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RESEARCH ARTICLE

The phylogenetic distribution and ecological role of carbon monoxide oxidation in the genus *Burkholderia*

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Keywords
Burkholderia; carbon monoxide; *coxL*.

Abstract

Burkholderia is a physiologically and ecologically diverse genus that occurs commonly in assemblages of soil and rhizosphere bacteria. Although *Burkholderia* is known for its heterotrophic versatility, we demonstrate that 14 distinct environmental isolates oxidized carbon monoxide (CO) and possessed the gene encoding the catalytic subunit of form I CO dehydrogenase (*coxL*). DNA from a *Burkholderia* isolate obtained from a passalid beetle also contained *coxL* as do the genomic sequences of species H160 and Ch1-1. Isolates were able to consume CO at concentrations ranging from 100 ppm (vol/vol) to sub-ambient (< 60 ppb (vol/vol)). High concentrations of pyruvate inhibited CO uptake (> 2.5 mM), but mixotrophic consumption of CO and pyruvate occurred when initial pyruvate concentrations were lower (*c.* 400 μM). With the exception of an isolate most closely related to *Burkholderia cepacia*, all CO-oxidizing isolates examined were members of a nonpathogenic clade and were most closely related to *Burkholderia* species, *B. caledonica*, *B. fungorum*, *B. oxiphila*, *B. mimosarum*, *B. nodosa*, *B. sacchari*, *B. bryophila*, *B. ferrariae*, *B. ginsengesoli*, and *B. unamae*. However, none of these type strains oxidized CO or contained *coxL* based on results from PCR analyses. Collectively, these results demonstrate that the presence of CO oxidation within members of the *Burkholderia* genus is variable but it is most commonly found among rhizosphere inhabitants that are not closely related to *B. cepacia*.

Introduction

The genus *Burkholderia* harbors over 40 formally described species that have been enriched and isolated under a variety of conditions from diverse sources (Vandamme *et al.*, 2007), many of which include plants and soil environments (e.g., Bramer *et al.*, 2001; Goris *et al.*, 2002; Caballero-Mellado *et al.*, 2004; Reis *et al.*, 2004; Sessitsch *et al.*, 2005; Chen *et al.*, 2007; Yoo *et al.*, 2007; Compant *et al.*, 2008; Otsuka *et al.*, 2010). Large, complex genomes appear to be typical of the genus (Chain *et al.*, 2006; Lessie *et al.*, 2006), which are responsible for their ability to consume a wide range of organic substrates, including various xenobiotics (Bedard *et al.*, 1986; Seeger *et al.*, 1995, 1999; Maltseva *et al.*, 1999; O'Sullivan & Mahenthiralingam, 2005). Although classified as heterotrophs, one isolate, *Burkholderia xenovorans* LB400, has been identified as a possible facultative lithotroph based on the presence in its genome of form I carbon monoxide dehydrogenase (*cox*) and ribulose-1,5-bisphosphate carboxylase/oxygenase (*cbb*) genes (King, 2003). This isolate and a closely related strain, *Burkholderia* sp. LUP, have been shown to oxidize but not grow on CO in culture (King, 2003).

With the exception of *B. xenovorans* LB400, *Burkholderia* sp. Ch1-1 and *Burkholderia* sp. H160, *cox* genes do not occur in the genome sequences of *Burkholderia* isolates, which as of February 2011 represent 23 different species (http://img.jgi.doe.gov/cgi-bin/w/main.cgi). This suggests that CO oxidation might be an unusual trait in the genus and confined to only a few strains.

However, results from molecular ecological studies reveal that CO-oxidizing *Burkholderia* may be more diverse and abundant *in situ* than genome studies would suggest (Weber & King, 2010a, b). For example, approximately 33% of *coxL* (large subunit of CO dehydrogenase)

sequences recovered from vegetated soils at sites on Kilauea volcano cluster most closely to sequences derived from *Burkholderia* and represent at least six species or operational taxonomic units (Weber & King, 2010b). A quantitative real-time PCR study conducted with these same soils demonstrated that *Burkholderia coxL* may be as abundant as 8.6×10^8 copies gdw^{-1} soil (Weber & King, 2010a). Collectively, these results offer a different perspective on the diversity and potential significance of CO-oxidizing *Burkholderia*.

Although molecular ecological data suggest that *Burkholderia* may be an important fraction of CO-oxidizing rhizosphere communities, their contribution to CO oxidation remains unknown. *Burkholderia* is well known for their associations with plant roots (e.g., Di Cello *et al.*, 1997; Chen *et al.*, 2006, 2007; Balandreau & Mavingui, 2007; Caballero-Mellado *et al.*, 2007) and their ability to consume organics from root exudates (Grayston *et al.*, 1998). This suggests that CO-oxidizing *Burkholderia* could play a role in oxidation of CO derived from live fine roots, which produce 170–260 Tg CO per year on a global basis (King & Crosby, 2002). However, their ability to consume environmentally relevant CO concentrations and how this ability might be impacted by an apparent preference to function heterotrophically using numerous substrates remain largely unknown.

Here, we report results from CO oxidation assays and analyses of *coxL* gene content for 14 *Burkholderia* isolates obtained from volcanic soils in Japan and Hawai'i, one isolate obtained from a passalid beetle gut, six isolates obtained from unvegetated alpine soils of Pico de Orizaba, Mexico and 10 *Burkholderia* type strains. We also describe results from a survey of 67 *Burkholderia* genomes for the presence of *cox* genes. In addition, for selected CO-oxidizing isolates, we also conducted physiological analyses to determine whether CO could be oxidized at environmentally relevant concentrations and under mixotrophic conditions.

Materials and methods

Isolate sources and identification

Fourteen *Burkholderia* were isolated from volcanic soils or cinders from previously described sites in Hawai'i and Japan (King & Weber, 2008; King *et al.*, 2008). Using standard plating and purification techniques, isolates were obtained from the enrichments of volcanic soils or cinders in dilute nutrient broth (0.08 g nutrient broth L^{-1}) with 500 μg mL^{-1} penicillin (DNBP) or from minimal media (pH = 7; Meyer & Schlegel, 1978) supplemented with yeast extract (YE; 0.01%) and one of the following primary carbon sources (25 mM unless otherwise noted): xylose, β-hydroxybutarate, methanol (1%), or pyruvate (Table 1). Ten type strains most closely related to these isolates were obtained from the Deutsche Sammlung von Mikroorganismen und Zellculturen (DSMZ, Braunschweig, Germany) or the Belgian Coordinated Collections of Microorganisms (BCCM/LMG, Ghent, Belgium): *Burkholderia terrae* (DSM17804^T), *Burkholderia unamae* (DSM17197^T), *Burkholderia mimosarum* (DSM21841^T), *Burkholderia sacchari* (DSM17165^T), *Burkholderia phytofirmans* (DSM17436^T), *Burkholderia hospita* (DSM17164^T), *Burkholderia ferrariae* (DSM 18251^T), *Burkholderia tropica* (DSM15359^T), *Burkholderia nodosa* (LMG 23741), and *Burkholderia ginsengisoli* (LMG 24044). Six additional *Burkholderia* isolates were obtained from a collection of heterotrophic bacteria enriched from alpine soils (0–5 cm depth) of Pico de Orizaba (elevation = 4357 m; GPS = 19°00′26.5″ N and 97°17′08.3″W), Mexico (gift of Dr F. Rainey, Louisiana State University, Baton Rouge, LA; Table 1). A DNA extract from a *Burkholderia* isolate from a passalid beetle gut was also assayed (gift of Dr M. Blackwell, Louisiana State University, Baton Rouge, LA; Table 1); this isolate had been lost from culture and was not available for physiological characterization.

Isolates were identified as *Burkholderia* based on traits typical for the genus (Vandamme *et al.*, 2007) and near full-length 16S rRNA gene sequences that were amplified by a standard PCR method using primers 27f and 1492r (Lane, 1991). The 16S rRNA gene amplicons were sequenced bidirectionally at the Louisiana State University Genomics Facility (Baton Rouge, LA). Bidirectional reads were assembled using SEQUENCHER 4.7 (Gene Codes Corporation, Ann Arbor, MI). Assembled sequences were aligned using the SILVA web interface (http://www.arb-silva.de/) and the SILVA 16S rRNA gene database (Pruesse *et al.*, 2007). Alignments were imported into ARB (Ludwig *et al.*, 2004) for editing and phylogenetic tree construction. Maximum likelihood trees were constructed using the PHYML (DNA) option as well as neighbor-joining methods. Phylogenetic trees were bootstrapped in PAUP v.4.0b (Swofford, 2002; Sinauer Associates, Sunderland, MA) using 1000 bootstrap replicates. Accession numbers for 16S rRNA genes are listed with the corresponding isolates in Fig. 1.

CoxL sequence analyses

PCR amplification of an approximately 1260 bp fragment of the form I *coxL* gene was attempted using DNA extracts from all isolates and type strains. Primers, conditions, and sequencing methods for amplicons have been described previously (King, 2003). All amplicons were sequenced bidirectionally by the Louisiana State University Genomics Facility and assembled using SEQUENCHER

Table 1. Isolation sources and medium (for isolates obtained in this study), culture collection (for isolates obtained from private collections), closest described relatives and maximum identity based on BLAST analysis and presence (+) or absence (−) of *coxL*

Isolate	Source	Isolation medium or culture collection	Closest named relative	Max. identity (%)	*coxL*
PP51-2	Volcanic soil, Kilauea, HI	MYE	*B. unamae* str. TR3.4 (AY391283)	98.3	+
PP52-1			*B. mimosarum* PAS44 (AY752958)	98.9	+
WA		XYE	*B. oxiphila* OX-01 (AB488692.1)	98.7	+
YA			*B. oxiphila* OX-01 (AB488692.1)	97.9	+
DNBP18		DNBP	*B. oxiphila* OX-01 (AB488692.1)	98.8	+
DNBP22			*B. sacchari* str. IPT10 (AB212237.1)	98.5	+
DNBP6-1			*B. bryophila* LMG 23644T (AM489501.1)	99.0	+
DNBP20			*B. sacchari* str. IPT10 (AF263278.1)	98.5	+
DNBP16			*B. oxiphila* OX-01 (AB488692.1)	98.7	+
CP11		PYE	*B. ferrariae* str. NBRC 106233 (AB537487.1)	98.3	+
I7		PYE (pH = 4)	*B. ginsengisoli* KMY03 (AB201286.1)	99.0	+
I2			*B. nodosa* str. Br3461 (AY773192.1)	99.0	+
Rim		PYE	*B. caledonica* LMG 19076 (NR_025057.1)	100	+
KP5Blue	Volcanic Soil, Miyak-jima, Japan	HBYE	*B. cepacia* str. 2EJ5 (GQ383907.1)	99.6	+
PO-04-17-38	Soil Pico de Orizaba, Mexico	F. Rainey (Louisiana State Univ. Baton Rouge, LA)	*B. cepacia* ATCC 35254 (AY741346.1)	97.7	+
PO-04-02-34			*B. sordidicola* BLN20 (GQ181055.1)	99.9	−
PO-04-17-25			*B. glathei* N15 (NR_037065.1)	98.6	−
PO-04-17-33			*B. cepacia* ATCC 55487 (AM741358.1)	98.9	−
PO-04-17-39			*B. bryophila* LMG 23648 (AM489500.1)	98.7	−
PO-04-17-44			*B. sordidicola* BLN20 (GQ181055.1)	97.8	−
E-B2	Passalid beetle hindgut	M. Blackwell (Louisiana State University, Baton Rouge, LA)	*B. fungorum* str. KN-08 (AB091189.1)	99.2	+
LUP	Lupine; Walpole, ME	PYE (King, 2003)	*B. xenovorans* LB400 (CP000271.1)	99.5	+

Medium abbreviations: DNBP, dilute nutrient broth (0.08 g L^{-1}) with 500 μg mL^{-1} penicillin; YE, minimal medium supplemented with 0.005% yeast extract (pH = 7); M, methanol (1%); X, xylose (25 mM); P, pyruvate (25 mM); and HB, β-hydroxybutarate (25 mM).

4.7 (Gene Codes Corporation). Inferred amino acid sequences were aligned using MUSCLE 3.6 (Edgar, 2004) and imported into ARB for neighbor-joining tree construction. Phylogenetic trees were bootstrapped using PAUP v. 4.0b (Swofford, 2002; Sinauer Associates) and 1000 bootstrap replicates. Sequence accession numbers are listed with the corresponding isolates in Fig. 2.

CO oxidation

Liquid cultures of all isolates and type strains were screened for their ability to consume CO. The cultures were grown in sealed serum bottles with about 100-ppm headspace CO concentrations (King, 2003). Uptake assays were conducted for > 48 h with repeated headspace sampling to allow for lags in expression of activity. CO concentrations were measured using gas chromatography as described previously (King, 2003). For a phylogenetically representative subset of CO-oxidizing isolates (CP11, I2, PP52-1, DNBP18, DNBP6-1), activity was also measured in the presence of atmospherically relevant concentrations of CO in the headspace (< 1 ppm).

Isolate PP52-1 was used to determine the effects of varied initial pyruvate concentrations on CO uptake rates. Briefly, PYE-grown cells were collected by centrifugation, washed twice with minimal medium, and resuspended in minimal medium. Washed cells were used to inoculate sealed triplicate 160 cm^3 serum bottles with 100 ppm CO in the headspaces and 4.5 mL of minimal medium amended with 0.005% yeast extract and pyruvate at concentrations of 0, 0.5, 2.5, 5, 10, or 20 mM. CO uptake was monitored through time as above.

The effect of organic substrate type on CO oxidation was assayed using both DNBP16 and PP52-1. PYE-grown cells of both isolates were collected by centrifugation, washed with minimal medium, and resuspended in a minimal medium containing 0.01% YE. The washed cells were used to inoculate quadruplicate 60 cm^3 serum

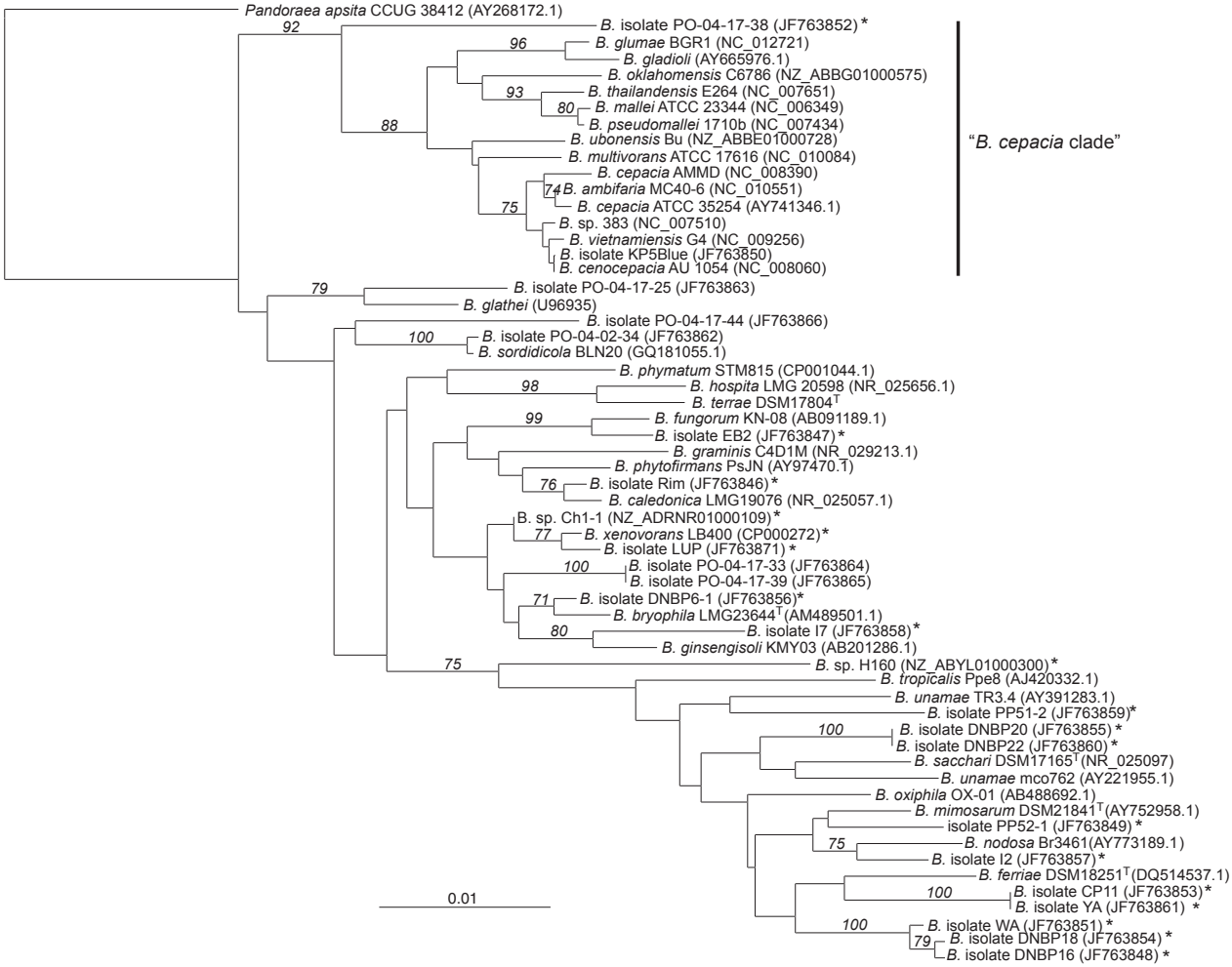

Fig. 1. A neighbor-joining phylogeny based on the 16S rRNA gene from *Burkholderia* isolates in this study, their nearest relatives and a phylogenetically representative subset of currently available genome sequences. Bootstrap values > 70 are displayed (1000 replicates). The tree is rooted with *Pandoraea apista* CCUG 38412. *Indicates the presence of *coxL*.

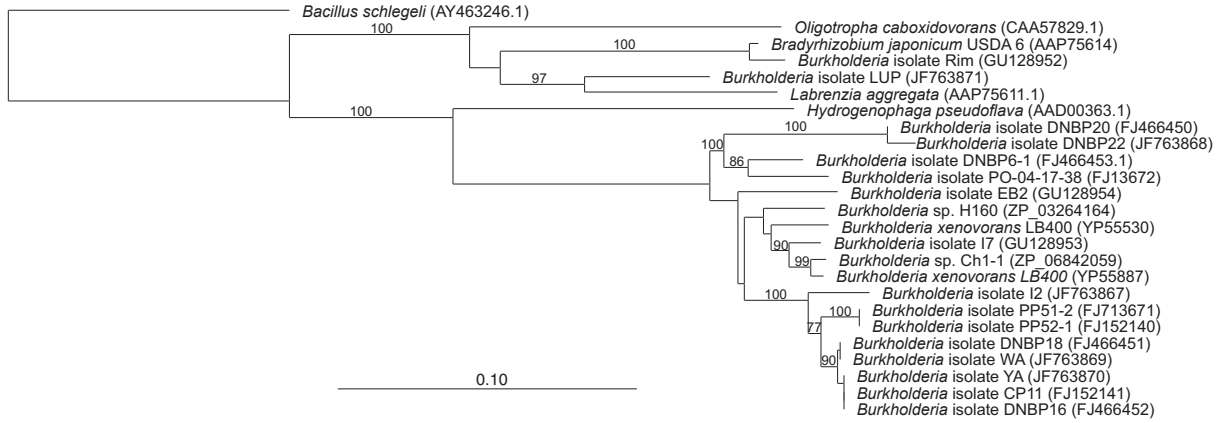

Fig. 2. Neighbor-joining tree of *Burkholderia coxL* inferred amino acid sequences. Bootstrap values > 70 are displayed (1000 replicates). The tree is rooted with a form I *coxL* gene from *Bacillus schlegeli*.

bottles containing 5 mL of minimal medium supplemented with 0.01% YE and one of the following carbon sources at a final concentration of 25 mM: acetate, gluconic acid, β-hydroxybutyrate, mannitol, methanol, methylamine, phthalate, proline, pyruvate, or sucrose. From each set of quadruplicates, two bottles were amended with CO (final concentration about 80 ppm) immediately after inoculation. Optical density measurements ($OD_{600\ nm}$) and CO concentrations were determined immediately after the inoculation and 24 h later and compared to quadruplicate control cultures that were prepared as above, but did not contain any carbon source in the medium.

The ability of DNBP18 to consume CO and pyruvate simultaneously, or mixotrophically, was assayed using two sets of triplicate gas-tight 60 cm^3 serum bottles containing 100 ppm CO in the headspace and 3-mL aliquots of PYE-grown cells that had been washed and resuspended in minimal medium. One set of bottles started with an initial pyruvate concentration of 0.4 mM, while the other did not contain pyruvate. Bottles were incubated with shaking at room temperature. CO uptake was monitored as above. Pyruvate concentrations were monitored by removing 0.1 mL of culture with a needle and syringe, pelleting the cells by centrifugation (10 000 ***g***) and immediately using a colorimetric microplate pyruvate assay kit (Biovision, Mountain View, CA) to determine the concentration in 25 μL of supernatant. Absorbance of the microplate wells was determined using a Gen5 Microplate Reader (Biotek Instruments, Winooski, VT).

Results and discussion

Phylogenetic distribution of CO oxidation within the genus *Burkholderia*

Based on phylogenetic analyses of the 16S rRNA gene, all isolates clustered within the *Burkholderia* genus. For each isolate, the nearest formally described taxon was a member of the *Burkholderia* having a sequence identity between 97.5% and 100% (Table 1). All 14 isolates from Hawai'i oxidized CO from atmospheres containing 100 ppm and had the *coxL* gene. The same was true for isolates LUP and PO-04-17-38. In addition, *coxL* genes were successfully amplified and sequenced from the DNA of isolate EB2; *coxL* genes were also documented in the genomes of *Burkholderia* sp. H160 and *Burkholderia* sp. Ch1-1. Additional isolates examined from Pico de Orizaba and Japan as well as all of the type strains from DSMZ and BCCM/LMG did not have *coxL* genes or oxidize CO. Maximum likelihood and neighbor-joining analyses of 16S rRNA genes from all isolates and type species yielded comparable phylogenies that revealed the following (Fig. 1): (1) the majority of the CO-oxidizing isolates (WA, YA, CP11, DNBP18, DNBP16, DNBP22, DNBP20, PP52-1, I2, PP51-2) and *Burkholderia* sp. H160 occurred within a clade encompassing formally described species from soils, rhizosphere, roots, and legume nodules (e.g., *B. sacchari*, *B. unamae*, *B. mimosarum*, *B. ferrariae*, and *B. nodosa*, *B. tropica*); (2) *Burkholderia* isolate LUP and *Burkholderia* sp. Ch1-1 were most closely related to *B. xenovorans* LB400, while isolates I7, DNBP6-1, RIM, and EB2 were most closely related to *B. ginsengesolis*, *B. bryophila*, *B. caledonica*, and *B. fungorum* LMG16225^T, respectively; (3) isolate PO-04-17–38 from Pico de Orizaba and KP5Blue from Japan (the latter did not oxidize CO) clustered with the major *Burkholderia* sub-group containing *B. cepacia*.

With the exception of the *coxL* gene from *Burkholderia* isolates Rim and LUP, all *coxL* gene sequences formed a single clade distinct from *coxL* sequences from other *Betaproteobacteria* CO oxidizers (e.g. *Hydrogenophaga pseudoflava*, Fig. 2). Within this clade, phylogenetic structure largely paralleled that for the 16S rRNA gene phylogeny. For example, the *coxL* sequences from *Burkholderia* isolates DNBP16, YA CP11, DNBP18, WA, PP51-2, PP52-1, and I2 clustered together as did 16S rRNA gene sequences for these isolates (Figs 1 and 2). A similar pattern occurred for *coxL* and 16S rRNA gene sequences from isolates I7, EB2, and *Burkholderia* sp. Ch1-1.

In contrast, *coxL* gene sequences from isolates DNBP20 and DNBP22 did not cluster with sequences from other isolates that are closely related according to the 16S rRNA gene phylogeny. In addition, the close phylogenetic relationship between *coxL* gene sequences from DNBP 6-1 and PO-04-17-38 was unexpected. Based on the 16S rRNA gene phylogeny, these isolates are most closely related to *B. bryophila* and *B. cepacia*, respectively, which are distantly related to one another. Further, the *coxL* gene sequence from isolates Rim and LUP clustered with *coxL* gene sequences from *Alphaproteobacteria* CO oxidizers.

These results suggest that while *coxL* and 16S rRNA gene sequences may often evolve in parallel, complete phylogenetic congruence cannot be assumed. The lack of congruence may be attributable to horizontal gene transfer (HGT). For example, isolate PO-04-17-38 represents the only known CO-oxidizing representative of the *B. cepacia* clade, but its *coxL* gene sequence is similar to that of isolates in a different *Burkholderia* clade, perhaps as a result of an HGT event between the members of the two groups. It is possible that the apparent scarcity of *coxL* in the *B. cepacia* clade could be partly due to the inability of our primer set to target the entire diversity of sequences present within the *Burkholderia* genus; however, evidence for the scarcity of *coxL* within the *B. cepacia*

clade is provided by a survey of the currently available *Burkholderia* genome sequences. As of February 2011, there were 67 *Burkholderia* genomes in the Joint Genome Institute's Intergrated Microbial Genomes Database (http://img.jgi.doe.gov/cgi-bin/pub/main.cgi) that encompass 23 species that are primarily pathogenic members of the *B. cepacia* clade (e.g., *B. cenocepacia*, *B. ambifaria*, *B. thailandensis*, *B. oklahomensis*). However, only three genomes contain authentic *coxL* genes, and these are the nonpathogenic *B. xenovorans* LB400 (previously noted by King, 2003), *Burkholderia* sp. H160, and *Burkholderia* sp. Ch1-1. HGT involving an *Alphaproteobacteria* CO-oxidizer may also account for the divergent *coxL* gene sequences recovered from isolates LUP and Rim. Consistent with this notion, *Alphaproteobacteria*-like *coxL* genes have been previously noted in several members of the *Gammaproteobacteria* (King & Weber, 2007).

Regardless of the impact of HGT, results from the isolate and genome survey reported here indicate that the capacity for CO oxidation occurs relatively infrequently among strains closely related to *B. cepacia* (e.g., KP5Blue, *B. cenocepacia* spp., *B. ambifaria* spp.) but is much more common among strains related to *B. sacchari* and *B. ferrariae* (e.g., DNBP16, CP11, WA, YA, DNAP20, DNBP22). Interestingly, none of the type strains analyzed in this study harbored form I *coxL* genes or oxidized CO, even though they were closely related to CO-oxidizing isolates described here. This 'patchy' distribution might be due to selective losses (or retention) of *cox* genes in descendents of ancestral CO-oxidizing *Burkholderia*. Specific factors, which might account for variable *cox* retention or loss, remain uncertain but could include the frequency of exposure to carbon starvation, which might result in different strategies for starvation survival (e.g., use of CO).

Atmospheric CO uptake

Although cultivation and molecular evidence has suggested that CO-oxidizing *Burkholderia* is relatively abundant *in situ* (Weber & King, 2010a, b), their capacity for oxidizing CO at environmentally relevant concentrations has only been documented for two isolates (i.e., *Burkholderia* strain LUP and *B. xenovorans* LB400; King, 2003). In this study, five arbitrarily selected isolates (CP11, I2, PP52-1, DNBP18, DNBP6-1) were tested for their ability to consume CO at concentrations typical of those found in the atmosphere (60–300 ppb; Crutzen & Gidel, 1983). All isolates were able to oxidize CO at sub-atmospheric concentrations, but rates varied significantly among them (Fig. 3). Isolate I2 and DNBP18 consumed CO most and least rapidly, respectively (Fig. 3).

The ability to use atmospheric and sub-atmospheric CO could provide a competitive advantage for CO-oxidizing *Burkholderia in situ* where substrate concentrations are chronically limiting (e.g., volcanic ash and cinders; King & Weber, 2008; King *et al.*, 2008), and in environments where CO might occur at elevated levels (e.g., the rhizoplane and rhizosphere; King & Crosby, 2002). The extent to which CO-oxidizing *Burkholderia* contributes to

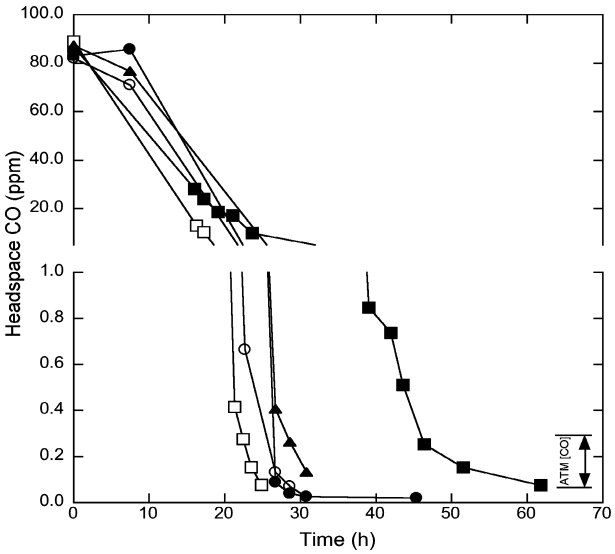

Fig. 3. CO uptake starting at about 80 ppm (vol/vol) and continuing at atmospheric concentrations (60–300 ppb (vol/vol); ATM [CO]) by *Burkholderia* isolates CP11 (○), I2 (□), PP52-1 (▲), DNBP18 (■), DNBP6-1 (●). Similar results were obtained for duplicate cultures, but only one replicate is shown for clarity. Note break in the *y*-axis between 1 and 10 ppm (vol/vol).

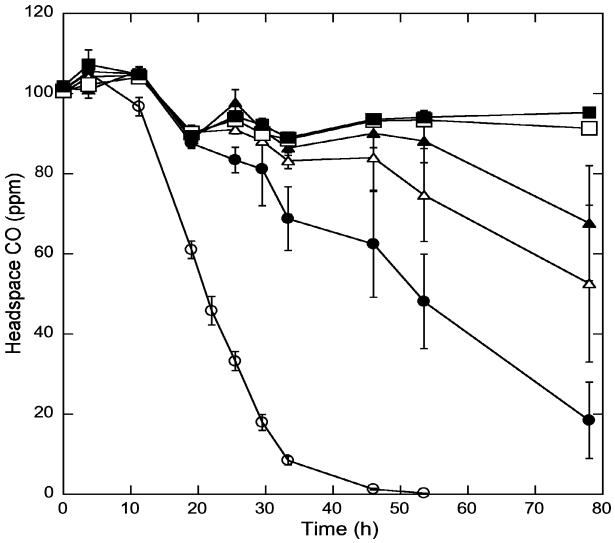

Fig. 4. CO consumption by PP52-1 incubated in the presence of 0 (○), 0.5 (●), 2.5 (△), 5 (▲), 10 (□), and 20 mM (■) pyruvate. Data points represent averages of triplicates (±1 SE).

Table 2. Growth ($\Delta OD_{600\ nm}$) and CO consumption (ΔCO) for isolates DNBP16 and PP52-1 in media containing various substrates (25 mM final concentrations with 0.01% yeast extract)

	DNBP16		PP52-1	
Substrate	$\Delta OD_{600\ nm}$	ΔCO	$\Delta OD_{600\ nm}$	ΔCO
Acetate	2.75 (1.07)	−0.05 (0.06)	2.48 (0.11)	0.05 (0.14)
β-hydroxybutyrate	6.53 (1.11)	0.11 (0.03)	7.13 (0.90)	−0.03 (0.02)
Gluconic acid	11.37 (0.78)	0.07 (0.00)	4.82 (0.02)	0.02 (0.02)
Mannitol	10.57 (1.57)	0.00 (0.02)	6.12 (0.53)	0.01 (0.03)
Proline	18.24 (1.24)	0.01 (0.04)	2.12 (0.09)	0.06 (0.03)
Pyruvate	11.89 (0.16)	−0.08 (0.08)	7.76 (0.11)	0.04 (0.05)
Methanol	0.73 (0.11)	0.77 (0.02)	0.16 (0.03)	0.69 (0.03)
Methylamine	0.60 (0.01)	0.78 (0.01)	0.01 (0.02)	0.59 (0.03)
Phthalate	0.33 (0.03)	0.77 (0.03)	0.08 (0.00)	0.60 (0.03)
Sucrose	0.59 (0.01)	0.83 (0.02)	0.11 (0.03)	0.72 (0.02)
Yeast extract (control)	0.56 (0.02)	0.77 (0.01)	0.21 (0.03)	0.76 (0.06)

Values for $\Delta OD_{600\ nm}$ represent the relative change in optical density after 24 h compared to controls. Values for ΔCO represent the fraction of CO removed from an initial starting concentration of about 80 ppm (vol/vol) (negative values indicate CO production) after 24 h relative to controls. All values are averages for duplicate cultures (±1 SE).

atmospheric CO uptake in terrestrial systems generally is unknown, but might be addressed in future studies using combinations of qPCR approaches, gene expression assays, and activity measurements (Weber & King, 2010a).

Impacts of organic substrate concentration and type on CO oxidation

The effects of heterotrophic substrates on CO oxidation by *Burkholderia* have not been explored previously. In this study, the rate of CO oxidation by PP52-1 was dependent on the concentration of pyruvate present in the medium, and only occurred with the concentrations < 2.5 mM (Fig. 4). At a pyruvate concentration of only 0.5 mM, CO oxidation by *Burkholderia* isolate PP52-1 was reduced by 72% compared to controls containing no pyruvate (Fig. 4). In similar experiments with *Labrenzia aggregata* and varying concentrations of glucose, 0.5 mM glucose reduced CO oxidation by a similar amount–about 40% (Weber & King, 2007). However, while 20 mM pyruvate completely inhibited CO oxidation by *Burkholderia* isolate PP52-1, activity was still observed for *L. aggregata.* These results suggest differential sensitivities to heterotrophic substrates and the possibility of a low level of constitutive *cox* expression for *L. aggregata*, but they also indicate that CO oxidation likely occurs *in situ*, where heterotrophic substrate concentrations are generally low.

A separate analysis with isolates DNBP18 and PP52-1 revealed that CO consumption depends not only on substrate concentration, but also on substrate type. CO was not consumed by either of the isolates within 24 h after inoculation in media containing any of the following growth-supporting substrates at final concentrations of 25 mM: proline, β-hydroxybutyrate, gluconic acid, acetate, pyruvate or mannitol. In contrast, both isolates consumed CO when inoculated into media containing the following substrates at 25 mM, which did not support growth: methylamine, methanol, phthalate, or sucrose. For isolates DNBP18 and PP52-1, no growth occurred in media containing only yeast extract, but CO was consumed rapidly. Collectively, these results indicate that CO is only consumed by *Burkholderia* when growth-supporting substrates are limiting (Table 2).

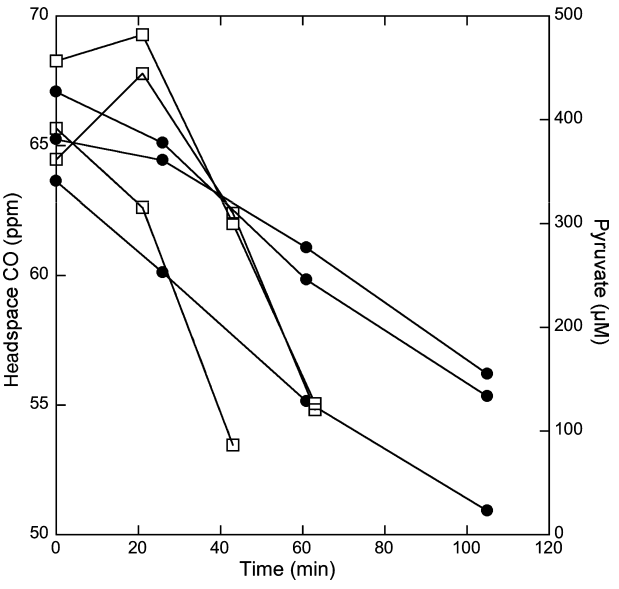

Fig. 5. Uptake of pyruvate (□) and CO (●) by triplicate cultures of DNBP18. Data for each replicate are shown.

Mixotrophic CO oxidation

Meyer & Schlegel (1983) indicated that mixotrophic growth by *Pseudomonas carboxydoflava* consuming CO and organic substrates simultaneously resulted in slightly higher growth yields, indicating that mixotrophic growth may result in a survival advantage *in situ* when heterotrophic substrates are limiting. Results described above indicate that CO consumption is impacted by organic substrate concentration and type. Knowing the ability of all isolates in this study to grow using pyruvate, we examined the ability of DNBP 18 to consume simultaneously CO and pyruvate with the latter at a low initial concentration (0.4 mM).

In this analysis, pyruvate concentrations declined rapidly to the levels that were undetectable after 1 h; during the same interval, CO concentrations also declined (Fig. 5). CO uptake rates in the presence and in the absence of pyruvate were essentially identical, 0.11 ± 0.01 and 0.11 ± 0.03 ppm min^{-1}, respectively. These results indicated that CO dehydrogenase in this isolate was induced in the presence of low substrate concentrations enabling mixotrophic metabolism; in addition, pyruvate at 0.4 mM did not decrease CO uptake rates.

Although the impact of substrates such as pyruvate on CO uptake activity undoubtedly varies among isolates, activity is likely expressed constitutively at the low micromolar concentrations that are typical of most substrates *in situ*. In this case, CO might serve as a supplemental energy substrate for the various CO-oxidizing *Burkholderia*. CO originating from roots may be especially significant (King & Crosby, 2002). *Burkholderia* is known to consume organic root exudates (Grayston *et al.*, 1998), and their ability to consume CO simultaneously could provide a competitive advantage. Although this has not yet been demonstrated, it is interesting to note that a previous study of volcanic soils along a vegetation gradient found that absolute numbers of *Burkholderia coxL* genes were higher in sites with extensive vegetation relative to nonvegetated sites (Weber & King, 2010a). These results suggest that the distribution and abundance of CO-oxidizing *Burkholderia* may co-vary with rhizosphere development and the availability of root-derived CO as a carbon and/or energy supplement.

Conclusion

Results presented here expand the known diversity of CO oxidizers in general and demonstrate that CO oxidation may be a common trait within one clade of the widely distributed genus, *Burkholderia*. Although heterotrophic substrates at high concentrations appear to repress CO oxidation, activity likely occurs constitutively under *in situ* conditions, resulting in mixotrophic metabolism. The degree to which mixotrophic metabolism enhances fitness and survival of CO-oxidizing *Burkholderia in situ* remains unknown and warrants further study.

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