henry et al., 2006; Kandeler et al., 2006). This indicates that CO oxidizers may be more abundant than other functional groups. Collectively, results suggest that CO-oxidizing *Burkholderia* are associated with the rhizosphere where they are abundant and contribute to CO oxidizer community expansion during biological succession on recent volcanic deposits.

Dissociation curve analysis, PCR amplification of *coxL* from known organisms as well as sequencing and phylogenetic analysis of clones indicate that primers F3 and R5 specifically amplify form I *coxL* genes from *Burkholderia*. The only *Burkholderia coxL* gene that did not amplify with the primers F3 and R5 is from strain Rim. Phylogenetic analysis of the Rim *coxL* gene that was amplified with the generic *coxL* primers (Sof and Psr, Chapter 2) revealed that it clustered with *coxL* genes from other α-Proteobacteria, such as *Bradyrhizobium* (Figure 4.2). Aside from this exception, the *Burkholderia coxL* phylogeny parallels the 16S rRNA gene phylogeny with the *Burkholderia coxL* sequences clustering together in the greater β-Proteobacteria *coxL* clade. This suggests that the Rim *coxL* gene may represent a case of horizontal gene transfer. Thus, the primers used in this study work effectively with the apparent majority, but not all, of the known CO-oxidizing *Burkholderia* and therefore likely underestimate *Burkholderia coxL* gene copy numbers to a limited extent.
Numbers of *Burkholderia* coxL genes in the Canopy site are significantly greater than in the Edge site when expressed per gdw soil (p = 0.0007). This increase is consistent with results from a previous study, which show that the percentage of β-Proteobacteria coxL sequences in clone libraries for the Bare, Edge and Canopy sites comprise 0%, 1.7% and 32.9 %, respectively, of the total number of sequences observed (Chapter 2). Collectively, these results suggest that β-Proteobacteria contribute to the expansion of CO oxidizer diversity with vegetation development, and that the genus, *Burkholderia*, is a particularly important contributor.

Numbers of β-Proteobacteria 16S rRNA genes gdw soil⁻¹ or gdw cinders⁻¹ increased 1400-fold from the Bare to the Canopy site, which was statistically significant and paralleled trends exhibited by previously generated 16S rRNA gene libraries for these sites (Chapter 2). The β-Proteobacterial fraction of previously generated 16S rRNA gene libraries for Bare, Edge and Canopy sites, was 0, 2.2 and 4 %, respectively (Chapter 2). Perhaps more notably, although β-Proteobacteria only occupied 4% of the Canopy 16S rRNA gene library, all of the sequences classified into the genus *Burkholderia*, indicating that *Burkholderias* were an important component of the β-Proteobacteria at this site (Chapter 2). Numbers of *Burkholderia* coxL and β-Proteobacteria 16S rRNA genes were statistically the same at the Edge and Canopy sites providing more evidence that *Burkholderias* are a dominant component of the β-Proteobacteria at these sites. In addition, the average number of β-Proteobacteria 16S rRNA genes expressed as a percent of 16S rRNA genes estimated for the Edge and Canopy site are 2 % and 1.5 %, which is consistent with the estimates from previously generated 16S rRNA gene libraries (Chapter 2). Consistency between the library and QPCR results provides evidence that the QPCR results are on the right order of magnitude; similarity of coxL and β-proteobacterial 16S
rRNA gene numbers truly suggests that burkholderias may dominate the β-Proteobacteria populations at these sites.

Copy numbers for coxL and 16S rRNA genes in individual genomes vary between 1-2 (King and Weber, 2007), and 1-15 (Fogel et al., 1999; Klappenbach et al., 2000), respectively. This constrains comparisons of gene numbers with cell numbers (Smith and Osborn, 2009). Comparisons of absolute gene numbers among studies are also limited by differences in DNA extraction methods and efficiencies (Smith and Osborn, 2009). Expressing functional gene numbers as a percentage of total 16S rRNA gene numbers, however, facilitates estimates of changes in relative abundance and comparisons among studies. Average numbers of Burkholderia coxL genes in the Edge and Canopy sites expressed as a percentage of the total 16S rRNA were 6.2 and 0.7 %, respectively, demonstrating that even though absolute numbers of coxL may increase with increasing vegetation, CO-oxidizing Burkholderias comprise a smaller fraction of total bacterial community in the Canopy than the Edge site.

These percentages are similar to or higher than those reported for other functional genes in several soil ecosystems indicating that relative abundance of coxL may be similar or higher than other functional genes. For instance, nirK and nosZ have been reported at levels from 0.1% to 6% of 16S rRNA gene copy numbers in agricultural, marsh and Himalyan soils (Henry et al., 2006). A study of denitrification gene abundance across a successional gradient on a glacial foreland has reported similar percentages for narG, nirK, nirS and nosZ, (Kandeler et al., 2006). Because Burkholderia genes are only a fraction of the cox gene pool, the total abundance of coxL must account for a somewhat larger percentage of 16S rRNA genes for Edge and Canopy sites than has been reported for other functional genes in other soil ecosystems.
Relative abundances of denitrifying genes across a successional gradient on a glacial foreland exhibited contrasting patterns to the relative abundances of *Burkholderia coxL* (Kandeler *et al.*, 2006). For instance, *nirK* and *nosZ* genes did not change in abundance across the gradient examined by Kandler *et al.* (2006), while *narG* and *nirS* genes contributed higher percentages in the younger sites (Kandeler *et al.*, 2006). In contrast, the relative abundance of *Burkholderia coxL* was greater in the presence of vegetation than in the Bare site. Kandeler *et al.* (2006) attributed the patterns they observed to the distribution of organic matter and differential substrate uptake abilities among different populations of denitrifiers. The distribution of organic matter may also explain the distribution of *Burkholderia coxL*. 

*Burkholderias* are well known for their ability to utilize a wide variety of substrates (Balandreau and Mavingui, 2007). In addition, they are abundant in the rhizosphere and are primary consumers of root exudates (Balandreau and Mavingui, 2007), which likely accounts for their relatively high numbers in Edge and Canopy sites. Their increased relative abundance in vegetated sites also indicates their ability to remain competitive at least into the intermediate stages of succession.

Assuming that CO-oxidizing *Burkholderia* have 1-2 copies of the *coxL* gene, the average number of *Burkholderia coxL* genes in the Canopy (8.60 x 10⁸ copies gdw⁻¹) corresponds to 4.30 x 10⁸ - 8.60 x 10⁸ organisms. In a previously generated *coxL* clone library for the Canopy site, β-*Proteobacteria* comprised 32.9% of the sequences (Chapter 2); assuming this fraction is entirely *Burkholderia*, total CO oxidizer abundance extrapolates as 1.30 x 10⁹ - 2.60 x 10⁹ gdw⁻¹. Because *Burkholderia* comprise only a fraction of the β-*Proteobacteria* CO oxidizers, this is a lower limit on Canopy CO oxidizer community size. This estimate is also lowered because the
primer set will not target *coxL* genes in *Burkholderia* that may have been horizontally transferred from other phyla.

However, even this underestimate of total CO oxidizers exceeds previous estimates of total CO oxidizer abundance in soils based on MPN assays (4.4 x 10^6 - 1.6 x 10^8 CO oxidizers gdw^-1) (Bender and Conrad, 1994; Chapter 2), and indicates that CO oxidizers are much more abundant than previously thought. Estimates based on MPN assays traditionally underestimate numbers of organisms because they depend on the growth of organisms under a defined set of conditions. In the case of CO oxidizers in particular, the sensitivity of CO oxidation to carbon rich conditions or possibly inhibitory concentrations of CO used in culture may have masked activity of some of community members.

Given that bacteria are known to contain 1-15 copies of the 16S rRNA gene per genome (Fogel *et al.*, 1999; Klappenbach *et al.*, 2000) the total Canopy 16S rRNA copy number per gdw soil may represent anywhere from 8.00 x10^9 to 1.20 x10^{11} organisms with an average of 6.40 x10^{10} organisms. The average number of total CO oxidizers estimated above (1.95 x10^9 organisms) would represent about 3% of the average estimated number of total organisms based on the 16S rRNA gene copy number for the Canopy site. Although only an estimate, it indicates the possibility that CO oxidizers occupy a significant fraction of the Canopy bacterial community as a whole.

The fact that a significant part of the community is comprised of facultative lithotrophs indicates that they can remain competitive during the latter stages of succession. *Burkholderia* in particular are known for their large genome sizes and ability to operate as opportunistic bacteria. Such opportunists, or R strategists are typically thought to be favored in during early succession when energy flow favors bacteria that are versatile and able to partition energy more
toward reproduction than survival. However, burkholderias are well known for the associations with plants, both symbiotic and pathogenic, and their versatility and connection with plants may promote their relatively high abundance in the rhizosphere, offsetting any disadvantage there might be from possessing large genomes.

In summary, the absolute abundance of Burkholderia coxL genes increases with increasing vegetation development, indicating that Burkholderia contribute to CO oxidizer community expansion during biological succession. Although Burkholderia-plant interactions, both pathogenic and symbiotic have long been documented, the recognition that many of these organisms may oxidize CO is new and provides fodder for asking questions regarding the role of CO in the bacterial-plant interactions. Although an underestimate, extrapolation from numbers of Burkholderia coxL genes to total CO-oxidizing organisms in the environment provide the first quantitative estimates of CO oxidizer abundances using molecular techniques and indicate that CO oxidizers may be more abundant in the rhizosphere than previously thought. In the future, development of other taxon specific QPCR assays as well as estimates of their activities will help link the newly expanded picture of CO oxidizer diversity and functional contributions of various taxa as well as refine quantitative estimates of CO oxidizer abundances in situ.
CHAPTER 5.
INSIGHTS INTO THE DISTRIBUTION AND ECOLOGICAL ROLE OF CARBON MONOXIDE OXIDATION IN THE GENUS *BURKHOLDERIA*
Introduction

The genus *Burkholderia* is metabolically versatile and occupies a wide variety of ecological niches. Members of the genus occur in the plant rhizosphere, endophytically in roots, soil, water, insect guts, in and on fungal mycelia, protozoans and mammalian macrophages, where they partake in a variety of interactions with other organisms ranging from beneficial to pathogenic (Vandamme *et al.*, 2007a). Many studies and recent genome sequencing efforts have focused on members of the *B. cepacia* complex, which are well-known as opportunistic pathogens in cystic fibrosis patients as well as two other animal pathogens, *B. mallei* and *B. pseudomallei*. However, the importance of *Burkholderia* as a member of the soil community is being increasingly recognized and the ecological roles of these organisms are receiving an increasing amount of attention, as they can potentially be used for bioremediation, biocontrol and promotion of plant-growth (Balandreau and Mavingui, 2007).

Members of the genus are known to carry out many biogeochemically important processes such as nitrogen-fixation (Perin *et al.*, 2006), inorganic phosphate solubilization (Delvasto *et al.*, 2009; Song *et al.*, 2008) and polyaromatic hydrocarbon degradation (Johnsen *et al.*, 2005; Kim *et al.*, 2003), but it was only recently discovered that *Burkholderia xenovorans* LB400, is capable of oxidizing carbon monoxide (King, 2003a). Microbial CO oxidation in soils serves as an important sink for atmospheric CO, consuming as much as 15% of annual CO emissions (Conrad, 1996; King, 1999b). This sink is important because CO reacts with and reduces the atmospheric pool of hydroxyl radical, which is a primary mediator of powerful greenhouse gases, like methane (Crutzen and Gidel, 1983; Khalil, 1999; Monson and Holland, 2001). Relatively few CO-oxidizing bacteria have been identified to date, but recent culture-independent assessments of CO oxidizer diversity and quantification of the *Burkholderia coxL*
gene (large subunit of carbon monoxide dehydrogenase) in soil, indicates that Burkholderia may be an important member of CO oxidizer communities in situ (Chapter 4).

Although the biogeochemical importance of biological CO oxidation is well-defined, the ecological role and regulation of bacterial CO oxidation in situ are not. While classic carboxydotrophs can grow using CO as the sole carbon and energy source (Meyer and Schlegel, 1983), many of the more recently discovered CO oxidizers lack suitable lithotrophic carbon-fixation pathways and appear to utilize CO only as a supplemental energy source (King and Weber, 2007). In a number of Burkholderia habitats such as roots and macrophages, CO is produced and may serve as a carbon and or energy source in these environments where a suitable supply of organic compounds may be lacking or CO may be used in conjunction with organic substrates as they become available. However, the response of CO-oxidizing burkholderias to environmentally relevant concentrations of CO and the ability to function mixotrophically has not been previously examined.

To date, the Burkholderia genus consists of over 40 formally described species but only one, B. xenovorans LB400, has been recognized as a CO oxidizer. However, recent efforts to isolate CO-oxidizing bacteria from volcanic soils resulted in the isolation of several burkholderias. To investigate the distribution of CO-oxidizing capabilities within the genus, these isolates as well as the nearest described relatives were screened for the presence of the coxL gene and the ability to consume CO at elevated and atmospheric concentrations. In addition, experiments were carried out to examine the impacts of heterotrophic substrate type and concentration on CO-oxidation in these isolates. Collectively, results presented here expand the known diversity and demonstrate that the trait may be widespread throughout the genus, but among root-associated species in particular. Physiological experiments demonstrate that CO-
oxidizing burkholderias are able to consume CO at sub-atmospheric concentrations of CO and can function mixotrophically in the presence of organic carbon concentrations \( \leq 500 \mu\text{M} \).

**Materials and Methods**

**Culture Sources and Isolation**

*B. phymatum* (DSM17167\(^T\)), *B. terrae* (DSM17804\(^T\)), *B. unamae* (DSM17197\(^T\)), *B. mimosarum* (DSM21841\(^T\)), *B. sacchari* (DSM17165\(^T\)), *B. phytofirmans* (DSM17436\(^T\)), *B. hospita* (DSM17164\(^T\)), *B. ferrariae* (18251\(^T\)) and *B. tropica* (DSM15359\(^T\)) were obtained from the Deutsche Sammlung von mikroorganismen und Zellculturen (DSMZ; Braunschweig, Germany). Cultures and/or DNA extracts from strains PO-04-17-38, PO-04-02-34, PO-04-17-25, PO-04-17-33, PO-04-17-39, PO-04-17-44, PO-04-19-36 and ME-04-02-52, which are soil isolates from Pico de Orizaba, Mexico (PO) or Mt. Evans (ME), were the gift of Fred Rainey (Louisiana State University, Baton Rouge, LA). A DNA extract from *Burkholderia* str. E-B2, an isolate from a passalid beetle hindgut, was the gift of Meredith Blackwell (Louisiana State University, Baton Rouge, LA).

Strains PP51-2, PP52-1, WA, YA, DNBP 18, DNBP22, DNBP6-1, DNBP20, DNBP16, CP11, I7, I2 were all enriched and isolated from soil located in the Canopy site on the 1959 Hawaiian volcanic deposit (Pu’u Puai) on the flank of Kilauea Volcano (19° 24’ 22.5” N X 155° 15’ 18.2” W), which was described previously (Chapter 2; King, 2003a; King and Weber, 2008). Four strains were enriched in basal salts medium (Meyer and Schlegel, 1978) containing 0.05% yeast extract (YE) and either 0.05% methanol (used to isolate PP51-2 and PP52-1) or 25 mM xylose (used to isolate WA and YA). Enrichments were assayed for CO oxidation by ammending the bottle headspaces to an initial CO concentration of 100 ppm, and monitoring headspace concentrations at suitable intervals using gas chromatography, as previously described.
Active enrichments were plated on their respective medium, solidified with agar (1.5%). Individual colonies were selected and used to inoculate small volumes of liquid media and were screened for CO oxidation as before. Active cultures were purified by plating and reexamined for activity. Strains designated DNBP were isolated from the canopy soil in a similar manner using dilute nutrient both (0.8 g L\(^{-1}\)) containing penicillin (500 \(\mu g\) ml\(^{-1}\)). Strains I7 and I2 were enriched using a pectin-YE medium adjusted to pH 3.5-4.

CP11 was enriched and isolated from pasteurized canopy soil in basal salts medium containing 0.05% YE and 25 mM pyruvate (PYE) as well as cyclohexamide to inhibit fungal growth (50 \(\mu g\) ml\(^{-1}\)). Soil was pasteurized by placing about 2 gram fresh weight (gfw) in 10 ml PYE and incubating at 62 °C for 30 min. The enrichment was then incubated with shaking at 30 °C until it became turbid. CO oxidation by the enrichment was confirmed using gas chromatography. CP11 was isolated on solid agar medium and purified as described above.

Strain Rim was enriched and isolated in PYE medium from volcanic soil on the Kilauea Caldera rim (19° 23’ 50.6” N x 155° 16’ 26.3” W). Physical, chemical and biological characteristics of this site has been previously described (Dunfield and King, 2004; Gomez-Alvarez et al., 2007; King, 2003c; King and Weber, 2008). Strain KP5Blue was isolated similarly in hydroxybutarate-YE medium from a 1983 volcanic deposit on Miyake-jima, Japan, a volcanic island southeast of Tokyo (latitude, longitude, International Terrestrial Reference Frame: 139° 30’ 4.4”, 34° 2’ 46.4”). This soil used in the isolation supported the growth of grasses and conifers.

**DNA Extraction, PCR, Sequencing and Phylogenetic Analysis**

Cells were harvested by centrigation at 10, 000 rpm for 1 minute from 2 ml of overnight cultures grown in PYE. Medium was discarded and cells were resuspended in 300 µl of Bead
Solution from the MoBio Ultraclean Microbial DNA Extraction Kit (MoBio Laboratories, Carlsbad, CA). Cell suspensions were subjected to three cycles of freezing and thawing at -80 °C and 65 °C, respectively. DNA was then extracted according to the manufacturer’s protocol.

DNA extracts were directly applied to 25 µl PCR reactions to amplify fragments of coxL (large subunit for carbon monoxide dehydrogenase) and 50 µl reactions to amplify 16S rRNA genes. The coxL fragment (form I; about 1,260 bp) was amplified in PCR reactions containing 12.5 µl of GoTaq Green Master Mix (Promega, Madison, WI) and 0.5 µM of primers (Sof and PSr; King, 2003a) using thermocycling conditions previously described (King, 2003a). The 16S rRNA genes were amplified as previously described using primers 1492r and 27f (Lane, 1991; Sambrook et al., 1989) in reactions containing 25 µl Go Taq Green Master Mix and 0.5 µM concentrations of each primer.

PCR products were visualized using gel electrophoresis and GelRed stain (Biotium, Inc., Hayward CA). Products of the correct size were purified using a MoBio Ultraclean PCR Cleanup Kit (MoBio Laboratories, Carlsbad, CA). PCR products were sequenced bidirectionally on an ABI model 3130XL sequencer using the amplification primers at the Louisiana State University Genomics Facility (Baton Rouge, LA). Sequences were assembled, edited and translated, where appropriate, using Sequencher version 4.8 (Gene Codes Corporation, Ann Arbor, MI).

Translated coxL sequences were aligned using ClustalX version 1.0.1 (Thompson et al., 1997). Phylogenetic analysis was performed in PAUP version 4.0b (Sinauer Associates, Inc., Sunderland, MA) using a neighbor-joining algorithm and 1000 bootstrap replicates. The 16S rRNA gene sequences were aligned with nearest neighbors, as determined by BLAST analysis, using the NAST alignment tool on the Greengenes webserver (greengenes.lbl.gov; DeSantis et
Alignments were adjusted manually, as necessary, using MacClade ver. 4.05 (Sinauer Associates, Inc., Sunderland, MA). Maximum likelihood analysis was performed using PHYML ([http://www.atgc-montpellier.fr/phyml/](http://www.atgc-montpellier.fr/phyml/)) with a HKY85 model for base substitution and 100 bootstrap replicates. The phylogenetic tree was visualized using MEGA software ([Kumar et al., 2008](#)). Also a neighbor-joining analysis using 1000 bootstrap replicates was carried out using PAUP (Sinauer Associates, Inc. Sunderland, MA).

**Maximum CO Uptake Velocities and Ambient CO Uptake Assays**

To determine the maximum potential CO uptake rates of the isolates, overnight cultures were grown in PYE, harvested by centrifugation, washed twice and resuspended in minimal medium. Ten ml aliquots were transferred to triplicate 160 cc bottles and the headspaces were immediately adjusted to a final concentration of approximately 200 ppm CO. CO concentrations were assayed immediately and at suitable intervals thereafter on an RGA-3 reduced gas analyzer (Trace Analytical Laboratories, Muskegon, MI), as previously described ([King, 1999b](#)). In between assays, cultures were incubated at 30 °C with shaking. After all measurements were completed, 2 ml samples of each culture were harvested for protein content determination by centrifuging them for 5 minutes at 10,000 rpm. Cells were resuspended in 500 µl basal salts medium containing 5 % tritonX 100 and subjected to five freeze thaw cycles at -80 °C and 65 °C, respectively. Protein concentrations were determined spectrophotometrically on a Gen5 Microplate reader (Biotek Instruments, Winooski, VT) using a Pierce Bicinchoninic Acid (BCA) Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer’s protocol.

Cultures examined for ambient CO consumption were grown for 48 hours in PYE, harvested and washed in a similar manner as described immediately above. For each culture, duplicate 160 cc bottles containing 10 ml of washed cells were spiked to a starting CO
concentration of about 85 ppm. Immediately after gas additions, bottle headspaces were sampled with a needle and syringe and assayed as described above. Headspace CO concentrations were monitored until they reached about 1 ppm. At suitable intervals thereafter, headspace CO concentrations were monitored using a Peak Performer 1 RCP gas chromatograph (Peak Laboratories, Mountain View, CA). Data were recorded and analyzed using PowerChrom Software (eDAQ, Inc., Colorado Springs, CO).

Growth on Heterotrophic Substrates in the Presence and Absence of CO

The ability to grow using heterotrophic substrates alone or in conjunction with CO was examined for strains DNBP16 and PP52-1 using basal salts medium supplemented with 0.01% yeast extract and various organic carbon substrates at concentrations of 25 mM. Substrates tested included proline, hydroxybutarate, gluconic acid, sucrose, acetate, mannitol, phthalate, methanol, pyruvate and methylamine. Cultures were grown overnight in PYE, harvested by centrifugation and washed twice in basal salts medium. Washed cultures were used to inoculate quadruplicate bottles for each organic carbon substrate, two of which were immediately spiked with CO to about 80 ppm CO. Optical densities were measured spectrophotometrically (600 nm) and CO concentrations were assayed using gas chromatography immediately after inoculation and 24 h later. Bottles were incubated with shaking at room temperature. Growth was determined relative to controls containing 0.01% yeast extract or 0.01% yeast extract with CO.

Impact of Heterotrophic Substrate Concentration on CO Uptake

An overnight culture of PP52-1 grown in PYE was harvested by centrifugation, washed twice in phosphate buffer and then resuspended in basal salts medium with 0.005% YE. Six sets of triplicate 160 cc serum bottles containing 4.5 ml of basal salts medium amended with 0.005% YE and 0, 0.5, 2.5, 5, 10 or 20 mM pyruvate were inoculated with 0.5 ml resuspended cells.
Immediately after inoculation, bottles were spiked to headspace concentrations of 100 ppm CO. Headspace CO concentrations were determined immediately and at suitable intervals thereafter.

In a separate experiment using cultures of DNBP18 that had been starved in basal salts medium for 3 months under a constant headspace CO concentration of about 200 ppm CO, CO uptake (initial concentration 100 ppm) and pyruvate uptake (initial concentration 400 µM) were examined simultaneously in triplicate cultures. In a duplicate set of cultures, CO uptake was examined in the absence of pyruvate. Three ml aliquots of culture were placed into sterile 60 cc gas tight serum bottles and incubated at room temperature with shaking. CO uptake was monitored as described above. Pyruvate concentrations were monitored through time by removing 0.1 ml samples of culture from the bottles. Samples were centrifuged at 10,000 rpm for 1 min to pellet the cells. Immediately following centrifugation pyruvate concentrations were determined in 25 µl samples of the supernatant using a microplate pyruvate assay kit (Biovision, Mountain View, CA) according to the manufacturer’s instructions. Microplates were read on a Gen5 Microplate Reader (Biotek Instruments, Winooski, VT). Cultures were incubated with shaking at room temperature in between samplings.

Results

Phylogenetic Analysis

BLAST analyses of 16S rRNA gene sequences from the isolates demonstrated that all shared > 97% maximum identity with 16S rRNA gene sequences of formally described Burkholderia species in the database. Phylogenetic analyses of isolate 16S rRNA gene sequences using maximum likelihood and neighbor-joining algorithms revealed the following consistencies: 1) the majority of the CO-oxidizing isolates (YA, CP11, DNBP18, DNBP22, DNBP20, PP52-1, I2, PP51-2) fall into the clade containing many rhizosphere, root and legume
nodule isolates and are most closely related to *B. sacchari*, *B. unamae*, *B. mimosarum* and *B. nodosa*, 2) Isolate PO-04-17-38 is the only CO-oxidizing isolate that is phylogenetically affiliated with the predominantly pathogenic clade containing *B. cepacia*, 3) strain Rim is most closely related to *B. caledonica* LMG 19076, 4) I7 clusters with *B. ginsengesolis* and 5) isolate KP5Blue is located within the cepacia complex (Figure 5.1). 6) isolate DNBP6-1 is most closely related to *B. bryophila*. The 16S rRNA gene sequence from isolate E-B2 was nearly identical to *B. fungorum* LMG16225T and is not displayed in the phylogenetic tree.

Form I *coxL* fragments were successfully PCR amplified and sequenced from strains I2, I7, PP51-2, PP52-1, CP11, DNBP16, DNBP18, DNBP20, DNBP6-1, DNBP22, WA, YA, PO-04-17-38, Rim, E-B2. Form I *coxL* and CO oxidation could not be detected in *B. phymatum* (17167T), *B. terrae* (17804T), *B. unamae* (DSM17197T), *B. sacchari* (DSM17165T), *B. phytofirmans* (DSM17436T), *B. hospita* (DSM17164T), *B. ferrariae* (DSM18251T) and *B. tropica* (DSM15359T), *B. bryophila* (LMG23644T), *B. mimosarum* (DSM21841T), PO-04-02-34, PO-04-17-25, PO-04-17-33, PO-04-17-39, PO-04-17-44, PO-04-19-36, ME-04-02-52 or KP5Blue. As of March 2009, *Burkholderia xenovorans* LB400, which has been previously noted as a CO oxidizer (King, 2003a) has the only *coxL* containing genome out of the 54 completed *Burkholderia* genomes in the IMG-JGI database that encompass 15 species (*B. xenovorans*, *B. vietnamensis*, *B. ubonensis*, *B. thailandensis*, *B. pseudomallei*, *B. phytofirmans*, *B. phymatum*, *B. oklahomensis*, *B. multivorans*, *B. mallei*, *B. graminis*, *B. dolosa*, *B. cepacia*, *B. cenocepacia*, *B. ambifaria*).

Phylogenetic analyses of inferred amino acid *coxL* sequences revealed that all sequences except that of strain Rim formed a tight cluster within the β-**Proteobacteria** *coxL* clade, but was distinct from the other β-**Proteobacteria** *coxL* sequences, *Hydrogenophaga pseudoflava* and
Figure 5.1. 16S rRNA gene phylogeny generated using a neighbor-joining method with 1000 bootstrap replicates. *Alcaligenes faecalis* ATCC 8750$^T$ (M22508) was used as an outgroup. Taxa in red type are CO oxidizers. Accession numbers: *B. ferrariae* str. FeG101 (DQ514537); *B. sacchari* DSM 17165$^T$ (AF263278); *B. mimosarum* str. PAS44 (AY752958); *B. silvatlantica* str. srmRH-20 (AY965240); *B. nodosa* str. R-22632 (AM284970); *B. tropica* str. Ppe8 (AJ420332); *B. unamae* str. MCo762 (AY221955); *B. unamae* str. MT1-641 (AY221956); *B. tuberum* str. STM678 (AJ302311); *B. kururiensis* (AB024310); *B. hospita* LMG 20598$^T$ (AY040365); *B. caribensis* MWAP64 (Y17009); *B. terrae* KMY02 (AB201285); *B. phymatum* STM815 (AJ302312); *B. graminis* C4D1M (ZP_02884902); *B. terricola* LMG 20594 (AY040362); *B. caledonica* LMG 19076 (AF215704); *B. fungorum* LMG 16225 (AF215705); *B. phytofirmans* str. PsJN (AY497470); *B. xenovorans* LB400 (NC_007951); *B. bryophila* LMG 23644 (AM489501); *B. ginsengesolis* KMY03 (AB201286); *B. phenzenium* LMG 2247$^T$ (U96936); *B. glathei* LMG 14190$^T$ (Y17052); “Candidatus B. calva” “19620512” (AY277697); “Candidatus B. kirkii” “19536779” (AF475063); *B. sordicola* S5-B$^T$ (AF512826); *B. phenoliruptrix* AC1100 (AY435213); *B. ubonae* (AB30584); *B. caryophili* ATCC 25418$^T$ (AB021423); *B. mallei* ATCC 23344 (NC_006348); *B. pseudomallei* str. 1026b (U91839); *B. thailandensis* E264 (U91838); *B. plantarii* LMG 9035$^T$ (U96933); *B. gladioli* ATCC 10248 (X67038); *B. glumae* LMG 2196$^T$ (U96931); *B. dolosa* LMG 18941 (AF175314); *B. vietnamiensis* str. G4 (CP000614); *B. multivorans* ATCC 17616 (AP009387); *B. cepacia* LMG 14294 (AF148554); *B. pyrrhocinia* ATCC 15958$^T$ (AB021369); *B. cepacia* str. AMMD (CP000440); *B. sp. 383 (CP000151); *B. ambifaria* mc40-6 (YP_001807657); *B. cepacia* ATCC 25416 (AY728197); *B. anthina* str. R-4183 (AJ420880); *Pandorea apista* CCUG 38412 (AY268172); YA, CP11, WA, DNBP18, DNBP22, DNBP20, PP51-2, PP, RIM, PO-04-17-33, PO-0417-39, DNBP6-1, I7, PO-04-17-25, PO-04-17-34, PO-04-17-38, KP5Blue (pending).
*Pseudomonas thermocarboxydovorans* (Figure 5.2). The *coxL* gene of strain Rim clusters with those of *Bradyrhizobium* within the larger α-Proteobacteria *coxL* clade (Figure 5.2).

Within the primary *Burkholderia coxL* clade, the phylogeny largely parallels the relationships demonstrated for the 16S rRNA gene phylogeny of these isolates. The *coxL* sequences from DNBP16, YA CP11, DNBP18, WA, PP51-2 and I2 cluster together (Figure 5.2), which is consistent with their 16S rRNA gene phylogeny, as their closest relatives are the closely related species of *B. nodosa*, *B. ferrariae*, *B. sacchari*, *B. unamae* and *B. mimosarum*. The *coxL* gene for DNBP6-1 falls phylogenetically outside of the primary *coxL* clade, which is also consistent with the 16S rRNA gene phylogeny (see Figure 5.1). The phylogenetic affiliation of the *coxL* genes from I7 and E-B2 is consistent with 16S rRNA gene phylogeny, as the closest realtives of these two isolates are *B. ginsengesolis* and *B. fungorum*.

Two close relatives of *B. sacchari*, DNBP20 and DNBP22 (>98% 16S rRNA gene identity), have *coxL* genes that do not cluster with the *coxL* genes from other close relatives of *B. sacchari*. In addition, the close phylogenetic relationship between the *coxL* genes of DNBP 6-1 and PO-04-17-38 is unexpected because these isolates are most closely related to distantly related described species (Figure 5.1).

**Maximum Potential CO Uptake Velocities and Atmospheric CO Consumption**

Isolates I2, I7, PP51-2, PP52-1, CP11, DNBP16, DNBP18, DNBP20, DNBP6-1, DNBP22, WA, YA, PO-04-17-38, and Rim all consumed CO at initial concentrations of 100 ppm (data not shown). Isolate E-B2 was not screened for activity because the original culture had been lost from isolation and only the DNA extract was available. Maximum CO uptake velocities expressed as nmol CO mg protein\(^{-1}\) hour\(^{-1}\) were (average ±1 s.e.): 32.2 ± 1.1 (n=3),
Figure 5.2. Inferred amino acid coxL phylogeny generated using a neighbor-joining method and 1000 bootstrap replicates. Only bootstrap values ≥ 70 are displayed. Accession numbers: *Pseudomonas thermocarboxydovorans* (X77931); *Hydrogenophaga pseudoflava* (HPU80806); *Alkalilimnicola ehrlichei* MLHE1 (YP742401); *Silicibacter pomeroyi* DSS-3 (YP166760); *Ruegeria* sp. WHOI JT-08 (AAW88347); *Pseudomonas carboxydohydrogena* (AY463247); *Oligotropha carboxidovorans* (CAA57829); *Stenotrophomonas* str. LUP (AY307920); *Stappia aggregata* IAM (AY307918); *Photobacterium* sp. COX (EF101508); *Shinella zoogloeoides* FG1M5 (FJ152138); *Carophilus carboxydus* (FJ466455); *Mesorhizobium* str. KP12W (FJ152139); *Burkholderia* str. RIM (pending); *Bradyrhizobium* sp. CPP (AY307911); *Bradyrhizobium japonicum* USDA6 (AY307921); *Burkholderia xenovorans* LB400 (YP558874). *Burkholderia* str. DNBP6-1 (FJ466453); *Burkholderia* str. PO-04-17-38 (FJ713672); *Burkholderia* str. DNBP20 (FJ466450); *Burkholderia* str. DNBP22 (pending); *Burkholderia* str. DNBP16 (FJ466452); *Burkholderia* str. YA (pending); *Burkholderia* str. CP11 (FJ152141); *Burkholderia* DNBP18 (FJ466451); *Burkholderia* str. WA (pending); *Burkholderia* str. PP51-2 (FJ713671); *Burkholderia* str. 12 (pending); *Burkholderia* str. 17 (pending); *Burkholderia* str. EB-2 (pending).
CP11; 32.1 ± 2.4 (n=3), DNBP22; 38.3 ± 1.5 (n=3), DNBP18; 74.5 ± 10 (n=2), I2; 35.2 ± 3.6 (n=3), DNBP20; 43.7 ± 2.4 (n=3), YA; 6.5 ± 0.1 (n=3), DNBP16. Maximum CO uptake velocities could not be expressed for the remainder of the isolates because they clumped and adhered to the side of the serum bottles, preventing accurate protein measurements. All five isolates examined (I2, DNBP18, PP52-1, DNBP6-1 and CP11), were able to consume CO at atmospheric concentrations (0.05-0.3 ppm; Figure 5.3).

**Figure 5.3.** CO uptake at atmospheric CO concentrations by isolates CP11 (■), I2 (▷), PP52-1 (▲), DNBP18 (●), DNBP6-1 (●). Duplicate cultures were used and results were similar for both, but only one set is shown for clarity.

**Impacts of Heterotrophic Substrates on CO Oxidation**

Without exception, isolates DNBP16 and PP52-1 only consumed CO in the presence of heterotrophic substrates (25 mM) that did not support growth (Figure 5.4); however, CO
consumption itself did not support growth over the 24-hour incubation, as there were not significant changes in optical density during this period (Figure 5.4). Growth yields within the 24 h period were generally higher for DNBP16 than PP52-1, but the substrate utilization profiles and trends in CO consumption were the same. Both isolates grew on proline, hydroxybutarate, gluconic acid, acetate, pyruvate, mannitol, but not methylamine, methanol, phthalate or sucrose.

**Figure 5.4.** Relative change in biomass vs. percent CO consumption (starting concentration approx 100 ppm) in 24 hours for two strains PP52-1 (a) and DNBP16 (b) grown on various organic substrates (25 mM) in the presence of CO. Note the change in scales on the x-axis.

A separate experiment was conducted to examine the impacts of pyruvate concentration on CO uptake in isolate PP52-1. The most rapid CO uptake was observed in the absence of pyruvate (Figure 5.5). A pyruvate concentration of 0.5 mM reduced the average CO uptake rate by almost 72% and increasing the pyruvate concentration to 5 mM reduced the average CO oxidation rate by 90%. No significant CO uptake was observed in the presence of 10 and 20 ppm.
mM (Figure 5.5), despite the fact that these treatments contained the most biomass, which was visually obvious; cultures formed clumps and adhered to sides of the bottles preventing accurate quantitative measurements of optical density or total protein measurement.

In another experiment using a starved DNBP18 culture, it was shown that pyruvate (400 µM initial concentration) and CO consumption (initial concentration 100 ppm) occurred simultaneously (Figure 5.6). A comparison of CO oxidation rates in the presence and absence of pyruvate were 0.11 ± 0.01 and 0.11± 0.03 ppm min⁻¹ indicating that this concentration of pyruvate did not impact CO oxidation (data not shown).

![Figure 5.5](image.png)

**Figure 5.5.** CO consumption by PP52-1 incubated in the presence of 0, 0.5, 2.5, 5, 10 and 20 mM pyruvate. Data points represent averages of triplicates (± 1 s.e.).
Figure 5.6. Simultaneous consumption of pyruvate (■) and CO (○) by DNBP18. All three replicates are shown.

Discussion

The Burkholderia genus is known for its metabolic versatility and numerous ecological roles which include plant pathogens, root endophytes and legume sybionts, animal pathogens, gut inhabitants and fungal symbionts (Balandreau and Mavingui, 2007; Vandamme et al., 2007b). Many of these habitats are characterized by fierce competition among bacteria and between bacteria and host organisms. The ability of Burkholderia to survive and compete under such conditions has been attributed to the many adaptations members of the genus possess, such as antibiotic production and resistance, type III and IV secretion systems, siderophore production, biofilm formation, quorum sensing and ability to rapidly consume a wide variety of heterotrophic substrates (Balandreau and Mavingui, 2007; Woods, 2007).
Their competitive nature and versatility is likely why they have been previously found to dominate the culturable fraction of numerous rhizosphere environments surveyed (Hebbar et al., 1992, 2002; Kokalis-Burelle et al., 2002; Tran Van et al., 1996; Vogel et al., 2002) and results presented here are no exception. Despite the fact that enrichment strategies utilized a variety of substrates at high and low concentrations, low pH and soil pasteurization, CO-oxidizing isolates were all members of the *Burkholderia* genus. Isolation of a *Burkholderia* from pasteurized soil, in particular, was remarkable because almost all other isolates from the enrichment were spore formers indicating that the pasteurization was largely successful. Isolate PO-04-17-38 was isolated at 10 °C (Rainey, personal communication) demonstrating that even though *Burkholderia* are mesophiles, they can grow at a wide range of temperatures. In addition, *Burkholderia coxL* was detected in a mixed culture derived from volcanic soil that had been desiccated (-150 MPa) for several weeks (Weber, unpublished).

Culture-independent assessments of CO-oxidizer diversity and QPCR estimates of *Burkholderia coxL* abundance indicate that *Burkholderia* is an important member of CO-oxidizing communities (Chapter 4), but prior to this study, only one CO-oxidizing member of the genus, *B. xenovorans* LB400, had been recognized (King, 2003a). Phylogenetic analysis of the 16S rRNA gene sequences of isolates obtained in this study indicate that CO-oxidizing capabilities may be widespread throughout the genus and are thus likely found in a number of habitats, but particularly in association with plant roots (Figure 5.1). Several of the isolate 16S rRNA gene sequences clustered phylogenetically in a clade containing *B. sacchari*, *B. unamae*, *B. tropica*, *B. mimosarum* and *B. nodosa*. *B. unamae* and *B. tropica* were originally isolated from Maize, teosinte plants and sugar cane across a wide geographical region including Mexico, Brazil and South Africa (Caballero-Mellado et al., 2004; Reis et al., 2004), where they are quite
abundant. *B. tropica* has been noted to reach population sizes up to $10^5$ g$^{-1}$ of fresh shoot in Maize grown in Mexico in the absence of fertilizer and pesticides (Estrada *et al.*, 2002). Likewise, *B. sacchari* was originally isolated from soil of a sugarcane plantation in Brazil (Braemer *et al.*, 2001). *B. mimosarum* and *B. nodosa* were originally isolated from root nodules of *Mimosa* spp. in Thailand (Chen *et al.*, 2006) and Brazil (Chen *et al.*, 2007), respectively.

The high abundance and beneficial interactions of burkholderias with roots has been well documented (Balandreau and Mavingui, 2007) and the close relatedness of several of the CO-oxidizing isolates to rhizosphere burkholderias provides evidence that CO-oxidizing *Burkholderias*, in particular, may partake in such interactions. CO production by roots provides CO inputs to soil that compare to diffusional inputs from the atmosphere (King and Crosby, 2002), and suggests that CO may play a role in root-CO oxidizer interactions. The fact that most soils are sinks for atmospheric CO, indicates that CO emitted from roots is consumed within the soil. *Burkholderias* may very well contribute to this internal sink, especially given their ability to consume CO at substmospheric concentrations (Figure 5.3). Furthermore, it has been proposed that CO oxidizers inside root nodules may alleviate inhibitory effects of root derived CO on the nitrogenase enzyme and assist indirectly in enhancing nitrogen-fixation (King and Weber, 2007; King and Crosby, 2002).

CO-oxidizing burkholderias are also likely involved in beneficial interactions with insects and fungi. Isolate E-B2, which was isolated from the gut of a passalid beetle, had a 16S rRNA sequence that was nearly identical to that of *B. fungorum*. Many plant-feeding animals such as *Riptortus calvatus, Leptocorisa chinesis*, termites and ant species, from which *B. fungorum* has also been isolated, contain *Burkholderia* symbionts in their midguts (Harazono *et al.*, 2003; Kikuchi *et al.*, 2005; Van Borm *et al.*, 2002). One species of ant in particular, *Atta*
sexdens rubripilosa, benefits from antifungal activities of burkholderias involved in a three-partner symbiosis with the ants and a fungus, Leucoagaricus gonglylophorus (Valmir Santos et al., 2004). These ants use glucose produced by the fungi and the antifungal activities of Burkholderia ward off fungal pathogens. CO-oxidizing burkholderias have not been isolated directly from fungi to date, but it might provide an interesting source for future efforts to enrich and isolate CO oxidizers.

The only CO-oxidizing isolate in this study that affiliated with the B. cepacia clade was PO-04-17-38, a soil isolate from Pico de Orizaba, Mexico. The affiliation of this isolate with the B. cepacia containing clade brings about several questions, particularly how this isolate may have acquired the trait. Screening of the 53 completed Burkholderia genome sequences aside from B. xenovorans LB400, which encompass 15 primarily pathogenic species revealed that none of these organisms contain authentic cox operons. In addition, isolate KP5Blue from a Japanese volcanic deposit, was most closely related to B. cenocepacia and did not oxidize CO. Thus far, it appears that CO-oxidizing contingent of the genus may be largely restricted to environmental isolates and largely dominated by soil and root inhabitants.

The evolutionary mechanism behind the distribution of aerobic CO oxidation among bacteria in general and within the Burkholderia genus specifically remains unexplored, but preliminary results here indicate that horizontal gene transfer may play a role in certain cases. The coxL phylogeny in general and within the Burkholderia genus largely parallels that of 16S rRNA gene phylogeny. However, there are a couple of exceptions where α-Proteobacteria-like coxL genes have been found in members of the γ-Proteobacteria (King and Weber, 2007). This appears to be the case for strain Rim, which has a coxL gene that clusters phylogenetically with the α-Proteobacteria-like coxL sequences (Figure 5.2).
In addition, it is interesting to note that despite the apparent close relationships between many of the CO-oxidizing isolates and described species (> 98% maximum identity of 16S rRNA), many of the type species screened, do not have coxL and do not oxidize CO. For instance, B. sacchari, B. unamae, B. tropica, B. ferrariae and B. mimosarum do not contain authentic coxL genes. It has been noted recently, however, that a species definition of 97% identity of 16S rRNA genes may be too liberal, especially for microorganisms like Burkholderia that have relatively large genomes (Konstantinidis et al., 2006). It has been demonstrated that 97% 16S rRNA gene sequence identity roughly correlates with $\geq 60\%$ average nucleotide identity (ANI) across the entire genome (Konstantinidis et al., 2006). However, even at 95% ANI, which correlated well with 70% similarity by DNA-DNA hybridization, the gene content of strains may differ by as much as 20%, meaning there may be a lot of functional heterogeneity encompassed within a given species based on current standards (Konstantinidis et al., 2006). This means that currently defined Burkholderia species could easily include both CO- and non-CO oxidizers; the use of coxL as a tool for distinguishing among isolates and redefining species requires further consideration.

Without exception, all strains possessing Form I coxL genes were able to consume CO from starting concentrations of about 150 ppm. Maximum CO uptake velocities calculated for six selected isolates ranged from $6.5 \pm 0.1$ to $74.5 \pm 10$ nmol CO mg protein$^{-1}$ h$^{-1}$. These maximum uptake velocities are low in comparison to those reported previously for members of the $\alpha$-Proteobacteria marine genus Stappia, which ranged from 65 to 510 nmol CO mg protein$^{-1}$ h$^{-1}$ (Weber and King, 2007) and a previous report of a maximum CO uptake velocity of 535 nmol CO mg protein$^{-1}$ h$^{-1}$ for B. xenovorans LB400 (King, 2003a). Nonetheless, five selected strains, which were phylogenetically representative of the isolates in this study, were all able to
consume atmospheric concentrations of CO (Figure 5.3). Typical atmospheric concentrations range from 50-300 ppb (Novelli et al., 1994a), and isolates consumed CO concentrations lower than 150 ppb (Figure 5.3). This indicates that these organisms have the ability to contribute to consumption of atmospheric concentrations in soil and likely also play a role in consuming CO emitted from roots.

Results reported here demonstrate that the extent of CO oxidation by members of the Burkholderia genus is highly dependent on the incubation conditions, particularly with respect to the type of available organic substrates. Similar observations have been made for isolates including Pseudomonas carboxydoflava (Kiessling and Meyer, 1982; Meyer and Schlegel, 1983), Pseudomonas thermocarboxydovorans (Pearson et al., 1994), Pseudomonas carboxydoflava (Meyer and Schlegel, 1983) and Hydrogenophaga pseudoflava (Kang and Kim, 1999). CODH is constitutively expressed in Pseudomonas carboxydoflava and Mycobacterium sp. strain JC1 and CO is consumed during heterotrophic growth (Kiessling and Meyer, 1982; Ro and Kim, 1993), but for the latter, CO consumption depends on organic substrate that is available (Ro and Kim, 1993). Mycobacterium sp. str. JC1 will consume CO while growing on glucose but not pyruvate (Ro and Kim, 1993). In the case of Pseudomonas carboxydoflava, it has been demonstrated that the extra energy derived from the oxidation of CO during heterotrophic growth enables the organism to assimilate more carbon than when grown on the heterotrophic substrate alone (Kiessling and Meyer, 1982). In many other cases, CODH is absent from heterotrophically grown cells (Meyer and Schlegel, 1983). For two strains in this study, CO consumption did not occur in the presence of growth supporting heterotrophic substrates present at 25 mM concentrations, but CO was consumed rapidly in the absence of suitable growth substrates (Figure 5.4).
However, CO oxidation is not only controlled by the type of carbon substrate present but is also controlled by the concentration of carbon substrate. In a separate experiment examining the impact of pyruvate concentration on CO uptake by strain PP52-1, CO uptake was most rapid in the absence of pyruvate, but was significantly reduced in the presence of 0.5 mM pyruvate and did not occur in the presence of 10 or 20 mM pyruvate (Figure 5.5). In a similar experiment carried out for the marine isolate *Stappia aggregata*, reduction of CO-oxidation rates in the presence of 0.5 mM glucose also occurred (Weber and King, 2007). Due to the fact that 0.5 mM glucose did not stimulate growth of *S. aggregata*, it was suggested that allosteric inhibition may be responsible for rate reduction; if glucose were only inhibiting CODH synthesis, the levels of CODH activity would have been the same in the 0 mM and 0.5 mM glucose treatments (Weber and King, 2007). This may also be the case for the reduced CO oxidation rates for PP52-1 in the presence of 0.5 mM pyruvate, which did not stimulate growth. With increasing concentrations of glucose (up to 20 mM), which stimulated growth in *Stappia aggregata*, CO oxidation rates were not further reduced and it was suggested that repression of CODH synthesis had occurred (Weber and King, 2007). In contrast, with increasing concentrations of pyruvate (up to 20 mM), which stimulated growth of PP52-1, CO oxidation was completely halted. This indicates that CODH synthesis is not only repressed, but the activity of existing CODH is completely inhibited. Although the mechanism is unknown, this demonstrates that there are some fundamental differences to the way in which CODH synthesis and activity is regulated among carboxydovores.

Many environments inhabited by CO-oxidizers are not typified by the carbon-rich conditions used to grow cultures in the laboratory, and consumption of CO in the absence of suitable organic growth substrates and at ambient CO concentrations, in particular, speaks to the
potential importance of this metabolism in situ. CO oxidation rates measured in situ support this notion. A recent survey of CO oxidizer activity measured across a vegetation gradient in a Hawaiian volcanic deposit indicated that CO oxidation may contribute as much as 10% to total energy flow in carbon-poor barren substrates, but contribute only 0.1% to total energy flow in carbon-rich sites (King, 2003c; King and Weber, 2008). Such observations in conjunction with observations made in culture-based physiological studies have lead to the hypothesis that CO-oxidation may serve as an important supplemental energy source in situ.

In cultures of DNBPI8 that had been starved for three months CO oxidation still occurred and continued uninhibited in the presence of 400 µM, which was consumed simultaneously (Figure 5.6). This demonstrates that at much lower concentrations of organic carbon, CO oxidation still occurs unabated and members of the Burkholderia genus may function mixotrophically in situ when organics become available. Whether or not simultaneous use of CO and organics provides a competitive advantage over non-CO-oxidizers at environmentally relevant concentrations, remains to be examined.

In summary, results presented here expand the known diversity of CO oxidizers in general and demonstrate that the trait may be widespread throughout the genus Burkholderia, particularly among root-associated species. The ability of isolates to consume atmospheric concentrations of CO demonstrates their potential to oxidize CO in situ, particularly in the absence of suitable heterotrophic substrates or in the presence of low concentrations of suitable heterotrophic substrates. Given the relatively high abundance of CO-oxidizing Burkholderia in situ, it is possible that this genus may be an important contributor to the soil CO sink.
CHAPTER 6.
FINAL REMARKS
CO-oxidizing bacteria readily colonize young substrates where they actively consume atmospheric CO, despite the challenges posed by environmental conditions on young volcanic substrates to the survival of early colonists. The ability of these organisms to utilize an atmospheric trace gas as a source of carbon seems an advantageous trait for carbon-poor environments characteristic of early succession; however, CO oxidizers encompass a phylogenetically broad group of versatile taxa that remain a competitive and active constituent of bacterial communities even as ecosystem development proceeds and organic carbon accumulates (Chapter 2). Chapter 2 and King et al. (2008) represent the first molecular ecological studies of CO oxidizers that use a newly defined coxL OTU (Appendix B), providing the first insights into the extent of CO oxidizer diversity in situ. A survey across the Pu’u Puai vegetation gradient demonstrated that both absolute diversity as well as diversity relative to that of the total bacterial community increased with increasing vegetation development (Chapter 2) and paralleled increases in abundance as shown by MPN and activity assays. These trends were accompanied by a distinct compositional shift from a community dominated by apparent Firmicutes-like CO oxidizers to a community dominated by proteobacterial CO oxidizers.

Many environmental factors such as carbon and nitrogen contents, pH and plant influence co-varied across the Pu’u Puai transect and likely collectively drive the changes in CO oxidizer community composition and diversity observed. Predominance of Proteobacteria in the rhizosphere have been linked to the presence of plants and increased labile carbon sources from root exudates (Lu et al., 2006; Marilley and Aragno, 1999; Olsson and Persson, 1999; Singh et al., 2007; Zul et al., 2007). Interestingly, the abundance of β-Proteobacteria coxL sequences showed the strongest increasing trend with increasing vegetation, which have been documented as primary consumers of root exudates and contain many root symbionts and endophytes.
In general, however, the increased availability of organics and increased abundance of CO oxidizers, in general, fits well with physiological data indicating their ability to function as preferential heterotrophs.

Rather surprisingly, the compositional shift in CO oxidizers across the transect does not appear to be driven by the different water regimes in the unvegetated and vegetated sites (Chapter 2). Although the general notion exists that Firmicutes, which dominated the Bare site would typically fare better in severely water-stressed environments than Proteobacteria, which dominated the Canopy site, communities in both sites appeared to be equally sensitive to water stress. Despite the sensitivity of CO oxidation to water stress, when communities at both sites were desiccated, even for extended periods of time, CO oxidation resumed rapidly upon rehydration. Such resilience is likely partly responsible for enabling CO oxidizers to colonize young substrates in which water availability can undergo dramatic diurnal oscillations. Furthermore, results of this study indicate that water stress in general is an important parameter controlling CO oxidation in situ and should be incorporated into future models of terrestrial CO dynamics.

Molecular assessments of coxL and the isolation of several CO-oxidizing β-Proteobacteria from the sites, all members of the genus Burkholderia, provided fodder for developing a quantitative PCR approach to determine the abundance of Burkholderia coxL genes in samples from the transect sites (Chapter 4) and exploring the distribution of the trait within the genus, which was previously unknown (Chapter 5). Absolute numbers of Burkholderia coxL genes increased substantially across the transect with increasing vegetation. Numbers of coxL genes relative to 16S rRNA gene copy numbers were lower in the Canopy than in the Edge, but still remained a significant part of the total community. Calculations based on the assumption
that all of the β-Proteobacteria in the Canopy site were *Burkholderia* and the numbers of *Burkholderia coxL* genes in the site provide a preliminary estimate that total CO oxidizers per in the Canopy soil could be as high as $2.6 \times 10^9$ gdw$^{-1}$. This estimate is the first estimate of CO oxidizer abundance based on molecular techniques and indicates that CO oxidizers may be more abundant than previously estimated from MPN assays (Bender and Conrad, 1994; Chapter 2). Future studies involving multiple primer sets targeting various taxonomic subgroups will aid in refining this estimate and understanding the structure of CO-oxidizing communities. Such primer sets provide tools for performing RT-qPCR and understanding not only abundance, but the dynamics of *coxL* gene expression as well.

In addition to being abundant in the rhizosphere, CO-oxidizing *Burkholderias* are diverse (Chapter 5). Prior to this study, only one species in the genus, *Burkholderia xenovorans* LB400, was recognized as a CO oxidizer (King, 2003a). CO-oxidizing *Burkholderia* isolates from volcanic deposits were especially prevalent among root-associated burkholderias. The interactions of members of the genus and plants has been well documented (Balandreau and Mavingui, 2007; Vandamme *et al.*, 2007), but the association of CO oxidizing isolates in particular provides an interesting subject for investigation. Production of CO by plant roots and now the demonstrated ability of burkholderias to consume environmentally relevant concentrations of CO (Chapter 5) indicates that CO may play a role in bacteria plant interactions, but the exact nature remains to be determined. An RT-qPCR approach using molecular tools designed in Chapter 4, may enable future research exploring possible interaction between *coxL* gene expression and CO emission from plant roots.

Further physiological examination of the *Burkholderia* isolates indicated that they are capable of mixotrophic metabolism utilizing CO and low concentrations of organics.
simultaneously, indicating that burkholderias may very well participate in consuming both CO and organic root exudates *in situ*. For one CO-oxidizer, *Hydrogenophaga pseudoflava*, it has been demonstrated that such mixotrophic metabolism resulted in higher growth yields than with the organic substrate alone (Kiessling and Meyer, 1982). If this is the case for CO-oxidizing burkholderias, CO oxidation may provide a competitive advantage in the rhizosphere and should be investigated in the future.

Although microbial CO oxidation has been known to exist since the early 1900s, studies of CO oxidizer ecology are still in their infancy. Research in the 1970s, 80s and 90s on classic carboxydotrophs made greatly advanced our knowledge of the physiology, enzymology and genetics of CO oxidation and established biological CO oxidation as a process of biogeochemical importance, but aerobic CO oxidizers have long been viewed as a somewhat obscure functional group that occupies a very specialized niche. This was largely due to the fact that early studies were predominantly focused on the use of CO as a sole carbon and energy source at concentrations that well exceed atmospheric concentrations. The relatively recent discovery of isolates that can oxidize CO at environmentally relevant concentrations of CO as well as molecular techniques based on the *coxL* gene have increased the rate of CO oxidizer discovery, expanded the known habitats in which they exist and provided the necessary stimulus to address ecological questions (King and Weber, 2007).

Application of these recently developed tools and those developed within this dissertation, made the work presented in Chapters 2, 4 and 5 as well as Appendix A possible. Collectively, results presented in this volume expand our knowledge of the known diversity and distributions of CO oxidizers and what environmental factors may be driving it, as well as provide physiological insights that should fuel research in the future. Continued expansion of
molecular tools to target newly discovered CO oxidizers and understand the abundance and gene expression of dominant groups in situ, as well as physiological studies aimed at understanding the role of CO in interactions among bacteria and between bacteria and higher organisms will greatly refine the current understanding of CO oxidizer ecology.
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APPENDIX A

PHYSIOLOGICAL, ECOLOGICAL AND PHYLOGENETIC CHARACTERIZATION
OF Stappia, A MARINE CO-OXIDIZING BACTERIAL GENUS*

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Abstract

Bacteria play a major role in marine CO cycling, yet very little is known about the microbes involved. Thirteen CO-oxidizing *Stappia* isolates obtained from existing cultures, macroalgae or surf samples representing geographically and ecologically diverse habitats were characterized using biochemical, physiological and phylogenetic approaches. All isolates were aerobic chemoorganotrophs that oxidized CO at elevated (1000 ppm) and ambient to sub-ambient concentrations (< 0.3 ppm). All contained the form I (OMP) *coxL* gene for aerobic CO dehydrogenase (CODH) and also the form II (BMS) putative *coxL* gene. In addition, some strains possessed *cbbL*, the large sub-unit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase (ruBisCO), suggesting the possibility of lithotrophic or mixotrophic metabolism. All isolates used a wide range of sugars, organic acids, amino acids and aromatics for growth, and grew at salinities from 5-45 ppt. All but one isolate denitrified or respired nitrate. Phylogenetic analyses based on 16S rRNA gene sequences indicated that several isolates could not be distinguished from *Stappia aggregata*, and contributed to a widely distributed species complex. Four isolates (GA15, HI, MIO and M4) were phylogenetically distinct from validly described *Stappia* species and closely related genera (e.g., *Ahrensia*, *Pannonibacter*, *Pseudovibrio*, and *Roseibium*). Substrate utilization profiles, enzymatic activity and membrane lipid composition further distinguished these isolates and support their designation as new *Stappia* species. The observed metabolic versatility of *Stappia* likely accounts for its cosmopolitan distribution and its ability to contribute to CO cycling as well as other important biogeochemical cycles.
**Introduction**

The marine genus, *Stappia*, encompasses four phylogenetically distinct chemoorganotrophic species in the α-2 sub-group of the *Proteobacteria* (9, 27, 38). *S. stellulata* and *S. aggregata* were originally isolated from coastal marine water column and sediment samples, assigned to *Agrobacterium* and subsequently transferred to *Stappia* (1, 29, 38). *S. alba* (27), *S. marina* (9) and various *Stappia*-like isolates have since been obtained from numerous widely distributed sources, including warm temperate surface and permanently cold deep-sea waters, sediments, phytoplankton, macroalgae, invertebrates and salt marshes (e.g., 2, 3, 5, 9, 13, 17, 26, 27, 33; Donachie et al., unpubl. results). In addition, the presence of *Stappia* or *Stappia*-like taxa in a similar range of habitats has also been inferred from cultivation-independent analyses (e.g., 3, 26; Donachie et al., unpubl. results).

Neither the original description (29), nor subsequent work by Uchino et al. (38) addressed the geographic distribution of *Stappia*, or its physiological and ecological attributes. Results of subsequent studies have shown that *Stappia*-like isolates can account for a significant percentage of cultivable α-*Proteobacteria* containing dioxygenase genes (3). *Stappia* or *Stappia*-like isolates have been reported to produce sodium channel-blocking proteins (5, 34) and a rhizobactin-like siderophore (21). *Stappia* are thus functionally versatile, occupy several ecological niches and participate in biogeochemical cycles and processes that are important on micro to global scales (e.g., CO oxidation, denitrification).

To date, all *Stappia* strains have been obtained from heterotrophic enrichments. Accordingly, the genus has been described as chemoorganotrophic (e.g., 38). Some strains, however, may function as facultative lithotrophs. All *Stappia* strains examined to date oxidize
carbon monoxide (CO) and possess the form I (OMP) \textit{coxL} gene encoding the large subunit of carbon monoxide dehydrogenase (CODH; 9, 13). Some also contain a gene for the large subunit of ribulose-1,1-biphosphate carboxylase/oxygenase (\textit{ruBisCO}; \textit{cbbL}) and may be able to couple CO utilization to CO$_2$ fixation (13).

In spite of their ubiquity, relatively little is known about phylogenetic and physiological relationships among \textit{Stappia} isolates, or responses to environmental variables that may affect their distribution and activity. We have enriched and isolated \textit{Stappia} from geographically diverse habitats and characterized novel strains using biochemical, physiological and molecular approaches. We have also determined the extent to which isolates oxidize CO at elevated and near ambient concentrations. Results collectively support designation of four new species: \textit{S. conradae}, \textit{S. meyerae}, \textit{S. carboxidovorans} and \textit{S. kahanamokuae}.

\textbf{Materials and Methods}

\textit{Culture sources and isolation.} \textit{Stappia aggregata} and \textit{Stappia} sp. strains CV902-700 and CV812-530 were obtained from the Damariscotta River, Maine (2). \textit{Stappia} sp. strain MIO was obtained from a marine methanotrophic consortium (gift of Dr. M. Takeuchi, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan). \textit{S. stellulata} was obtained from the Deutsche Sammlung für Mikroorganizmen und Zellkulturen (Braunschweig, Germany). \textit{Stappia} sp. strains SE 09 and SE11 were obtained from the Duplin River, Georgia (Dr. A. Buchan, University of Tennesee; 3). \textit{Stappia} strains M4 and M8 were obtained from enrichments based on marine macroalgae as previously described (13).

Additional \textit{Stappia} strains were obtained from seawater and macroalgae by incubating 20-100 ml basal salts medium (PYE; 21) containing 0.01-0.05\% yeast extract and 25 mM pyruvate with 1 ml of surf water samples from Ka Lae, HI or 1-5 g fresh weight of macroalgae:
Gracilaria sp., Ulva sp., Caulerpa sp. or an unidentified, tube-shaped green alga, Ascophyllum nodosum or Ulva lactuca (the latter from the Damariscotta River, Walpole, ME). CO was added at 100-1000 ppm concentrations to enrichment flask headspaces and uptake was monitored using gas chromatography (11). Enrichments positive for CO oxidation were diluted serially and plated onto PYE prepared with artificial seawater (MPYE). Colonies were selected arbitrarily and transferred to liquid MPYE and grown to stationary phase. Cultures that oxidized CO were purified by plating serial dilutions on solid MPYE, and transferred to liquid media.

Morphological, physiological and biochemical characterization. Growth supporting substrates were examined using a basal salts medium supplemented with 0.05% yeast extract (20) and various carbon substrates. Growth was assayed spectrophotometrically after 48 h and compared to controls containing 0.05% yeast extract only. Selected biochemical traits were assayed using API 20 NE Strips according to the manufacturers instructions (Biomerieux Inc., France). Catalase and oxidase tests and Gram staining were performed using standard methods (4). Major membrane lipids of S. aggregata and strains BrT4, GA15, HI, M4, M8, and MIO were obtained from MicrobialID, Inc. (Newark, DE). A Zeiss Axioscope fitted with a Neofluor 100x objective and an Axiokam MmR digital camera was used to obtain images of logarithmically-growing cells for size estimates and to determine motility.

G+C determination. Nucleotides were hydrolyzed enzymatically from RNA-free genomic DNA, then dephosphorylated to produce nucleosides. Nucleosides were separated by HPLC using a C-18 column and an isocratic mobile phase of ammonium phosphate and methanol and detected by UV absorption (254 nm). Reference standards include genomic DNA from E. coli, Burkholderia xenovorans and Mycobacterium smegmatis for which % G + C contents are known from genomic sequences.
**CO utilization.** To determine maximum CO uptake velocities, *Stappia* isolates were grown to stationary phase in MPYE, harvested by centrifugation, washed with buffered artificial seawater (ASW), and resuspended to an OD of 0.5 in buffered ASW containing mineral salts (20) and 0.005% yeast extract. Five ml cell suspensions for each isolate were transferred to triplicate 160 cm$^3$ serum bottles. CO was added to bottle headspaces (1,000 ppm) and cultures were incubated with shaking (200 rpm) at 30 °C. CO uptake was monitored at intervals by gas chromatography. Maximum uptake velocities were expressed per mg protein after measuring culture protein contents using the bicinchoionic acid assay kit (Pierce, Inc.).

The ability of *Stappia* isolates to consume CO at ambient concentrations was assessed using triplicate 10 ml cultures of strains M4, GA15, MIO and HI that were grown to stationary phase and transferred to 160 cm$^3$ serum bottles. The cultures were sealed and incubated at 30 °C with rotary shaking (200 rpm) with initial CO concentra-tions of about 2 ppm. Headspace sub-samples were obtained at intervals by needle and syringe for analysis using an RGA-3 reduced gas analyzer (Trace Analytical, Inc.).

**Heterotrophic substrate effects on S. aggregata CO uptake.** *S. aggregata* grown overnight on MPYE was harvested by centrifugation, washed with buffered ASW, and resuspended in basal salts with 0.005% yeast extract. Six sets of sealed triplicate 160 cm$^3$ serum bottles containing 4.5 ml of marine basal salts (20) with glucose concentrations of 0, 0.5, 2.5, 5.0, 10 or 20 mM were inoculated by syringe and needle with 0.5 ml of washed culture. CO was added to the bottle headspaces (1000 ppm) and absorbance (OD 600 nm) and headspace CO concentrations were monitored at suitable intervals. CO uptake rates were determined from analyses of CO concentrations over time using Kaleidagraph software (ver. 4.0.5, Synergy Software, Inc.) as
described by King (6, 12). Cell biomass was estimated from absorbance using the following empirical expression after correcting for differences in absorbance at 600 and 660 nm (16):

\[
\mu \text{g dry weight ml}^{-1} = (364.74)(\text{Abs}_{660\text{nm}}) + (6.7)(\text{Abs}_{660\text{nm}}).
\]

**Growth response to varied salinities.** Growth responses to salinities ranging from 0-45 ppt were determined for strains GA15, HI, M4 and MIO by growing the isolates in 250 ml Erlenmeyer flasks containing 50 ml basal salts medium (20) in artificial seawater supplemented with 0.05% yeast extract; salinities were adjusted to 5, 15, 25, 35 and 45 ppt. Non-marine media were created using deionized water. A sodium-free medium was created by using non-sodium salts (from 20) and deionized water. Filter-sterilized glucose (25 mM) was added to sterile basal media. All flasks were inoculated with fresh cultures (< 24 h) grown on MPYE. Cultures used for sodium-free medium were harvested by centrifugation (1 min, 13,000 rpm), washed in buffered ASW and resuspended in sodium-free medium prior to inoculation. Culture absorbance (OD 600 nm) was monitored immediately following inoculation and at intervals thereafter. Specific growth rate constants were determined by fitting absorbance data to a modified Gompertz equation (40) using Kaleidagraph software (ver. 4.0.5, Synergy Software) to obtain the fitting parameters. All assays were conducted in triplicate.

**DNA extraction, polymerase chain reaction, sequencing and analysis.** Genomic extracts were obtained using UltraClean Microbial DNA Isolation Kits (MoBio Laboratories Inc., Carlsbad CA.) as described by King (13). 16SrRNA genes were amplified using primers 27f and 1492r (18, 30). PCR reactions were carried out using 50 µl volumes (13). A 492-495 bp *cblL* fragment was amplified and sequenced using primers K2 and V2 as described in Nanba et al. (22). OMP *coxL* genes (form I; about 1260 bp) and putative *coxL* genes (BMS, form II; about 1260 bp), were also amplified from the isolates and sequenced as described by King (13). PCR
products of the correct size were purified using MoBio Ultraclean PCR Cleanup Kit (MoBio Inc. Carlsbad, CA.) and then sequenced bidirectionally with an ABI model 377 sequencer at the University of Maine DNA Sequencing Facility using the amplification primers (Orono, ME.).

**Phylogenetic analysis.** 16S rRNA gene sequences for *Stappia* isolates and for various phylogenetic neighbors were obtained from the Ribosomal Database Project (www.rdp.cme.msu.edu). Alignments were imported into ClustalX, and further aligned manually as necessary. Aligned sequences were analyzed using maximum parsimony and distance (neighbor joining) algorithms as implemented in PAUP (ver. 4.0b, Sinauer Associates, Inc., Sunderland, MA). Maximum likelihood analyses were implemented using PHYML (http://atgc.lirmm.fr/phyml/) with an HKY model for base substitution and 100 bootstrap replicates.

**BOX PCR.** Genomic extracts were amplified using the BOXAR1 primer (19) in 50 µL PCR reactions containing recommended concentrations of deoxynucleoside triphosphates, buffers, Mg$^{2+}$, 1.25 U MasterTaq DNA polymerase (Brinkmann Inc., Westbury, NY.), DMSO (2.5 µl) and template. Amplification conditions were as described by Louws et al. (19). PCR products were electrophoresed at 70 V on a 1.25% agarose gel for 4 h at 4 ºC and visualized with Gelred (Biotium, Inc. Hayward, CA.). Molecular weights of fragments were determined using Kodak Imaging Software. A similarity index was created by dividing two times the number of shared fragments by the total number of fragments of a given pair of strains.

**Results**

**Morphological, physiological and biochemical characterization.** All strains were Gram-negative, non-sporing, motile rods with average dimensions of about 2 µm x 0.8 µm (l x w; Table 1). All strains form irregular to star-shaped aggregates in liquid culture. Strain M4 also produced irregular forms in older cultures, including cells fused into a large spherical aggregate,
and cells that ballooned centrally or terminally. All strains formed circular, entire, smooth, slightly convex colonies with a light tan color on MPYE agar.

All strains grew on acetate, fumarate, citrate, succinate, pyruvate, β-hydroxybutarate, malate, glucose, fructose, maltose and proline. Strain M8 also grew on 4-hydroxybenzoate (4-HBA), which supported weak growth by S. aggregata and inhibited growth of S. stellulata and strain M4 (Table 1). A purple metabolite accumulated transiently during incubation of S. aggregata and S. stellulata with 4-HBA, indicating at least partial uncoupling of reactions in 4-HBA transformation (not shown). Terephthalate supported growth by some isolates (e.g., those of strains GA15, HI and MIO), but inhibited the growth of strain M4. Isophthalate supported only weak or no growth, while phthalate supported growth by only strain GA15. None of the isolates grew on methylamines, methanol, isopropanol, acetone, or glycerine, which proved inhibitory for S. aggregata (Table 1).

API test results were identical for strains GA15, M4 and M8 (Table 1). Strain MIO differed from the others in its ability to respire nitrate but not denitrify; strain BrT4 neither respired nitrate nor denitrified; strain HI differed in its ability to hydrolyze gelatin (Table 1). All strains were urease, β-glucosidase, and β-galactosidase positive; all strains were negative for indole production and arginine dihydrolase. Only weak evidence of fermentation was observed for strains GA15, M4 and M8.

All isolate membrane lipids were dominated by the fatty acid, 18:1ω7c, followed by 18:0 and 11-methyl,18:1ω7c (Table 2). Similarity indices did not provide definitive matches with isolates in the existing MicrobialID database, but did differentiate strains M4 and M8 from S. aggregata and strains GA15, HI and MIO (not shown). Individual isolates were differentiated
Table 1: Substrate utilization profiles for *S. aggregata* (SA), *S. stellulata* (SS), and strains MIO, HI, BrT4, GA15, M4 and M8. Growth and no growth is indicated by “+” and “−” respectively. “W” indicates where there was weak growth, “I” indicates the substrate was inhibitory and “NT” indicates that the substrate was not tested. Asterisks indicate data obtained from Uchino et al. (40).

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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Aspartate</td>
<td>+</td>
<td>W</td>
<td>W</td>
<td>+</td>
<td>R</td>
<td>+</td>
<td>W+</td>
<td>W+</td>
</tr>
<tr>
<td>Serine</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>I</td>
<td>+</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>+</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>W</td>
<td>W</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benzoate</td>
<td>I</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Methylamine</td>
<td>W</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>Dimethylamine</td>
<td>W</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>W</td>
<td>I</td>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Betaine</td>
<td>+</td>
<td>W</td>
<td>W</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>G+C content (%)</td>
<td>59%*</td>
<td>59%*</td>
<td>58.3</td>
<td>60.9</td>
<td>56.8</td>
<td>57.9</td>
<td>57.4</td>
<td>57.8</td>
</tr>
</tbody>
</table>

from each other by the presence of 17:1ω7c in strains GA15 and BrT4 only, by the presence of 19:0 in all strains except MIO, BrT4 and M8, by the presence of 20:4ω6,9,12,15c in strain HI only, and by the presence of 14:0 and 18:0iso in strains BrT4 and MIO, and by 20:0iso and 20:1ω9c in strain MIO only (Table 2). G+C contents ranged from 56.8-61.2 % (Table 1). G+C contents for *E. coli*
strain DH5α, *Burkholderia xenovorans*, and *Mycobacterium smegmatis* were 50.8%, 65.7% and 65.2%, respectively. Reported values from genomic sequences for several *E. coli* species and the latter two isolates were 50-51%, 62.6% and 67.4%, respectively (www.ncbi.nlm.nih.gov/genomes/lproks.cgi).

**Table 2**: Fatty acid composition (%) for *S. aggregata* (SA) and strains MIO, HI, BrT4, GA15, M4 and M8.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>SA</th>
<th>MIO</th>
<th>HI</th>
<th>BrT4</th>
<th>GA15</th>
<th>M4</th>
<th>M8</th>
</tr>
</thead>
<tbody>
<tr>
<td>17:1 ω 7c</td>
<td>0.6</td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1ω 5c</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1ω7c</td>
<td>71.7</td>
<td>69.4</td>
<td>72.6</td>
<td>80.7</td>
<td>71.7</td>
<td>83.3</td>
<td>86.3</td>
</tr>
<tr>
<td>20:1 ω 9c</td>
<td>1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:4 ω 6, 9, 12, 15c</td>
<td></td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11-methyl 18:1ω7c</td>
<td>8.6</td>
<td>7.7</td>
<td>4.5</td>
<td>0.6</td>
<td>9.6</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>12:0</td>
<td></td>
<td></td>
<td></td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td></td>
<td>0.2</td>
<td></td>
<td>0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>1.3</td>
<td>3.0</td>
<td>0.7</td>
<td>2.5</td>
<td>0.7</td>
<td>0.5</td>
<td>1.4</td>
</tr>
<tr>
<td>17:0</td>
<td>1.3</td>
<td>0.1</td>
<td>0.4</td>
<td>0.3</td>
<td>1.8</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>18:0</td>
<td>8.7</td>
<td>10.3</td>
<td>10.5</td>
<td>5.5</td>
<td>9.2</td>
<td>7.9</td>
<td>7.8</td>
</tr>
<tr>
<td>19:0</td>
<td>1.4</td>
<td>0.4</td>
<td></td>
<td>1.4</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:0</td>
<td>1.6</td>
<td>3.4</td>
<td>7.1</td>
<td>0.3</td>
<td>1.6</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>18:0-3OH</td>
<td>0.9</td>
<td>0.9</td>
<td>0.6</td>
<td>1.1</td>
<td>0.4</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>18:0 iso</td>
<td>0.9</td>
<td></td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20:0 iso</td>
<td></td>
<td>0.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Growth response to salinity**. Strains GA15, HI, M4 and MIO grew exponentially when incubated in MPYE with salinities ranging from 5-45 ppt (Table 3). ANOVA revealed significant differences in growth rate constants as a function of strain and salinity (p <0.0001). For strains GA15, M4 and MIO, maximum growth rate constants decreased at 45 ppt relative to
growth rate constants at optimum salinities of 15, 15, and 25 ppt, respectively (Table 3). Strain HI exhibited the highest optimum salinity, 35 ppt. Growth rate constants were greater for strain M4 than for strains GA15 and MIO at all salinities, and greater than values for strain HI from 5-15 ppt. In contrast, values for strain HI were greatest at seawater and hypersaline salinities (35-

Table 3. Growth rate constants (h\(^{-1}\); average of triplicate growth rate constants ± 1 standard error) versus salinity (parts per thousand, ppt); strains were grown in MPYE.

<table>
<thead>
<tr>
<th>Salinity (ppt)</th>
<th>HI</th>
<th>MIO</th>
<th>M4</th>
<th>GA15</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.134 (0.004)</td>
<td>0.136 (0.011)</td>
<td>0.273 (0.016)</td>
<td>0.224 (0.005)</td>
</tr>
<tr>
<td>15</td>
<td>0.176 (0.023)</td>
<td>0.178 (0.006)</td>
<td>0.330 (0.006)</td>
<td>0.231 (0.001)</td>
</tr>
<tr>
<td>25</td>
<td>0.221 (0.019)</td>
<td>0.164 (0.006)</td>
<td>0.336 (0.006)</td>
<td>0.218 (0.004)</td>
</tr>
<tr>
<td>35</td>
<td>0.229 (0.006)</td>
<td>0.148 (0.006)</td>
<td>0.265 (0.017)</td>
<td>0.201 (0.003)</td>
</tr>
<tr>
<td>45</td>
<td>0.256 (0.009)</td>
<td>0.097 (0.005)</td>
<td>0.220 (0.008)</td>
<td>0.159 (0.006)</td>
</tr>
</tbody>
</table>

45 ppt). All strains grew slowly (linearly) in a non-marine, basal salts medium with lower growth yields than in marine media (not shown). All strains grew negligibly after inoculation into sodium-free media, even though residual sodium may have been present from the washing step (not shown).

Carbon monoxide utilization. All isolates oxidized carbon monoxide at concentrations < 1,000 ppm (0.1%), but higher concentrations proved inhibitory (Fig. 1). At concentrations < 1000 ppm, CO uptake was consistent with Michaelis-Menten kinetics (e.g., Fig. 1), and apparent maximum uptake rates values ranged from about 270-510 nmol CO mg protein\(^{-1}\) h\(^{-1}\) (Table 1). CO was also consumed at ambient concentrations by strains GA15, HI, M4 and MIO (Fig. 2). Uptake rate constants were similar for the four strains, and within 17 h all were able to reduce
CO concentrations to values comparable to those reported for the marine water column.

Continued incubation resulted in headspace concentrations of 30-40 ppb.

CO consumption by *S. aggregata* was partially inhibited by incubation with > 0.5 mM exogenous glucose (Fig. 3). Consumption rates decreased by up to 40% when expressed per unit volume of culture, even though *S. aggregata* density increased 2- to 3-fold for glucose.

**Figure 1.** CO uptake (ppm h⁻¹) as a function of initial headspace CO concentration for *Stappia* sp str. KB902. Data are means of triplicates (± 1 standard error).

concentrations ≥ 2 mM. When expressed per unit cell biomass, increases in cell density were accompanied by up to a 58% decrease in uptake rates (Fig. 3).

**Phylogenetic analyses.** Phylogenetic trees derived from analyses of 16S rRNA gene sequences using maximum likelihood, maximum parsimony and distance (neighbor-joining) models were topologically similar and characterized by the following consistent patterns (Fig. 4): 1) numerous isolate sequences and clone sequences from uncultured bacteria formed a cluster
closely related to *S. aggregata*; 2) 16S rRNA gene sequences from *S. aggregata* were clearly distinct from the sequence for *S. stellulata*; 3) 16S rRNA gene sequences from strains GA15, HI, M4 and MIO were distinct from both *S. aggregata* and *S. stellulata* as well as from *S. marina*

![Graph](image)

**Figure 2.** Headspace CO concentrations in cultures of strains GA15 (●), HI (■), MIO (□), and M4 (○) at near ambient to sub-ambient concentrations versus time (h).

and *S. alba* and other genera (e.g., *Pannonibacter*, *Pseudovibrio* and *Roseibium*), which were not distinctly resolved from *Stappia* phylogenetically (Fig. 4). Other marine bacterial genera that form aggregates in liquid culture, e.g., *Ruegeria*, also appeared polyphyletic, but distinct from *Stappia* (Fig. 4). The phylogenetic distinctness of strains GA15, HI, M4 and MIO was consistent with 16S rRNA gene sequence similarities between these taxa and validly described *Stappia* sp., which ranged from 93.1-97.7%.
Form I (OMP) *coxL* sequences were obtained from all isolates examined in this study, while form II (putative, BMS) *coxL* sequences were obtained from all *Stappia* isolates but not from a Hawaiian *Photobacterium* isolate, *M. marinum*, or a *Ruegeria* isolate (Fig. 5). Phylogenetic analysis revealed distinct OMP and BMS clades, within which sequences from *Stappia* were differentiated from those of other marine and non-marine CO oxidizers (Fig. 5).

![Figure 3](image.png)

**Figure 3.** CO oxidation rates (●, nmol [mgdw biomass]⁻¹ h⁻¹; ○, nmol [ml culture]⁻¹ h⁻¹) for triplicate cultures of *Stappia aggregata* as a function of initial medium glucose concentration.

The topology of the form I *Stappia coxL* cluster was generally similar to that for the form II cluster, with differences primarily in the location of branches for *Stappia* sp. str. MIO and M8. Within the form I and form II clades, sequences from several *Stappia* isolates were identical or nearly identical. OMP sequences from *S. aggregata, S. marina*, and *S. stellulata* were distinct from sequences for strains HI, MIO and M4. An OMP *coxL* sequence from *M. marinum*, a
marine actinobacterium, was distantly related to the proteobacterial sequences. In contrast, an OMP coxl sequence from a *Photobacterium* (γ-Proteobacteria) was remarkably similar to that of *Stappia* isolates (Fig. 5).

**Figure 4.** Maximum likelihood analysis of partial 16S rRNA gene sequences (100 bootstrap replicates) implemented with phyml using an HYK correction. The phylogenetic tree was visualized with NJplot. Numbers at nodes indicate bootstrap support; values < 70% are not shown. Known CO-oxidizing bacteria are indicated with italics. *Photobacterium* sp. str. COX was used as an outgroup.
Figure 5. Phylogram from neighbor-joining analysis (1000 bootstrap replicates) of inferred OMP and BMS putative coxL amino acid sequences implemented with PAUP. *Mycobacterium marinum* coxL was used as an outgroup. Numbers at nodes indicate bootstrap support; values < 70% are not shown.
Figure 6. Phylogram from neighbor-joining analysis (1000 bootstrap replicates) of aligned nucleotide sequences implemented in PAUP for bacterial form I $cbbL$ with bootstrap support indicated at nodes (values < 70% not shown). *Prochlorococcus marinus* was used as an outgroup to root the tree for visual representation.
Partial sequences were obtained for the cbbL gene from a sub-set of *Stappia* isolates, including *S. aggregata* and strains BrT7, CV812, GA15, and MIO. PCR products were not obtained from *S. stellulata*, nor from strains BrC2, BrG2, BrT4, CV902, HI, M4, and M8. All of the *Stappia cbbL* sequences clustered with representatives of the form IC rubisco clade based on a phylogenetic analysis (Fig. 6). Sequences from *S. aggregata*, and strains BrT7, CV812 and MIO were nearly identical and clearly distinct phylogenetically from the strain GA15 sequence. *Stappia cbbL* sequences were not closely related to form IC sequences from other *Proteobacteria*, including several CO-oxidizing isolates.

BOX-PCR banding patterns were distinctly different for the various *Stappia* isolates examined, and exhibited little similarity (Table 4). Similarity indices (S\textsubscript{AB}) for pairwise comparisons varied between 0.0-0.4, which indicated substantial differentiation among taxa (Table 3). S\textsubscript{AB} values derived from comparisons of *S. aggregata* with all other strains, including

<table>
<thead>
<tr>
<th>Strain</th>
<th>SA</th>
<th>M4</th>
<th>M8</th>
<th>CV902</th>
<th>BrT4</th>
<th>SS</th>
<th>GA15</th>
<th>MIO</th>
<th>HI</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>0</td>
<td>0</td>
<td>0.17</td>
<td>0.11</td>
<td>0.13</td>
<td>0.12</td>
<td>0.11</td>
<td>0.10</td>
<td>0.11</td>
</tr>
<tr>
<td>M8</td>
<td>0</td>
<td>0.17</td>
<td>0.17</td>
<td>0.15</td>
<td>0.15</td>
<td>0.29</td>
<td>0.13</td>
<td>0.12</td>
<td>0.4</td>
</tr>
<tr>
<td>CV902</td>
<td>0.11</td>
<td>0.13</td>
<td>0.15</td>
<td>0.15</td>
<td>0.15</td>
<td>0.13</td>
<td>0.30</td>
<td>0.35</td>
<td>0.13</td>
</tr>
<tr>
<td>BrT4</td>
<td>0.13</td>
<td>0</td>
<td>0</td>
<td>0.15</td>
<td>0.15</td>
<td>0.29</td>
<td>0.13</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>SS</td>
<td>0.12</td>
<td>0.29</td>
<td>0</td>
<td>0.13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GA15</td>
<td>0.11</td>
<td>0</td>
<td>0.15</td>
<td>0.13</td>
<td>0.30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.13</td>
</tr>
<tr>
<td>MIO</td>
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<td>0.12</td>
<td>0.13</td>
<td>0.22</td>
<td>0.13</td>
<td>0.35</td>
<td>0.22</td>
<td>0</td>
<td>0.11</td>
</tr>
<tr>
<td>HI</td>
<td>0.11</td>
<td>0.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.13</td>
<td>0.13</td>
<td>0</td>
<td>0.11</td>
</tr>
</tbody>
</table>
$S.\ stellulata$, were $\leq 0.13$; values for comparisons of all strains with $S.\ stellulata$ were $\leq 0.36$. In a number of instances, $S_{AB}$ values were 0 due to the absence of shared bands. The greatest similarities were observed for comparisons of strains HI and M4 ($S_{AB} = 0.4$) and strains MIO and $S.\ stellulata$ ($S_{AB} = 0.36$).

**Discussion**

Our results and those of others indicate that *Stappia* are readily isolated from many marine environments (1, 5, 9, 17, 27, 33). Though *Stappia* have been considered “marine” based on sodium requirements and weak growth in non-marine media (1, 27, 38), three of four isolates in this study (strains GA15, MIO and M4) exhibit growth optima in dilute seawater (Table 3). Only strain HI grows optimally in full-strength seawater (35 ppt). While strain GA15, MIO and M4 occur in coastal environments, isolate growth optima do not reflect *in situ* salinity regimes, since all are derived from systems with relatively constant salinities greater than 30 ppt. Although $S.\ stellulata$ was originally described as an obligate halophile (1), salinity optima have not been reported for $S.\ aggregata$ and $S.\ alba$. Thus, it is not clear to what extent adaptation to seawater varies within the genus, or how *Stappia* compares in its salinity tolerance with other “marine” genera.

All *Stappia* isolates examined to date oxidize CO and contain form I $coxL$ (Fig. 5 and Tables 1, 3; 13). CO uptake capacity has not been reported for $S.\ alba$ or $S.\ marina$, but $S.\ marina$ possesses a form I CODH gene (9), a strong predictor of its physiological capacity. All *Stappia* isolates examined to date also contain form II putative $coxL$ (Fig. 5). The form II protein appears to function as a CODH, but may have a reduced capacity for CO oxidation and use an alternate substrate preferentially (15). CO utilization by $S.\ aggregata$ conforms to a simple Michaelis-Menten kinetic model for low to moderate concentrations, but relatively high
concentrations result in inhibition (Fig. 1), which may account for the inability of *Stappia* isolates to grow as typical carboxydrotrophs.

In addition to using super-ambient concentrations (e.g., 1000 ppm), strains GA15, HI, MIO and M4 oxidize CO at concentrations significantly lower than reported for seawater (e.g., Fig. 2; 37). Similar results have been obtained for other *Stappia* isolates (not shown). This suggests that CO may serve as a substrate for some *Stappia* under *in situ* conditions, perhaps supplementing uptake of heterotrophic substrates, which typically occur at only nM-low µM concentrations.

*In vitro* CO consumption by *Stappia* and other CO oxidizers depends on incubation conditions (7, 8, 24, 28, 31). In *Pseudomonas carboxydoflava*, form I CODH is expressed constitutively, and CO uptake occurs during batch heterotrophic growth on pyruvate (8). CODH is also expressed constitutively in *Mycobacterium* sp. str. JC1 and CO uptake occurs in the presence of several heterotrophic substrates, including glucose, but not pyruvate (28). Variability in expression and uptake also occurs for *Pseudomonas thermocarboxydovorans* (24), *Hydrogenophaga pseudoflava* (7) and *Oligotropha carboxidovorans*. Results presented here (Fig. 3) reveal partial inhibition of activity by glucose addition to *Stappia aggregata* cells that are actively oxidizing CO. This effect appears due to allosteric regulation by glucose or glucose metabolites, repression of CODH synthesis, or both, and is consistent with results from *O. carboxidovorans* CODH expression studies (31).

Decreased CO oxidation due to addition of 0.5 mM glucose (Fig. 3), which did not stimulate *S. aggregata* growth (not shown), supports a role for allosteric inhibition. Repression of synthesis alone would leave cells with CODH levels (and presumably activities) similar to treatments without glucose. Repression of CODH synthesis by glucose concentrations > 0.5 mM
is indicated by the fact that CO uptake per unit volume with 20 mM glucose is similar to that with 0.5 mM glucose, but cell density for the former increased by about 2.6-fold relative to the latter. An increase in cell density but not activity is consistent with maintenance of pre-existing CODH without new synthesis. Regardless of the mechanism, inhibition is only partial, which suggests that under carbon-limited conditions in situ, CODH may be expressed and active while diverse heterotrophic substrates are also used.

The metabolic versatility of Stappia is illustrated by growth with a wide range of sugars, organic acids, aromatics and amino acids, among others, and nitrate respiration or denitrification (Table 1; 1, 9, 14, 27, 29, 38). The potential for aromatic utilization reported here is consistent with prior analyses of Stappia-like isolates by Buchan et al. (3), who documented protocatechuic acid (PCA) degradation. In addition, some Stappia isolates (S. aggregata and strains BrT7, CV812, GA15, and MIO, and M4) possess cbbL (Fig. 6) and may be able to fix CO₂ via the Calvin cycle, possibly in conjunction with CO oxidation. The extent to which these two processes are coupled under in situ conditions remains to be determined.

Variability in substrate utilization and other phenotypic and biochemical traits (Table 1, 2), along with phylogenetic analyses, support designation of four new species. 16S rRNA gene analyses demonstrate that strains CV812, CV902, M8, BrC2, BrG2, BrT4 and BrT7 cannot be resolved from S. aggregata (Fig. 4), and contribute to a “species complex” that also includes isolates and uncultured bacterial clone sequences obtained from geographically and ecologically diverse sources. Strains GA15, HI, MIO, and M4 are distinct from this complex. Strain GA15 shares < 97% 16S rRNA gene sequence similarity with S. aggregata, S. alba, S. marina and S. stellulata, supporting its designation as a new species, Stappia meyerae sp. nov. It is united with Stappia through growth substrates, lipid profiles, polar flagellation and enzymatic activities.
Although its closest phylogenetic relatives include two *Roseibium* isolates, phylogenetic distance from them, growth substrates, gelatinase activity, and the absence of distinct pink pigmentation and peritricious flagellation differentiate *Stappia meyerae* sp. nov. from *Roseibium* (35).

Strain MIO 16S rRNA sequence is 96.2 and 97.6 % similar to sequences from its closest phylogenetic neighbors, *S. aggregata* and *S. marina*, respectively. Phylogenetic distance (Fig. 4), lipid composition (Table 2), and the ability to denitrify and grow on terephthalate (Table 1) differentiate strain MIO from *S. aggregata*. Lipid composition and growth with glucose, mannose, mannitol, maltose, gluconate and citrate differentiate strain MIO from *S. marina*. In addition, previous analyses involving *S. alba* (27) and *S. marina* (9) have shown that even 16S rRNA gene sequence similarities as high as 98.9% are associated with DNA-DNA hybridization values $< 70\%$ for congeneric *Stappia* species. Thus, these collective observations support designation of a new species, *Stappia conradae* sp. nov.

Strain HI and M4 16S rRNA gene sequences share $> 97\%$ similarity with the *S. aggregata* 16S rRNA gene sequence (97.6% and 97.7%, respectively), but both strains are clearly distinct from *S. aggregata* and all other validly described *Stappia* based on 16S rRNA and *coxL* phylogenies (Fig. 4, 5), lipid profiles (Table 2), substrate utilization and the presence of *cbbL* (and presumably the Calvin cycle) in *S. aggregata* but not strains HI or M4. The latter strains also denitrify, while *S. marina* does not. In addition, BOX-PCR results show substantial divergence between strains HI and MIO and all other *Stappia* isolates (Table 4). Collectively, these observations support two new species, *Stappia kahanamokuai* sp. nov. for strain HI and *Stappia carboxidovorans* sp. nov. for strain M4.
Growth substrate profiles not only provide valuable taxonomic information, but also offer important ecological insights. For instance, aromatic use, though variable among strains, is consistent with isolation of *Stappia* from phaeophyte macroalgae, which produce relatively high concentrations of polyphenols (25, 39). Recent identification of putative dehalogenase genes in the draft genome of *S. aggregata* (King et al., unpubl. data) suggests possible use for growth of various alkyl or aryl halides, which are produced commonly by phytoplankton, macroalgae, and marine invertebrates (10, 23, 32, 36). The variable presence of *cbbL* genes indicates that some *Stappia* strains can function mixotrophically, supplementing organic carbon and energy sources with CO$_2$ fixation, presumably driven by CO oxidation. Other strains may simply use CO as a supplemental energy source.

In summary, results presented here indicate that in addition to its heterotrophic metabolism, the genus *Stappia* is characterized by its ability to oxidize CO at sub-ambient to super-ambient levels, with or without mixotrophic CO$_2$ fixation. The ability of *Stappia* isolates to respire nitrate or denitrify, use numerous organics and function in low to moderate salinities facilitates participation in both aerobic and anaerobic processes in carbon and nitrogen cycling in a wide range of marine environments. Results also support establishment of four new species derived from geographically diverse habitats.

**Description of *Stappia meyerae* sp. nov.**

*Stappia meyerae* (meyerae. L. fem. adj. *meyerae* of Meyer, honoring fundamental contributions by Prof. Ortwin Meyer, University of Bayreuth, to the physiology, biochemistry and molecular biology of CO-oxidizing microbes).

Cells are aerobic Gram-negative, non-sporing, motile rods with a single polar flagellum; 2.0 ± 0.03 µm in length and 0.8 ± 0.03 in width µm. Colonies circular, entire, slightly convex,
smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase positive; aesculin hydrolysis, gelatinase, indole production from tryptophan and arginine dihydrolase negative.

Nitrate reduced to gas. Sodium required, optimum salinity for growth 5-25 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c; G+C content 57.8 ± 0.6%. Oxidizes carbon monoxide; contains large sub-unit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase.

Grows with acetate, alanine, aspartate betaine, citrate, fumarate, fructose, galactose, glucose, gluconate, glucuronate, glutamate, glycerol, β-hydroxybutyrate, lactate, lactose, malate, malonate, maltose, mannitol, mannose, phthalate, propionate, pyruvate, proline, ribose, succinate, terephthalate, and valine. Does not grow with acetone, benzoate, 4-hydroxybenzoate, formate, glycine, isophthalate, isopropanol, methanol, tartrate, or mono-, di- and trimethylamine. Weak growth on phenylalanine.

The type strain, GA15, was isolated from Ascophyllum nodosum in the Damariscotta River (Maine, USA).

**Description of *Stappia conradae* sp. nov.**

*Stappia conradae* (conradae. L. fem. adj. conradae of Conrad, honoring important contributions by Prof. R. Conrad, Max-Planck-Institute for Terrestrial Biogeochemistry, Marburg, Germany, to the physiology and ecology of CO-oxidizing microbes in soil and aquatic environments).

Cells are aerobic Gram-negative, non-sporing, motile rods with a single polar flagellum; 1.8 ± 0.1 μm in length and 0.7 ± 0.1 in width μm. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase positive; aesculin hydrolysis, gelatinase, indole production from tryptophan and arginine dihydrolase negative.

Nitrate respired to nitrite; gas not produced. Sodium required, optimum salinity for growth 15-35 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c; G+C content 58.2 ± 0.5%.
Oxidizes carbon monoxide; contains large sub-unit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, alanine, citrate, fumarate, fructose, glucose, gluconate, glucuronate, glutamate, β-hydroxybutyrate, lactate, lactose, malate, maltose, mannitol, mannose, propionate, pyruvate, proline, succinate, and terephthalate. Does not grow with acetone, benzoate, 4-hydroxybenzoate, glycine, isophthalate, isopropanol, methanol, phenylalanine, or mono-, dimethylamine. Weak growth on aspartate, betaine, formate, galactose, glycerol, malonate, phthalate, ribose, tartrate, and valine. Inhibited by trimethylamine.

The type strain, MIO, was isolated from a methanotrophic enrichment based on sediment obtained from 160 m depth in Kagoshima Bay, Japan.

**Description of *Stappia kahanamokuae* sp. nov.**


Cells are aerobic Gram-negative, non-sporing, motile rods with a single polar flagellum; 2.1 ± 0.1 μm in length and 0.7 ± 0.1 in width μm. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase, gelatinase positive; aesculin hydrolysis, indole production from tryptophan and arginine dihydrolase negative.

Nitrate reduced to gas. Sodium required, optimum salinity for growth 35 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c; G+C content 61.2 ± 0.1%. Oxidizes carbon monoxide; does not contain large sub-unit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, aspartate, betaine, citrate, fumarate, fructose, galactose, galacturonate, glucose, gluconate, glucuronate, glycerol, glycolate, β-hydroxybutyrate, lactate, lactose, malate, maltose, mannitol, mannose, propionate, pyruvate, proline, ribose,
succinate, sucrose, terephthalate and valine. Does not grow with acetone, alanine, benzoate, ethanol, formate, 4-hydroxybenzoate, glutamate, glycine, isophthalate, isopropanol, malonate, methanol, phenylalanine, phthalate, serine, or mono-, di- and trimethylamine. Weak growth on tartrate. The type strain, HI, was isolated from a surf water sample at South Point (Ka Lae), Hawai’i.

**Description of *Stappia carboxidovorans* sp. nov.**

*Stappia 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-negative, non-sporing, motile rods with a single polar flagellum; 1.6 ± 0.1 μm in length and 0.8 ± 0.1 in width μm. Colonies circular, entire, slightly convex, smooth, cream to tan. Oxidase and catalase positive. Urease, β-galactosidase, gelatinase positive; aesculin hydrolysis, indole production from tryptophan and arginine dihydrolase negative. Nitrate reduced to gas. Sodium required, optimum salinity for growth 15-25 ppt. Principle fatty acids are 18:1ω7c and 11-methyl,18:1ω7c.; G+C content 57.4 ± 0.3%. Oxidizes carbon monoxide; does not contain large sub-unit gene for ribulose-1,5-bisphosphate carboxylase/oxygenase. Grows with acetate, alanine, aspartate, betaine, citrate, fumarate, fructose, galactose, galacturonate, glucose, gluconate, glucuronate, glutamate, glycerol, glycolate, β-hydroxybutyrate, lactate, lactose, malate, malonate, maltose, mannitol, mannose, propionate, pyruvate, proline, ribose, succinate, sucrose, and valine. Does not grow with acetone, ethanol, formate, glycine, isophthalate, isopropanol, methanol, phenylalanine, phthalate, serine, tartrate or mono-, di- and trimethylamine. Inhibited by 4-hydroxybenzoate, serine and terephthalate.
The type strain, M4, was isolated from *Ascophyllum nodosum* from the Damariscotta River, Walpole, ME.

**Acknowledgments**

We thank K. Boettcher for cultures of *S. aggregata* and *Stappia* strains CV-812, and CV902. We thank Dr. M. Takeuchi for the gift of strain MIO. We thank Ms. H. Crosby for help with the initial isolation and characterization of strains M4 and M8. We thank Ms. K. Johnston and Mr. W. Yeung for technical support. This research was funded by National Science Foundation awards OCE-0425579 and MCB-0348100. Ms. C.F. Weber and H. Crosby were partially supported by NSF-REU funds.

**Literature Cited**


**Figure B.1.** Derivation of the OTU definition for *cox*-*L*. For known CO-oxidizing cultures, *cox*-*L* inferred amino acid sequence similarities were plotted vs. 16S rRNA gene similarities. Comparisons of *cox*-*L* genes were all greater than 90 % similar using a 16S rRNA gene based OTU definition of 97% similarity.
Table B.1. Diversity indices and phylogenetic subgroup composition of the *Acidobacteria* 16S rRNA gene sequences. All diversity estimates are based on an OTU definition using an evolutionary distance of ≤ 0.03. The 95% confidence intervals are listed in parentheses where applicable.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Bare</th>
<th>Edge</th>
<th>Canopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diversity Estimates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of OTUs</td>
<td>21</td>
<td>31</td>
<td>41</td>
</tr>
<tr>
<td>$S_{ace}$</td>
<td>30.6 (23.7, 54.9)</td>
<td>39.7 (33.7, 59)</td>
<td>73.4 (54.3, 119.9)</td>
</tr>
<tr>
<td>Chao1</td>
<td>30 (23.0, 61.5)</td>
<td>35.5 (32. 50.6)</td>
<td>69.9 (51.1, 123.8)</td>
</tr>
<tr>
<td>Shannon (H)</td>
<td>2.78 (2.56, 2.99)</td>
<td>3.09 (2.92, 3.25)</td>
<td>3.39 (3.20, 3.58)</td>
</tr>
<tr>
<td>Simpson (1/D)</td>
<td>16.3</td>
<td>19.8</td>
<td>28</td>
</tr>
<tr>
<td>Phylogenetic Subgroup (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>53</td>
<td>88</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>25</td>
<td></td>
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<td>3</td>
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</tr>
<tr>
<td>13</td>
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<td></td>
<td>1</td>
</tr>
</tbody>
</table>

Table B.2. Homologous and heterologous converages as determined by LIBSHUFF analyses of *Acidobacteria* sequences in the 16S rRNA gene libraries. Coverages are listed as percentages for an evolutionary distance of 0.03. All comparisons between homologous and heterologous coverages were determined to be statistically significant at the p values listed.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>$C_x$</th>
<th>$C_{xy}$</th>
<th>$C_{yx}$</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Bare (x) vs. Edge (y)</td>
<td>86</td>
<td>29.8</td>
<td>18.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Edge (x) vs. Canopy (y)</td>
<td>93</td>
<td>66.4</td>
<td>56.7</td>
<td>0.001</td>
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<tr>
<td>Canopy (x) vs. Bare (y)</td>
<td>74</td>
<td>8.2</td>
<td>7.0</td>
<td>0.001</td>
</tr>
</tbody>
</table>
Figure B.2 Neighbor-joining tree (1000 bootstrap replicates) of 16S rRNA clone sequences from the Bare site identified as “possible Chloroflexus” using RDPII classifier and their closest relatives. *Aquifex pyrofilus* str. Ko15a (M83548) was used as the outgroup. Accession numbers: Uncultured chloroflexi bacterium clone B12_WMSP2 (DQ450726); uncultured bacterium clone 1921-3 (AY425700); uncultured bacterium clone 1921-4 (AY425780); Uncultureds bacterium JG30a-KF-32 (AJ536876); uncultured *Chloroflexi* bacterium clone F03_WMSP1 (DQ450723); uncultured bacterium clone 1790-3 (AY425771); uncultured bacterium clone 1921-7 (AY425783); uncultured bacterium clone 1790-3 (AY425771); uncultured bacterium clone 1921-6 (AY425782); uncultured bacterium clone 1921-2 (AY425778); *Chloroflexi* bacterium Ellin7237 (AY673403); *Chloroflexus aurantiacus* (M34116); *Chloronema giganteum* clone Gnsb-1 (AF345825); *Roseiflexus castenholzii* (AB041226); *Thermomicrobium roseum* (M34115); uncultured soil bacterium clone ABS-6 (AY289487); B26 (FJ466013); B32 (FJ465997); B155 (FJ466066); B21 (FJ466084); B68 (FJ466031); B149 (FJ466038); B176 (FJ466020); B61 (FJ466045); B125 (FJ466021); B138 (FJ466006); B187 (FJ465993); B414 (FJ466072); B73 (FJ466010); B183 (FJ466015); B425 (FJ466082); B415 (FJ466054); B143 (FJ466068); B416 (FJ466007); B424 (FJ465969); B418 (FJ466052); B151 (FJ466064); B28 (FJ465994); B423 (FJ466026); B170 (FJ466012); B417 (FJ465986); B422 (FJ466079); B118 (FJ466059); B156 (FJ466033); B421 (FJ465980); B420 (FJ466058); B166 (FJ466039); B178 (FJ466001).
Table B.3. Unique and shared *Acidobacteria* OTU’s (evolutionary distance of 0.03) among the Bare, Edge and Canopy 16S rRNA gene libraries.

<table>
<thead>
<tr>
<th></th>
<th>Bare</th>
<th>Edge</th>
<th>Canopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Edge</td>
<td>3</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>Canopy</td>
<td>0</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>

Table B.4. Unique and shared *Proteobacteria* OTU’s (evolutionary distance of 0.03) among the Bare, Edge and Canopy 16S rRNA gene libraries.

<table>
<thead>
<tr>
<th></th>
<th>Bare</th>
<th>Edge</th>
<th>Canopy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Edge</td>
<td>0</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Canopy</td>
<td>1</td>
<td>9</td>
<td>27</td>
</tr>
</tbody>
</table>
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VITA

Carolyn Frances Weber was born to Scott and Lynn Weber in February 1983 in Barrington, Illinois. In 2000, she graduated as a junior from Prairie Ridge High School in Crystal Lake, Illinois. After attending McHenry Community College in Crystal Lake, Illinois, for one year, Carolyn moved to Mount Vernon, Iowa, in August 2001 to continue undergraduate study at Cornell College. In May 2004, Carolyn graduated summa cum laude with distinction in biochemistry and molecular biology (BMB) from Cornell College with Bachelor of Arts degrees in chemistry and BMB. In Summer 2004, she began graduate study in microbiology at the University of Maine (Walpole, Maine) in the laboratory of Dr. Gary M. King. In January 2007, Carolyn relocated to Baton Rouge, Louisiana, to continue graduate study under the direction of Dr. Gary M. King at Louisiana State University. Carolyn will receive the degree of Doctor of Philosophy at the August 2009 commencement.