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Tree phyllospheres are a habitat for diverse populations of CO-oxidizing bacteria

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Summary

Carbon monoxide (CO) is both a ubiquitous atmospheric trace gas and an air pollutant. While aerobic CO-degrading microorganisms in soils and oceans are estimated to remove ~370 Tg of CO per year, the presence of CO-degrading microorganisms in above-ground habitats, such as the phyllosphere, and their potential role in CO cycling remains unknown. CO-degradation by leaf washes of two common British trees, *Ilex aquifolium* and *Crataegus monogyna*, demonstrated CO uptake in all samples investigated. Based on the analyses of taxonomic and functional genes, diverse communities of candidate CO-oxidizing taxa were identified, including members of Rhizobiales and Burkholderiales which were abundant in the phyllosphere at the time of sampling. Based on predicted genomes of phyllosphere community members, an estimated 21% of phyllosphere bacteria contained CoxL, the large subunit of CO-dehydrogenase. In support of this, data mining of publicly available phyllosphere metagenomes for genes encoding CO-dehydrogenase subunits demonstrated that, on average, 25% of phyllosphere bacteria contained CO-dehydrogenase gene homologues. A CO-oxidizing Phyllobacteriaceae strain was also isolated from phyllosphere samples which contains genes encoding both CO-dehydrogenase as well as a ribulose-1,5-bisphosphate carboxylase-oxygenase. These results suggest that the phyllosphere supports diverse and potentially abundant CO-oxidizing bacteria, which are a potential sink for atmospheric CO.

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Introduction

The phyllosphere, defined as the above ground parts of plants, is a vast microbial habitat covering an estimated surface area of around 1 billion km^2 (Woodward and Lomas, 2004; Vorholt, 2012). It is colonized by diverse microorganisms including fungi, archaea, protists, viruses and bacteria, of which bacteria are the most abundant group, with an estimated 10^6–10^7 bacterial cells per cm^2 of leaf (Lindow and Brandl, 2003). Phyllosphere microbial communities have taxonomically diverse bacterial communities that vary seasonally, geographically and with plant host (Whipps *et al*., 2008; Redford *et al*., 2010; Knief *et al*., 2012; Vorholt, 2012; Kembel and Mueller, 2014).

The phyllosphere is characterized as a harsh microbial environment, with exposure to UV radiation and fluctuations in water availability and temperature. In addition, nutrient sources on the leaf surface are highly spatially heterogeneous, with most areas of the leaf harbouring such low quantities of nutrients they are considered oligotrophic, resulting in the majority of phyllosphere colonists being limited by carbon availability (Mercier and Lindow, 2000; Leveau and Lindow, 2001; Lindow and Brandl, 2003). Phyllosphere bacteria have been implicated in plant health, and interactions with the plant host range from symbiotic to pathogenic (Leveau and Lindow, 2001; Remus-Emsermann *et al*., 2012). Phyllosphere bacteria also contribute to biogeochemical cycling and degradation of pollutants, as demonstrated by the degradation of plant or atmospherically derived organic compounds such as methanol (Knief *et al*., 2010), phenol (Sandhu *et al*., 2009), 4-chlorophenol (Scheublin and Leveau, 2013), benzene (Jindachot *et al*., 2018), polycyclic aromatic hydrocarbons (PAHs) (Yutthammo *et al*., 2010), methyl chloride (Nadalig *et al*., 2011) isoprene (Crombie *et al*., 2018; Carrión *et al*., 2020) and diesel (Imperato *et al*., 2019). In doing so, activities of phyllosphere bacteria affect atmospheric chemistry and the fate of atmospheric pollutants and may thus contribute to critical ecosystem services of the phyllosphere in mitigation of air pollution, a major global public health problem causing around 5.5 million premature deaths worldwide (Lelieveld *et al*., 2015).

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Carbon monoxide (CO) is a ubiquitous component of the atmosphere. It is toxic to humans and even small changes in CO concentration in urban areas can have an impact on human health, with a 1 mg/m^3 increase in CO being associated with a 4.4% increase in cardiovascular disease hospital admissions (Liu *et al*., 2018). Atmospheric CO also negatively impacts the environment by reducing concentrations of hydroxyl radicals and thus increasing the residence times of greenhouse gases such as methane (Daniel and Solomon, 1998), contributing to the increase in ground-level ozone (Reeves *et al*., 2002) and leading to the formation of photochemical smog (Westberg *et al*., 1971).

Environmental sources of atmospheric CO include volcanic eruptions, bushfires, photochemical reactions and photolysis of marine coloured dissolved organic matter (CDOM) (Badr and Probert, 1994; Stubbins *et al*., 2006). In addition, both live and dead plant matter have been shown to emit CO due to photochemical transformations within living leaf tissue or by UV-induced photoproduction by dead leaf tissue. This results in an estimated 50–200 Tg yr^{-1} of CO and 60–90 Tg yr^{-1} of CO being produced from live plant tissues and dead plant matter, respectively (Seiler and Giehl, 1977; Tarr *et al*., 1995).

The major global source of CO, however, is anthropogenic combustion processes, which contribute more than half of annual CO emissions (approx. >1200 Tg per year) (Khalil and Rasmussen, 1994; King and Weber, 2007). CO concentrations are highest in urban areas, ranging from 1.1 to 2.5 mg/m^3 in the United Kingdom (DEFRA, 2014), compared with the atmospheric background of roughly 50–150 ppb (0.058–0.17 mg/m^3) (Khalil and Rasmussen, 1984; Novelli *et al*., 1998; Ou-Yang *et al*., 2014). However, in polluted cities such as Beijing, CO concentrations as high as 17.1 mg/m^3 have been reported (Xue *et al*., 2006).

Most CO in the atmosphere is converted to CO_2 via tropospheric hydroxylation (Badr and Probert, 1995), but significant amounts of CO are removed by microbial oxidation. Approximately 10%–15% of the global CO flux is consumed by soil microbiota (King, 1999) which remove approximately 300 Tg of CO per year (King and Weber, 2007); marine microbiota are estimated to oxidize approximately 70 Tg of CO per year (Conte *et al*., 2019), preventing the release of 85% of CO produced in the oceans into the atmosphere (Tolli and Taylor, 2005; Xie *et al*., 2005). Despite the fact that a significant amount (110–290 Tg yr^{-1}) of CO is produced by living and dead plant matter, it is currently unknown whether plant-associated microorganisms play a role in the mitigation of CO from this source.

Autotrophic bacteria using CO as a carbon and energy source, the so-called carboxydotrophs, typically utilize high concentrations of CO (>1%), assimilating the CO_2 produced via the Calvin cycle. However, carboxydotrophs have a low affinity for CO, preferring other organic substrates (King and Weber, 2007). Heterotrophic CO-oxidizers, known as carboxydovores, cannot grow at elevated CO concentrations but oxidize CO up to 1000 ppm. Carboxydovores have a high affinity for CO but cannot generate biomass when CO is the sole carbon source; they likely use CO as a supplementary energy source (King and Weber, 2007).

Aerobic CO-oxidizing bacteria oxidize CO to CO_2 using the enzyme carbon monoxide dehydrogenase (CODH) of which two forms, referred to as form I and form II, have been described. Both enzymes are composed of three subunits, encoded by *coxL* (large subunit), *coxM* (medium subunit), and *coxS* (small subunit) (Moran *et al*., 2004). The form I *cox* operon is transcribed in the order M, S, L whereas the form II operon is transcribed in the order S, L, M. Form I CODH has been described as the 'definitive' form and has been well characterized in several carboxydotrophs (Meyer and Schlegel, 1983). A form II enzyme was shown to be a functional CODH in *Bradyrhizobium japonicum* USDA 110 (Lorite *et al*., 2000) and *Kyrpidia spormannii* (Hogendoorn *et al*., 2020), but the inability to oxidize CO by a range of bacteria harbouring exclusively form II CODH (Cunliffe, 2011) suggests that some aspects of form II CODH function or regulation remain unknown. Carboxydotrophs and carboxydovores are taxonomically diverse, including members of the Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi and Bacteroidetes identified in marine (King, 2003; Tolli *et al*., 2006; Cunliffe, 2011), soil (King, 2003; Park *et al*., 2003; Weber and King, 2017) and rhizosphere environments (Lorite *et al*., 2000; King, 2003). The presence of *coxL* genes in a wide range of environments and in representatives of uncultivated clades of bacteria indicates that the diversity of CO-oxidizing bacteria is greater than those currently identified by cultivation (Hardy and King, 2001; Venter *et al*., 2004; Tolli *et al*., 2006; Weber and King, 2010; Yang *et al*., 2015; Lalonde and Constant, 2016; Wu *et al*., 2017; Hernández *et al*., 2020). CO oxidation is known to enhance the long-term survival of some bacteria, supporting their persistence in deprived or changeable environments (Cordero *et al*., 2019) and recent research indicates that CO oxidation may be a more generalist function than previously assumed, with 56% of soil bacteria predicted to be capable of CO oxidation (Bay *et al*., 2021). It is therefore possible that CO oxidation may be a widespread function in a range of microbial environments.

CO emissions from plant matter and anthropogenic sources such as vehicle exhaust fumes likely provides a constant input of CO to the leaf environment, suggesting that CO could be a nutrient exploited by bacteria in the

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nutrient-limited habitat of the leaf surface. Here, we assessed the potential of phyllosphere microbial communities to degrade CO and identified CO-degrading microorganisms using a combination of cultivation-dependent and cultivation-independent approaches. We show that tree phyllosphere samples are capable of CO oxidation and that some samples oxidized CO even when no other organic carbon sources were provided. The phyllosphere communities of *Ilex aquifolium* (holly) and *Crataegus monogyna* (hawthorn) contained diverse populations of CO degrading bacteria based on high-resolution microbial community analysis coupled to multivariate statistical analyses and functional marker diversity of *coxL*. A Phyllobacteriaceae strain able to oxidize CO was also isolated from phyllosphere samples. Overall, these results identify tree phyllospheres as a habitat for CO-oxidizing bacteria and suggest that phyllosphere microorganisms contribute to global cycling of CO.

Results

Leaf wash communities are capable of CO oxidation

Leaf material was sampled from two different sites, one next to a polluted roadside where hawthorn (sample ID: HtR) trees were sampled and the other in a less polluted woodland where both hawthorn (sample ID: HtW) and holly (sample ID: HlW) trees were sampled. Surface material was extracted from leaf samples by washing, followed by filtration. Leaf wash filters were then used as an inoculum for CO enrichment cultures containing 800 ppm CO plus an additional carbon source of yeast extract to encourage the growth of heterotrophic CO oxidizers. Incubation of leaf wash samples from all three sample types, woodland hawthorn (HtW), roadside hawthorn (HtR) and woodland holly (HlW) in mineral medium amended with yeast extract resulted in the degradation of 800 ppm CO (Fig. 1). On average, it took 33 days for CO to be degraded beyond the detectable limit (<50 ppm) by all phyllosphere samples ($n = 12$), although there was variation in different replicate samples (range of 17–52 days).

The length of lag phases between samples also varied (Fig. 1A–C), with five of the eight hawthorn samples (HtW and HtR) showing long lag phases between 17 and 30 days, whereas some hawthorn samples (3/8) and all holly samples had short lag phases of 2–5 days before degradation of CO began. Once degradation of CO began, woodland samples degraded CO faster (average 45 ppm and 39 ppm CO per day for HtW and HlW samples, respectively), than the roadside hawthorn samples (24 ppm CO per day).

As shown in Fig. 1D, woodland holly (HlW) samples degraded the CO the fastest (average 18.5 days), followed by woodland hawthorn (HtW) samples (average 37.25 days). Roadside hawthorn (HtR) samples were the slowest (average of 43 days). Wilcoxon significance tests between sample types showed that there was no significant difference between CO degradation rates of HtW and HtR samples ($P = 0.49$). However, HlW samples degraded CO significantly faster than HtW ($P = 0.0037$) and HtR ($P = 0.013$) samples.

Once each sample had been sub-cultured three times upon CO degradation, the samples were inoculated into minimal media with CO but without an additional carbon source to potentially indicate the presence of autotrophic CO oxidizers. Only half of all samples subcultured in this way continued to fully degrade three spikes of 800 ppm CO, suggesting that some of the enrichments may contain carboxydotrophs whereas the other half of the samples could not continuously oxidize CO as a sole carbon source, indicating that only carboxydovores may have been present (Supporting Information Fig. S1). It is noteworthy that those cultures which continued to degrade CO generally showed increasing rates of CO degradation which might indicate increased abundance of autotrophic CO oxidizers.

Candidate CO oxidizers in the phyllosphere include rare and abundant community members

Following complete CO degradation of three consecutive sub-cultures or re-spikes, the diversity of the enrichment cultures and no-substrate controls was assessed by high-throughput sequencing of 16S rRNA genes. Analyses of the bacterial community composition outlined below revealed how leaf surface communities responded to CO during the incubations and identified candidate CO oxidizers.

Analysis of similarity (ANOSIM) statistics showed that CO enrichment cultures were weakly but significantly dissimilar to their corresponding no-substrate controls, irrespective of whether they contained additional yeast extract as carbon source or not ($R = 0.31$, $P = 0.001$ and $R = 0.24$, $P = 0.02$, respectively), as shown in Fig. 2. Differences in bacterial community composition between CO enrichment cultures and controls at the OTU level are shown in the Supporting Information Figs S2 and S3. While the original leaf wash bacterial communities at the time of sampling were significantly dissimilar dependent on tree species ($R = 1$, $P = 0.035$) and sampling location ($R = 0.88$, $P = 0.033$), sample type did not significantly impact the bacterial community composition following cultivation with CO (data not shown).

SIMPER analysis identified those OTUs that drove the dissimilarity between the leaf wash cultures enriched with CO and no-substrate controls, thus highlighting candidate CO oxidizers. For this purpose, we initially considered

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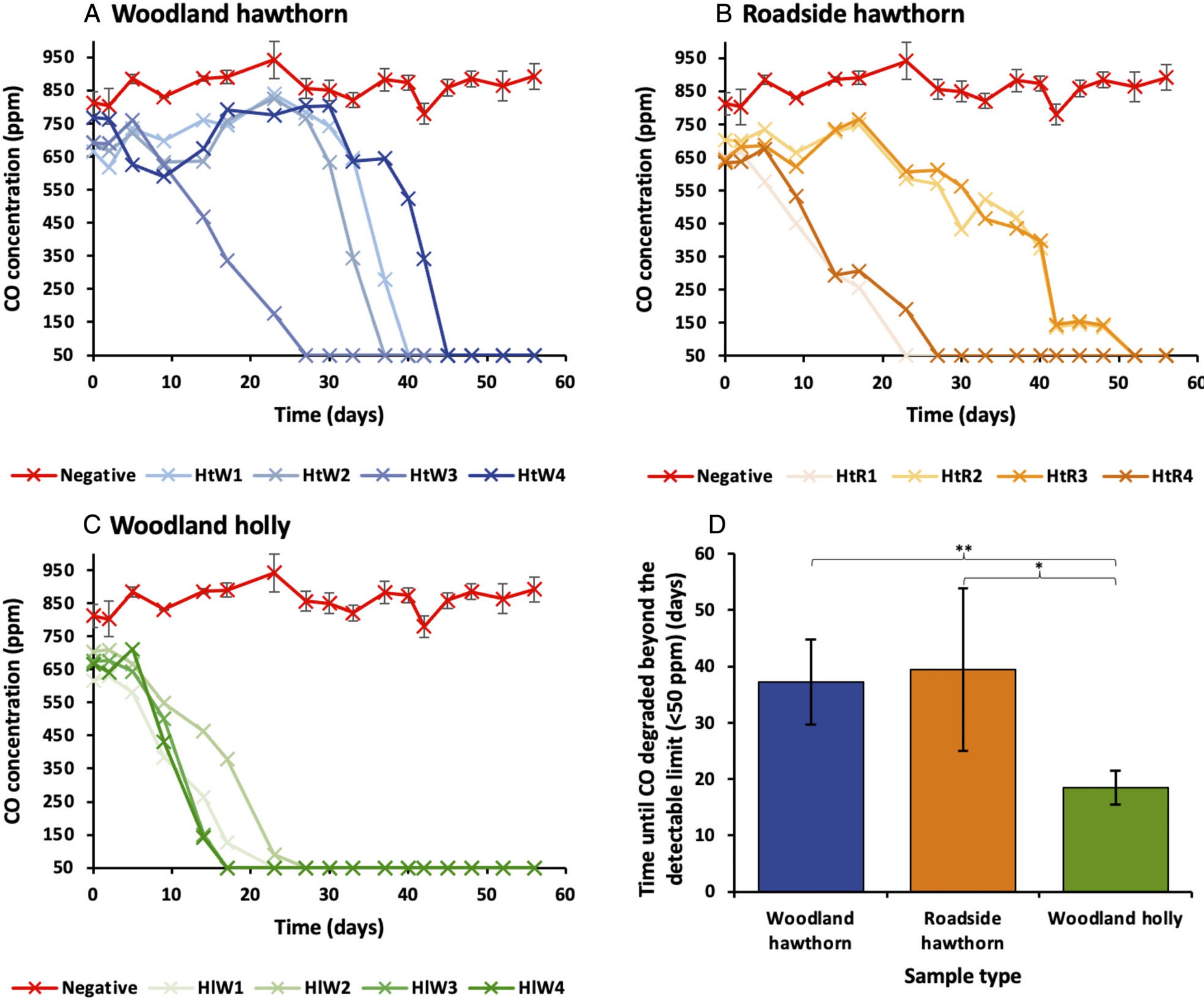

Fig. 1. Degradation of CO by holly and hawthorn leaf wash communities cultivated in media with added yeast extract. Enrichment culture medium contained 800 ppm CO with 0.25 g l^{-1} yeast extract.
A. Woodland hawthorn (HtW) samples.
B. Roadside hawthorn (HtR) samples.
C. Woodland holly (HlW) samples.
D. Average time taken for CO degradation between sample types (***P*-value of <0.001, **P*-value of <0.05, error bars indicate standard deviation). Negative controls ($n = 4$) did not contain leaf wash as an inoculum. CO concentrations displayed in this figure are those of enrichment cultures where original leaf wash samples were used as an inoculum. CO oxidation data from subsequent sub-cultures are not shown. [Color figure can be viewed at wileyonlinelibrary.com]

candidate CO oxidizers as those OTUs that were amongst the top 10 OTUs driving dissimilarity between CO and no-substrate controls, and which showed >1% increase in relative abundance in the CO enrichment cultures versus the no-substrate controls (Table 1). The majority of these OTUs were present at low relative abundances in the unenriched leaf wash samples. However, three candidate CO oxidizing OTUs (OTU9 (*Pseudomonas*), OTU36 (Oxalobacteriaceae) and OTU10 (Comamonadaceae)) together comprised >0.5% to 5.5% of total leaf wash community reads, on average. Strikingly, OTU10 (Comamonadaceae) had high relative read abundance (range 2.1%–10.1%) in all unenriched leaf wash communities, suggesting it could be an abundant colonizer of the phyllosphere.

Using the Deseq2 package, we further considered as potential candidate CO oxidizers all of those OTUs which showed a log2-fold increase of >1 and a *P* value of <0.05 in cultures enriched with CO versus no-substrate controls (Table 2 and Supporting Information Fig. S4). Four of these OTUs (OTU792 (*Arthrobacter*), OTU90 (Bradyrhizobiaceae), OTU33 (*Caulobacter*) and OTU5 (*Luteimonas*)) had high read abundances (range 3.5%–6.7%) in the CO enrichment cultures and had also been

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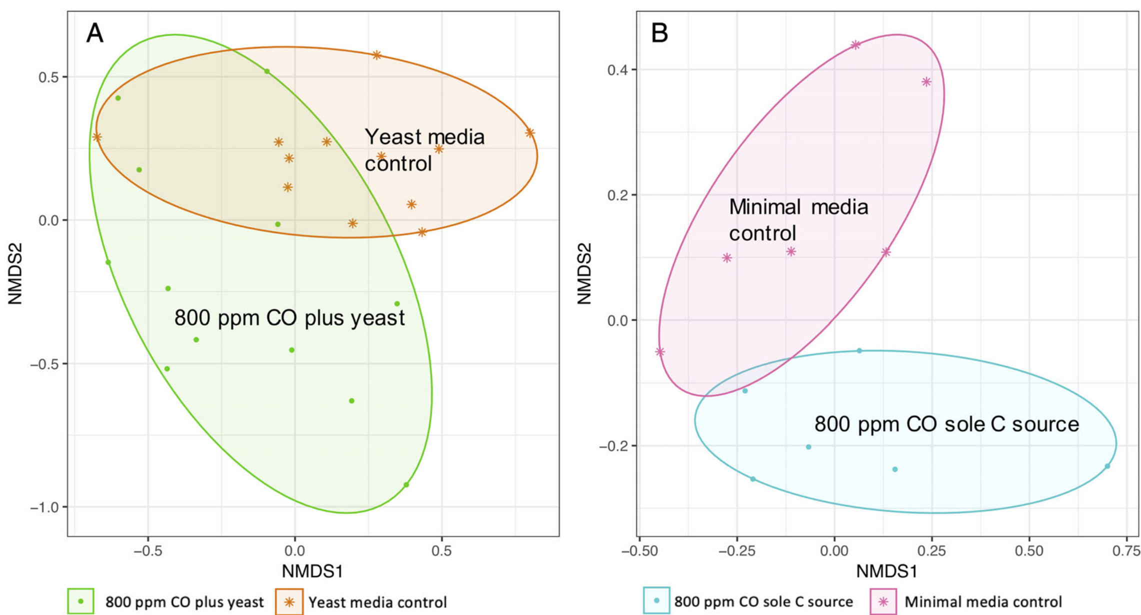

Fig. 2. NMDS ordination of variation between bacterial OTUs of CO enrichment culture samples and no-substrate control incubations. NMDS analyses were derived from Bray–Curtis similarity matrices constructed with relative read abundance data of OTUs present in each sample.
A. Variation between leaf wash samples cultivated with or without 800 ppm CO with the addition of another carbon source (yeast extract) ($n = 24$).
B. Variation in subcultures from samples shown in 2A which were then able to oxidize 800 ppm of CO without additional yeast extract (50% of subcultures oxidized CO as a sole carbon source, $n = 12$). [Color figure can be viewed at wileyonlinelibrary.com]

Table 1. Candidate CO-oxidizing OTUs based on SIMPER analyses.

						Unenriched leaf wash		
	OTU ID	Contribution to dissimilarity (%)	Greengenes assignment	CO (%)	Control (%)	HtW (%)	HtR (%)	H1W (%)
CO plus yeast extract	OTU34	6.38	f__Xanthomonadaceae; g__Dokdonella	8.004	3.425	0	0.006	0
	OTU792	4.59	f__Micrococcaceae; g__Arthrobacter	6.693	0.140	0	0	0
	OTU9	2.38	g__Pseudomonas; s__viridiflava	3.166	0.624	1.475	0.108	0.35
	0TU36	1.12	o__Burkholderiales; f__Oxalobacteraceae	1.611	0.087	0.164	0.403	1.294
	OTU128	1.09	f__Sphingobacteriaceae; g__Sphingobacterium	1.578	0.001	0	0	0
CO as a sole C source	OTU97	5.03	f__Xanthomonadaceae; g__Rhodanobacter	8.217	0.444	0.003	0.000	0.039
	OTU72	3.31	o__Rhizobiales; f__Phyllobacteriaceae	5.789	0.850	0.006	0.003	0.006
	OTU121	3.17	o__Xanthomonadales; f__Xanthomonadaceae	3.898	2.230	0.000	0.003	0.000
	OTU90	3.06	o__Rhizobiales; f__Bradyrhizobiaceae	5.285	0.178	0.000	0.000	0.003
	OTU33	2.62	g__Caulobacter; s__henricii	4.470	0.065	0.003	0.000	0.000
	OTU5	2.05	f__Xanthomonadaceae; g__Luteimonas	3.478	0.059	0.003	0.008	0.047
	OTU194	1.34	g__Nocardioides; s__plantarum	2.261	0.000	0.069	0.014	0.022
	OTU10	1.11	o__Burkholderiales; f__Comamonadaceae	2.163	0.374	4.186	10.122	2.150
	OTU57	0.84	g__Sphingopyxis; s__alaskensis	1.533	0.311	0.000	0.000	0.008

The 'CO' column represents enrichment cultures containing 800 ppm CO [either with and additional carbon source of yeast extract ($n = 12$) or as a sole carbon source ($n = 6$)]. The 'control' column represents sample control cultures that contained identical media [additional yeast extract ($n = 12$) or minimal media ($n = 6$)] and inoculum as CO cultures but without the addition of CO. Values represent relative abundance. Where Greengenes assignments are indicated, o_ = order, f_ = family, g_ = genus and s_ = species. Most detailed taxonomic lineages were assigned via UCLUST with a cut-off of ≥90% of sequences per OTU. Where values for unenriched leaf wash samples are indicated, HtW = woodland hawthorn samples, HtR = roadside hawthorn samples and HlW = woodland holly samples. OTUs shown in bold indicate those which were identified as candidate CO oxidizers via significant log2fold change (Table 2) in addition to SIMPER.

identified by SIMPER analysis. Therefore, in addition to contributing to the most dissimilarity between CO enrichment culture and control groups, these OTUs also showed a significant log2-fold change. All other candidate CO oxidizing OTUs identified using this approach were not identified by SIMPER analysis and were present in CO enrichment cultures at low relative read abundances, which were generally <1% [except OTU87 (Comamonadaceae), 2.1%]. Similar to

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Table 2. Candidate CO-oxidizing OTUs indicated by those which showed the most significant log2 fold change between CO enrichment cultures and controls.

	OTU ID	Greengenes assignment	CO (%)	Control (%)	Log2 fold change	*P*-value	Unenriched leaf wash HtW (%)	Unenriched leaf wash HtR (%)	Unenriched leaf wash H1W (%)
CO plus yeast extract	**OTU792**	f_Micrococcaceae; g_Arthrobacter	6.689	0.140	4.836	0.000	0.000	0.000	0.000
	OTU325	o_Saprospirales; f_Chitinophagaceae	0.265	0.003	3.768	0.008	0.000	0.000	0.000
	OTU114	c_Deltaproteobacteria; o_Myxococcales	0.311	0.015	3.302	0.002	0.000	0.000	0.003
	OTU355	c_Gemmatimonadetes; o_Ellin5290	0.161	0.010	3.234	0.009	0.000	0.000	0.000
	OTU351	o_Rhodospirillales; f_Rhodospirillaceae	0.120	0.006	3.003	0.015	0.000	0.000	0.000
	OTU4556	f_Sphingobacteriaceae; g_Pedobacter	0.023	0.001	2.971	0.026	0.000	0.000	0.000
	OTU279	o_Saprospirales; f_Chitinophagaceae	0.683	0.000	2.487	0.000	0.000	0.000	0.000
	OTU5	f_Xanthomonadaceae; g_Luteimonas	0.467	0.104	2.419	0.027	0.003	0.008	0.047
	OTU423	o_Rhodospirillales; f_Acetobacteraceae	0.044	0.004	2.372	0.048	0.000	0.000	0.000
	OTU67	f_Flavobacteriaceae; g_Flavobacterium	0.377	0.017	2.348	0.020	0.003	0.003	0.003
	OTU235	o_Saprospirales; f_Chitinophagaceae	0.097	0.015	2.058	0.040	0.000	0.000	0.000
	OTU87	o_Burkholderiales; f_Comamonadaceae	2.066	0.730	1.818	0.038	0.356	0.242	0.508
	OTU20	g_Janthinobacterium; s_lividum	0.156	0.024	1.741	0.003	0.217	0.381	0.128
	OTU62	f_Deinococcaceae; g_Deinococcus	0.344	0.183	1.536	0.003	0.000	0.022	0.006
CO as a sole C source	OTU267	o_Rhodobacterales; f_Hyphomonadaceae	0.617	0.006	4.603	0.007	0.000	0.000	0.000
	OTU90	o_Rhizobiales; f_Bradyrhizobiaceae	5.285	0.178	4.162	0.002	0.000	0.000	0.003
	OTU269	f_Hyphomicrobiaceae; g_Rhodoplanes	0.222	0.000	4.136	0.021	0.000	0.000	0.000
	OTU13	f_Pseudonocardiaceae; g_Actinomycetospora	0.200	0.000	4.043	0.023	1.989	1.372	4.331
	OTU359	o_Acidimicrobiales; f_Microthrixaceae	0.661	0.011	3.864	0.010	0.000	0.000	0.000
	OTU33	g_Caulobacter; s_henricii	4.470	0.065	3.735	0.010	0.003	0.000	0.000
	OTU355	c_Gemmatimonadetes; o_Ellin5290	0.126	0.000	3.654	0.039	0.000	0.000	0.000
	OTU5	f_Xanthomonadaceae; g_Luteimonas	3.478	0.059	3.52	0.037	0.003	0.008	0.047
	OTU1705	f_Hyphomicrobiaceae; g_Devosia	0.539	0.011	3.33	0.034	0.000	0.000	0.003

The 'CO' column represents enrichment cultures containing 800 ppm CO [either with and additional carbon source of yeast extract ($n = 12$) or as a sole carbon source ($n = 6$)]. The 'control' column represents sample control cultures which had identical media [additional yeast extract (n = 12) or minimal media (n = 6)] and inoculum as CO cultures, but without the addition of CO. Values represent relative abundance. Where Greengenes assignments are indicated, o_ = order, f_ = family, g_ = genus and s_ = species. Most detailed taxonomic lineages were assigned via UCLUST with a cut-off of ≥90% of sequences per OTU. Where values for unenriched leaf wash samples are indicated, HtW = woodland hawthorn samples, HtR = roadside hawthorn samples and HlW = woodland holly samples. OTUs shown in bold indicate those which were identified as candidate CO oxidizers via SIMPER analyses change (Table 1) in addition to significant log2 fold changes.

candidate CO-oxidizing OTUs identified by SIMPER analysis, most of the OTUs identified in Table 2 had low relative read abundance (<0.1%) in the unenriched leaf wash samples. Noteworthy exceptions include OTU20 (*Janthinobacterium*) and OTU87 (Comamonadaceae), which comprised 0.26% and 0.37% of unenriched leaf wash reads on average, respectively. Additionally, OTU13 (*Actinomycetospora*), which showed a significant log2-fold change in the 'autotrophic' CO enrichment culture was abundant in the leaf wash samples, comprising an average of 2% of reads of woodland hawthorn samples, 1.4% of roadside hawthorn samples and 4.3% of woodland holly samples. In total, 31 OTUs were identified as candidate CO oxidizers, representing 10% of total reads of the unenriched leaf wash community, on average.

Candidate CO oxidizers in the phyllosphere include diverse bacterial taxa

The candidate CO oxidizing OTUs identified above (Tables 1 and 2) included a diverse range of bacteria belonging to five different phyla (Fig. 3). The majority of candidate CO oxidizers were Proteobacteria (19/31), followed by Bacteroidetes (6/31), Actinobacteria (4/31), Deinococcus-Thermus (1/31) and Gemmatimonadetes (1/31). Three orders of bacteria contained the highest number of candidate CO-oxidizing OTUs, four OTUs each were members of Burkholderiales, Xanthomonadales and Rhizobiales.

There were two groups of OTUs which clustered within their treatment group [CO plus yeast extract (Fig. 3, labelled in green) or CO only (Fig. 3, labelled in blue)]. The four Rhizobiales OTUs (OTU90, OTU269, OTU1705 and OTU72) were all enriched on CO when it was the sole carbon source, whereas all six Bacteroidetes OTUs (OTU279, OTU235, OTU325, OTU67, OTU128 and OTU4556) were identified in CO plus yeast extract incubations. Two OTUs were identified in both types of enrichments (Fig. 3, labelled purple).

Identification of potential CO-oxidizing bacteria based on cox *gene functional markers*

Analysis of *coxL* diversity within CO enrichment cultures was achieved by PCR amplification, cloning and

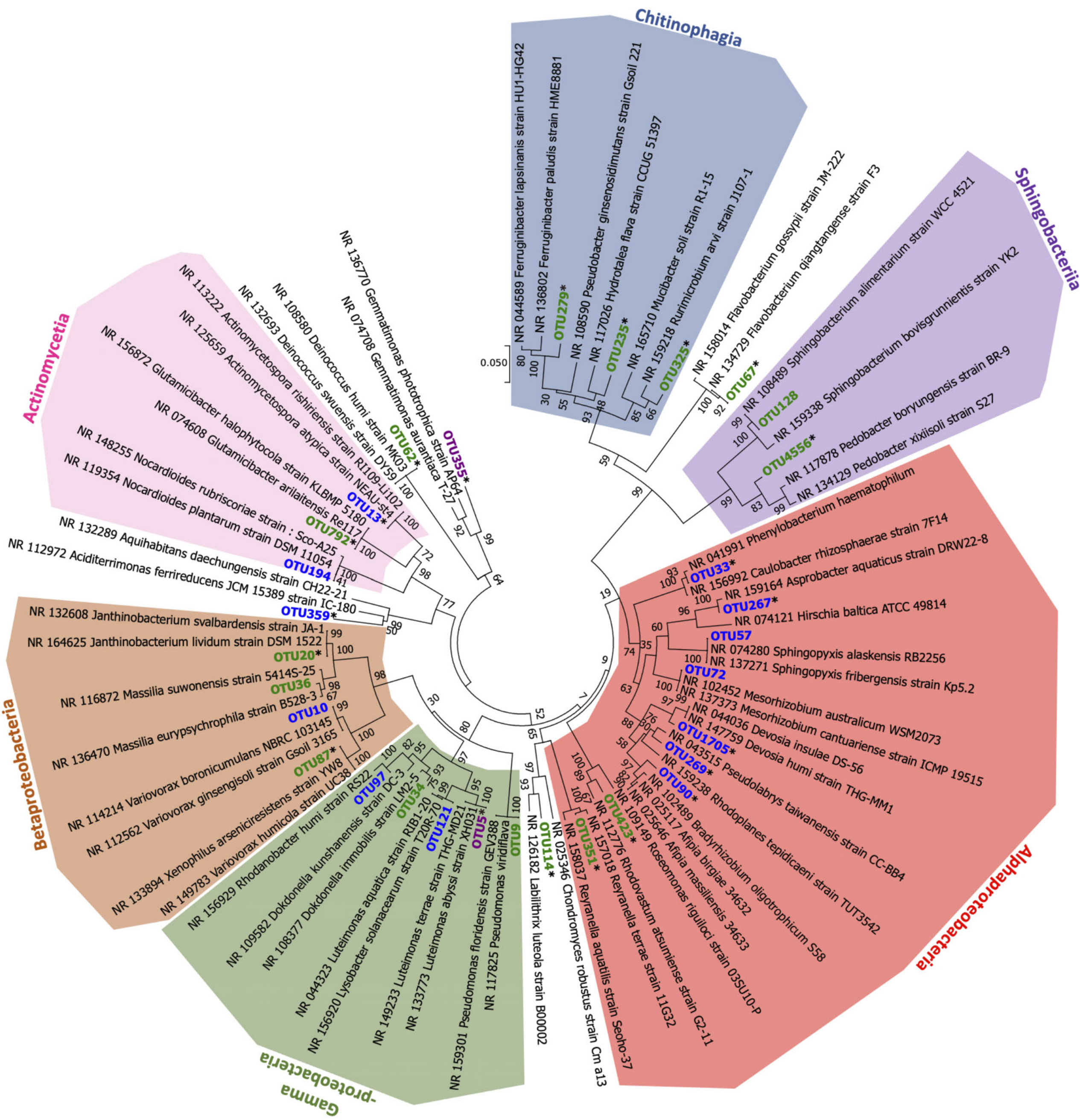

Fig. 3. 16S rRNA phylogeny of candidate CO-oxidizing bacteria from phyllosphere samples. OTUs were identified as potential CO-oxidizing species from SIMPER and log2 fold change analyses between leaf wash samples cultivated with CO and substrate controls without CO. OTUs indicated in green text are those which were enriched in cultures where yeast extract was provided as a carbon source in addition to CO. OTUs indicated in blue text are those which were enriched in cultures where CO was the sole carbon source. OTUs indicated in purple text are those which were enriched in both cultures where yeast extract was provided in addition to CO, as well as in subcultures where CO was a sole carbon source. OTUs indicated with an Asterisk (*) are those which had a significant (P-value = <0.05) log fold change between CO enrichment cultures and the control group without CO. Average amplicon size is 254 nucleotides. Phylogeny of OTUs and BLAST hits was inferred using the Neighbour-Joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The scale bar denotes the number of nucleotide differences per site. [Color figure can be viewed at wileyonlinelibrary.com]

sequencing of *coxL* using three CO-enriched samples (one per sample type) with added yeast extract. Ten transformant colonies containing an insert were used in subsequent *coxL* PCRs and sent for Sanger sequencing, resulting in 28 of the 30 high-quality sequences (Fig. 4).

Altogether, nine different clades of *coxL* sequences were identified. The majority of sequences obtained from

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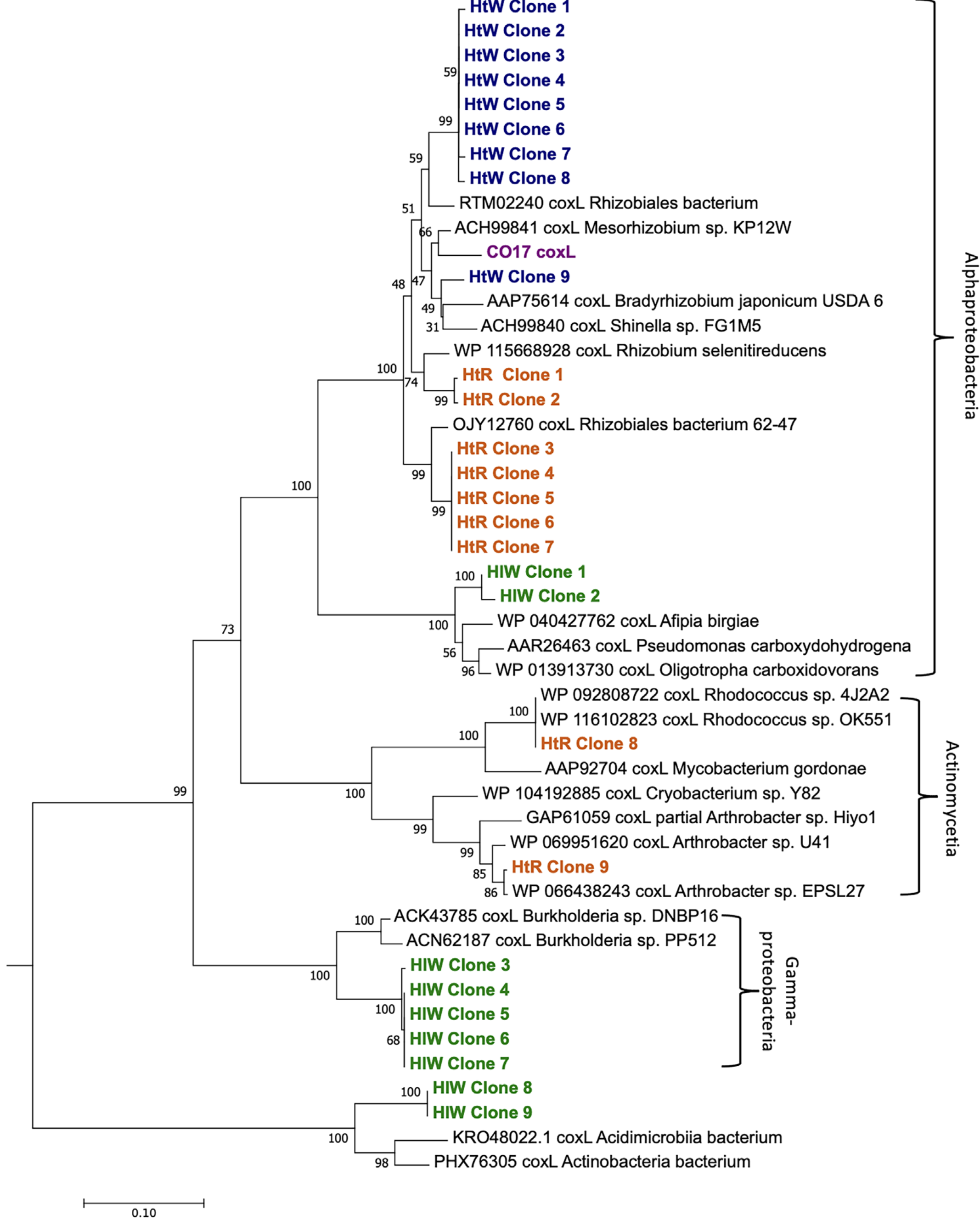

Fig. 4. Phylogenetic tree of *coxL* PCR product sequences from CO-enrichment culture clones. Phylogeny of sequences and BLAST hits were inferred using the Neighbour-Joining method of amino acid sequences in MEGA7. Form II *coxL* sequences were used as an outgroup. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Accession numbers are indicated before sequence names. HtW = woodland hawthorn samples (blue), HtR = roadside hawthorn samples (orange), HlW = woodland holly samples (green). For comparison, the *coxL* amino acid sequence from isolate CO17 (purple) was also included. Average amplicon size is 228 amino acids. The scale bar denotes the number of amino acid differences per site. [Color figure can be viewed at wileyonlinelibrary.com]

woodland hawthorn (HtW) enrichments (8/9) were highly similar, being most closely related to *coxL* genes from a Rhizobiales bacterium and one sequence having the highest similarity to a *Bradyrhizobium coxL* gene. Four different clades of *coxL* originated from roadside hawthorn (HtR) samples, two of which were most similar to Rhizobiales, e.g. *Rhizobium selenitireducens* (2/9) and ‘Rhizobiales bacterium 62–47’ (5/9). A further two *coxL* types detected in the HtR sample were most closely related to *coxL* from *Rhodococcus* and *Arthrobacter*

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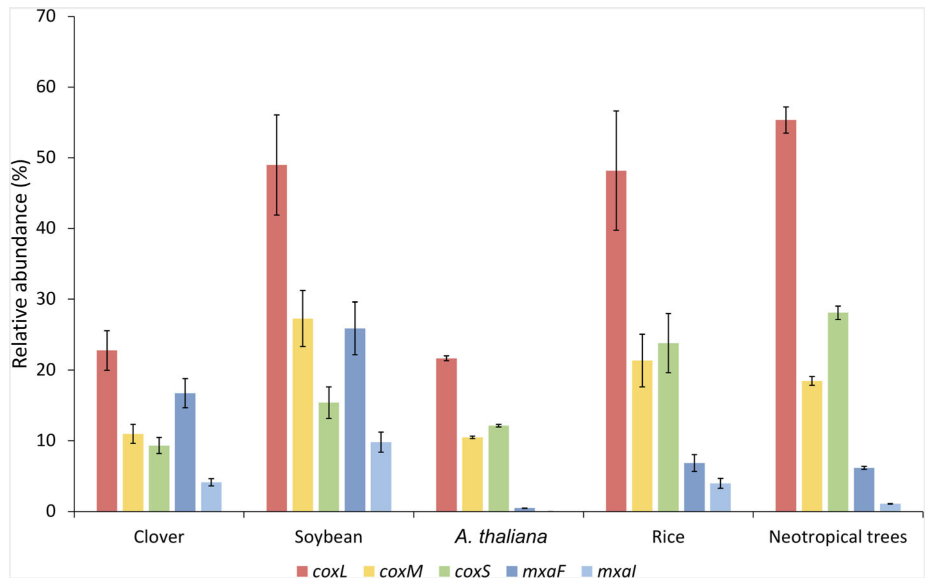

Fig. 5. Abundance of *cox* gene homologues in phyllosphere metagenomes available on MG-RAST. Abundance of the large (*coxL*), medium (*coxM*) and small (*coxS*) subunits of CODH are shown in comparison with methanol dehydrogenase subunits (*mxaF* and *mxaI*), as methanol utilization is a common phyllosphere function. All functional genes were normalized using three single-copy housekeeping genes: *recA*, *rho* and *rpoA*. [Color figure can be viewed at wileyonlinelibrary.com]

species, respectively. The majority of woodland holly (HlW) *coxL* sequences (5/10) were most similar to *Burkholderia coxL* genes. The remaining sequences were most similar to *coxL* from *Afipia* (2/10) or an *Acidimicrobiia* species (2/10). One of the sequenced clones had the highest similarity to a xanthine dehydrogenase rather than a *coxL* gene and so was omitted from the tree. Thus, despite the limited depth of sequencing, the analysis of cloned *coxL* amplicons revealed a relatively high diversity of phyllosphere bacteria with the potential to degrade CO in the enrichment cultures. It is noteworthy that the closest relatives of *coxL* identified were in good agreement with some candidate CO oxidizers identified based on analysis of the 16S rRNA sequencing data, i.e. members of the Rhizobiales, Burkholderiales, and Actinobacteria. None of the cloned *coxL* sequences were identical to the *coxL* gene of CO17, a CO-oxidizing phyllosphere isolate (discussed below).

PICRUSt2 was used to predict the relative abundance of OTUs in the leaf wash samples which were most closely related to a species in the genome database which contained *coxL*. A nearest sequenced taxon index (NSTI) cut-off of 0.05 was used, meaning that only OTUs which were approximately 95% similar to a species which had a genome within the database were used in the analyses. Of the 10 649 leaf wash OTUs, 2896 (27%) had a closely related genome available in the database. Of these OTUs, 513 (18%) were predicted to contain a *coxL* homologue. The average NSTI of this subset of OTUs was 0.029. Nine out of 31 of the candidate CO-oxidizing OTUs identified previously were included in this subset (OTU13, OTU351, OTU423, OTU267, OTU72, OTU269, OTU90, OTU87 and OTU10). The relative abundance of the OTUs predicted to contain *coxL* in the leaf wash samples was 24% for hawthorn woodland, 21% for roadside hawthorn and 19% for woodland holly, on average. There were no significant differences between the relative abundances of OTUs predicted to contain *coxL* between the sample types. Overall, these analyses suggest that an estimated 21% of phyllosphere bacteria contain *coxL*, on average. These results, along with those where a more stringent NSTI of 0.03 were implemented, are summarized in the Supporting Information Table S1.

Furthermore, the relative abundance of *cox* gene homologues (normalized with three single-copy housekeeping genes) within phyllosphere metagenomes available on the MG-RAST database (clover, soybean, *Arabidopsis thaliana*, rice and neotropical trees) was determined using functional abundance data available on MG-RAST. As displayed in Fig. 5, the relative abundance of *cox* gene homologues ranges between 21.7% and 55.3% for *coxL*, 10.4% and 27.3% for *coxM* and 9.3% and 28.1% for *coxS* genes. The abundance of CODH genes was compared with those involved in methanol metabolism (*mxaF* and *mxaI*), as methanol metabolism is a thoroughly researched and dominant function of phyllosphere microbiota, with methylotrophic species being ubiquitous and abundant colonizers of phyllosphere samples (Vorholt, 2012). By comparison, the relative abundance of *mxaF* and *mxaI* in this study were between 0.5%–25.9% and 0%–9.8%, respectively. Overall, the average relative abundance of CODH genes (*coxL*, *coxM* and *coxS*) (25%) was higher than methanol dehydrogenase genes (7.5%) in the phyllosphere metagenomes which were analysed.

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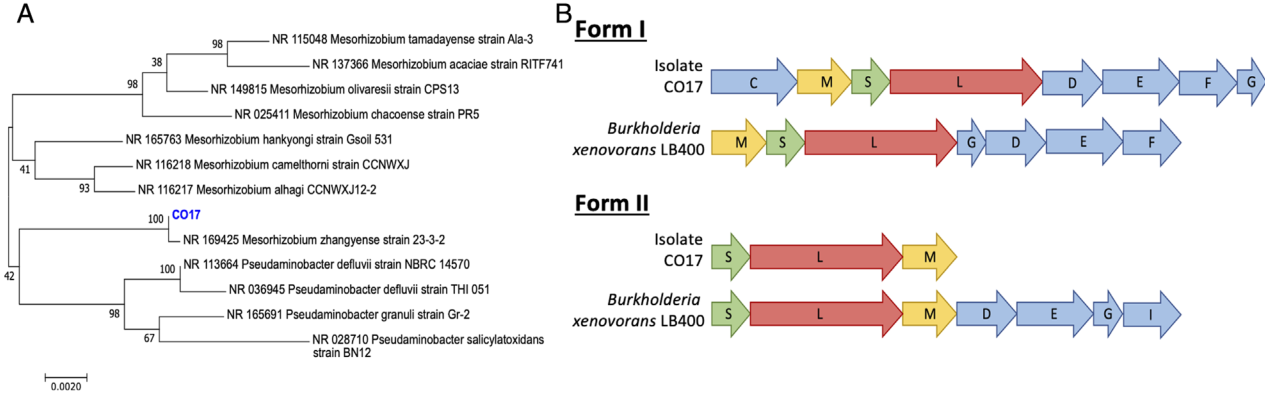

Fig. 6. Characterization of CO-oxidizing isolate CO17.
A. 16S rRNA phylogeny of isolate CO17. Phylogeny of isolate and BLAST hits was inferred using the Neighbour-Joining method of nucleotide sequences in MEGA7. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Accession numbers are indicated before genus names. Average amplicon size is 1344 nucleotides. The scale bar denotes the number of nucleotide differences per site.
B. Arrangement of form I and form II *cox* genes in isolate CO17 and *Burkholderia xenovorans* LB400. The genes encoding the small (*coxS*), medium (*coxM)* and large (*coxL*) subunits are indicated in green, yellow and red, respectively. Accessory genes are indicated in blue. [Color figure can be viewed at wileyonlinelibrary.com]

A Phyllobacteriaceae isolate, strain CO17, isolated from phyllosphere samples is capable of CO oxidation

Over 20 isolates were obtained from CO enrichment cultures and screened for their ability to degrade CO, and the presence of a *coxL* gene. Only one of these isolates, designated CO17, was confirmed to oxidize CO. This strain was isolated from material originating from woodland hawthorn leaf wash following incubation with 800 ppm as a sole carbon source. CO17 oxidized 800 ppm CO in the presence of yeast extract as an additional carbon source and while CO17 also oxidized 800 ppm of CO without an additional carbon source, autotrophic growth on CO as a sole carbon source could not be confirmed. Sequencing of the 16S rRNA gene identified isolate CO17 as closely related to *Mesorhizobium zhangyense*, with a 99.7% 16S rRNA sequence identity (Fig. 6A). The 16S rRNA gene of isolate CO17 is distinct from that of OTU72 (a *Mesorhizobium* species identified in MiSeq analysis as a candidate CO oxidizer), sharing only 95% sequence identity. The 16S rRNA gene of CO17 was 100% similar to OTU1995 which was detected in two woodland hawthorn leaf wash samples, representing 0.02%–0.03% of reads. A draft genome sequence was obtained for isolate CO17 consisting of an assembly size of 7 199 351 bp (number of contigs: 132, N50: 165,070; GC content: 61.1%). The genome assembly of strain CO17 has operons encoding both form I and form II CODH, the arrangements of which are typical of *cox* genes in other CO-oxidizing bacterial species (Fig. 6B). In addition, the genome of CO17 contained homologues of both the small (*cbbS*) and large (*cbbL*) ribulose-1,5-bisphosphate carboxylase-oxygenase (RubisCO) subunits. The *cbbL* homologue of CO17 clustered within *cbbL* genes, which encode the definitive form I red-like RubisCO proteins, as shown in the Supporting Information Fig. S5.

Discussion

Although CO oxidizers have been identified previously in a range of environments, their presence in the phyllosphere has only been speculative (King and Weber, 2007). This work provides the first evidence that there are microorganisms in the phyllosphere which are capable of CO oxidation, with all leaf wash samples in this study oxidizing 800 ppm CO when cultivated with an additional carbon source (Fig. 1A–C). Furthermore, there may be differences in the CO-oxidizing potential of different phyllosphere communities, as holly phyllosphere samples oxidized 800 ppm of CO significantly faster than hawthorn samples (Fig. 1D). It is therefore possible that tree species has a greater impact on the ability of phyllosphere communities to oxidize CO than location. The phyllosphere communities of evergreen tree species such as holly may be more efficient CO oxidizers as they do not senesce in autumn/winter months which may allow for a more established and thus specialized phyllosphere community to develop over time than deciduous trees in which the phylloplane is re-colonized each spring. Alternatively, differences in leaf physiology between the tree species may form selective pressures on the phyllosphere community, thus altering the community composition and functional capabilities of the phyllosphere. These initial data support that further work

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should include a wider range of tree species in order to investigate potential correlations between tree species and the CO-oxidizing potential of the phyllosphere.

Surprisingly, the phyllosphere communities sampled from roadside trees did not oxidize CO more rapidly than those sampled in the woodland (Fig. 1D). This indicates that phyllosphere communities exposed to higher concentrations of CO may not be enriched with greater populations of CO oxidizers. A similar result was reported by Imperato *et al*. (2019) who found that phyllosphere communities sampled from the city centre harboured fewer bacteria capable of diesel degradation than those in a primeval forest.

When CO enriched leaf wash cultures were subcultured into media where CO was a sole carbon source, only 50% of samples were able to consistently oxidize CO (Supporting Information Fig. S1). This could indicate that microorganisms capable of autotrophic CO oxidation (carboxydotrophs) are not as abundant as heterotrophic CO oxidizers (carboxydovores). This is not surprising as atmospheric CO would be available to phyllosphere microorganisms at low concentrations (~1–2 ppm in the United Kingdom) and therefore may better support microbial populations as a supplementary carbon source. Recent findings by Cordero *et al*. (2019) showed that CODH expression is upregulated in response to starvation and, due to the abundance of CODH in soil and marine samples, they predicted that CO supported the persistence of aerobic heterotrophic bacteria in nutrient limited environments. Thus, as results from the present study indicate that heterotrophic CO oxidation may be more common than autotrophic CO oxidation in phyllosphere communities, the CO survival-based model of CO oxidation proposed by Cordero *et al*. (2019) could also be an ecological adaptation enhancing survival in the phyllosphere, which is known to be deprived of energy sources (Mercier and Lindow, 2000; Leveau and Lindow, 2001; Lindow and Brandl, 2003).

The presence of CO in leaf wash cultures significantly impacted the bacterial community (Fig. 2) and although some of the dissimilarity between CO enrichment cultures and no-substrate controls may be due to toxic impacts of CO on the bacterial community, multiple bacterial species were identified which were potentially enriched on CO (Fig. 3). Three candidate CO-oxidizing OTUs where phylogenetically similar to genera which contain species proven to oxidize CO (Meyer and Schlegel, 1983; Lyons *et al*., 1984; Lorite *et al*., 2000; King, 2003). These are OTU9 (a *Pseudomonas* species), OTU72 (a *Mesorhizobium* species) and OTU90 (similar to *Bradyrhizobium* species). The majority of candidate CO oxidizers therefore present species for which CO oxidation would be a novel observation for the genus they belong to. Notable examples include an *Actinomycetospora* species (OTU13), a *Phenylobacterium* species (OTU33), a *Janthinobacterium* species (OTU20) and a *Luteimonas* species (OTU5). All of which were significantly enriched in the presence of CO, indicating that CO oxidation is a likely function of these species. In addition, candidate CO-oxidizing OTUs were identified within classes of bacteria which are not yet documented to contain CO-oxidizing species. Species within the Acidimicrobia, Deinococci, Deltaproteobacteria, Flavobacteriia, Gemmatimonadetes, Chitinophaga and Sphingobacteriia classes were identified as being potential CO oxidizers, indicating that the diversity of bacteria capable of CO oxidation may be higher than currently realized. Analysis of the functional diversity of CO oxidizers based on the *coxL* gene provided further support that some of these candidates are indeed capable of CO oxidation (Fig. 4). Four of the candidates identified by high-throughput sequencing of 16S rRNA genes were Rhizobiales species and the majority of the *coxL* sequences were most similar to those of Rhizobiales species, with five different clades of *coxL* likely belonging to this order. It is therefore likely that these candidates (OTU90, OTU269, OTU1705 and OTU72) are capable of CO oxidation. In addition, four species of Burkholderiales (OTU87, OTU10, OTU36 and OTU20) were identified in the 16S rRNA-based analyses. Interestingly, all of these OTUs were abundant in the phyllosphere at the time of sampling, making up 0.12%–10% of total reads in the original leaf wash samples. A cluster of *coxL* homologues sequenced from the clone library were most similar to, but distinct from, *coxL* genes from a *Burkholderia* species. It is possible that this *coxL* gene is from one of the Burkholderiales candidates.

In a parallel study investigating the degradation of 4-nitrophenol by phyllosphere microbiota (Palmer *et al*., unpublished), OTU10 (*Variovorax* sp.) and OTU13 (*Actinomycetospora* sp.) were also enriched in the presence of 4-nitrophenol. Metagenomes from samples enriched with these OTUs allowed for the assembly of metagenome assembled genomes (MAGs) of a *Variovorax* and *Actinomycetospora* species which likely correspond to OTU10 and OTU13, respectively. These draft genomes contained the form II arrangement of *cox* genes, providing further evidence that some abundant phyllosphere colonizers are likely to be capable of CO oxidation. Additional evidence from PICRUSt2 analyses supports this, with OTU10 and OTU13 being predicted to contain CoxL based on their closest relatives in the IMG database. In addition, PICRUSt2 analyses showed that 21% of bacteria within phyllosphere samples in this study estimated to contain CoxL, on average. This is likely an underestimation due to the majority of leaf wash OTUs (73%) which did not have a closest relative of over 95% identity in the IMG database. While this study provides

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initial evidence of which bacterial species are likely capable of CO oxidation in the phyllosphere, functional predictions based on taxonomic analyses are limited. Further evidence, for example, from metagenomic and metatranscriptomic data, is needed to confirm whether these candidate CO oxidizing species are capable of CO oxidation and whether they are actively oxidizing CO in the phyllosphere.

Mining of CODH sequences from publicly available phyllosphere metagenomes on MG-RAST further indicates that CODH genes may be abundant within the phyllosphere microbial community. Numerous CODH homologues were detected in all the phyllosphere metagenomes available (Fig. 5). Furthermore, when normalized with three housekeeping genes, it is estimated that up to 55% of the phyllosphere microbial community contained *coxL* gene homologues, or an average of 25% contained *cox* genes (*coxL*, *coxM* and *coxS*), whereas only up to 25.9% contained mxa*F* gene homologues, or an average of 7.5% from both *mxaF* and *mxaI*. Methanol metabolism is a dominant function of the phyllosphere due to the high availability of plant-produced methanol as a substrate on the leaf surface, which supports an abundant community of methylotrophs, impacting the flux of methanol into the atmosphere (Abanda-Nkpwatt *et al*., 2006; Delmotte *et al*., 2009; Knief *et al*., 2012; Vorholt, 2012). Our preliminary data suggest that the abundance of bacteria capable of oxidizing CO may be greater than those capable of methanol degradation which provides an initial indication that CO oxidation has the potential to be a common and previously unrecognized function of phyllosphere microbiota and therefore may also have an impact on the cycling of CO in the atmosphere. The estimated abundance of bacteria capable of CO oxidation in the phyllosphere in this study complements recent estimates that 56% of soil bacteria are capable of CO oxidation (Bay *et al*., 2021). It is possible that the phyllosphere and soil may harbour similar communities of CO-oxidizing bacteria as these biomes seed one another by aerial dispersal, leaf fall, precipitation runoff and plant seedling inoculation. Due to the short sequences of the phyllosphere metagenome *cox* gene homologues, which were available on MG-RAST, many of the CODH may be form II types of which the function is not fully known. Therefore, further research into the activity of form II CODH in CO oxidation, along with additional meta-omics investigations of phyllosphere communities, is necessary before the potential of phyllosphere microbiota to oxidize CO is understood.

The phyllosphere CO-oxidizing isolate CO17, a member of the *Phyllobacteriaceae*, was most closely related to *Mezorhizobium zhangyense* (Xu *et al*., 2018). Some *Mesorhizobium* species are already known to oxidize CO (King, 2003) and are common phyllosphere colonizers (Vogel *et al*., 2012). However, isolate CO17 and *Mesorhizobium zhangyense* do not cluster with other *Mesorhizobium* species (Fig. 5A). CO17 was also identified as an 'unclassified Phyllobacteriaceae' according to the RDP classifier (Wang *et al*., 2007), whereas *Mesorhizobium zhangyense* is not currently listed as a validated *Mesorhizobium* species in the LPSN (Parte *et al*., 2020), indicating that the taxonomic classification of both of these strains requires further attention. Likewise, it is noteworthy that isolate CO17 was isolated from a CO enrichment culture where CO was the sole carbon source and its genome contained genes encoding RubisCO (*cbbL* and *cbbS*) (Supporting Information Fig. S6). Whether this is driving the assimilation of CO_2 from CO oxidation remains to be determined; previously identified CO-oxidizing *Mesorhizobium* species did not grow on CO as a carbon source (King and Weber 2007).

Conclusions

Overall, this work demonstrates that the phyllosphere is host to diverse microorganisms capable of oxidizing CO. Some of the candidate CO-oxidizing species identified here are abundant members of the phyllosphere microbial community and the preliminary functional analyses of phyllosphere communities indicate an average of 25% of bacterial community colonizing the phyllosphere contain *cox* (*coxL*, *coxM* and *coxS*) gene homologues and therefore may be capable of CO oxidation. Recent research has suggested that carboxydovores capable of CO oxidation at sub-atmospheric concentrations are abundant in both marine and soil samples (Cordero *et al*., 2019; Bay *et al*., 2021). As the leaf surface is a nutrient-poor environment with direct exposure to atmospheric CO, we propose that CO oxidation may be a widespread function of the phyllosphere. Further work is required to establish the contribution of phyllosphere microbiota to the global CO cycling and mitigation of this toxic air pollutant.

Experimental procedures

Sample collection and processing

Two species of trees, hawthorn (*Crataegus monogyna*) and holly (*Ilex aquifolium*) were selected for investigation as they are common British trees and they represent both a deciduous and evergreen species. Tree branches were sampled in Coventry, United Kingdom on 06 October 2017 and four individual trees were sampled per site or species ($n = 12$). Both a woodland and a roadside site were chosen for sampling in order to investigate whether increased roadside pollutant exposure impacted the CO-oxidizing capabilities of phyllosphere microbiota.

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Hawthorn trees were sampled at both the woodland site (sample code 'HtW') (52.376248, −1.551322) and roadside site (sample code 'HtR') (52.394371, −1.554182), whereas holly trees were sampled at the woodland site (sample code 'HlW') (52.376248, −1.556771) only. At each site, four trees were selected and whole twigs were removed at random from 1.5 to 2 m high and were placed into sterile, polyethylene zip-lock bags to be transported to the lab where they were processed within 24 h.

Phyllosphere communities were collected by adapting a leaf washing method by Atamna-Ismaeel *et al*. (2011). In summary, 5 g of leaves per sample was weighed into sterile 250 ml Erlenmeyer flasks, to which 150 ml sterile phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was added. Microorganisms and other particulates were then dislodged from the leaf surface by sonication for 2 min in a water bath, followed by 10 s of vortexing and a 5-min resting period. The vortex and resting stages were repeated six times to thoroughly dislodge leaf surface material into the buffer. Leaf wash was filtered through 0.22 μm pore size membrane filters (PVDF membrane; Millipore, USA). Leaf wash filters were cut in half and either immediately used to determine their potential for CO oxidation or immediately frozen and stored at −80°C for future DNA extraction.

Assessment of leaf wash CO oxidation potential and cultivation of phyllosphere microbial communities enriched with CO

Sterile mineral salts medium was prepared for use in CO degradation experiments and enrichment of CO-degrading microorganisms as described by Meyer and Schlegel (1983). Analytical grade chemicals were dissolved in Milli-Q water before sterilization by autoclaving at 121°C for 15 min. To encourage the heterotrophic oxidation of CO, 0.25 g l^{-1} of yeast extract and 1 ml of filter sterilized vitamin solution (Kanagawa *et al*., 1982) was added to the mineral salts medium. Solid media for isolation of CO-oxidizing bacteria were prepared accordingly with addition of 1.5% (w/v) BactoAgar (Becton Dickinson, USA). Liquid media (25 ml) added to 125 ml serum vials were inoculated by adding half of a leaf wash filter per vial. Vials were sealed with a butyl plug, crimp-sealed, and CO (MQ 200; ≥99%; Sigma-Aldrich, USA) was injected into the headspace to a concentration of approximately 800 ppm. Sterile controls were prepared without inoculum and no-substrate controls were prepared containing the microbial inoculum but without addition of CO. Enrichment cultures and controls were incubated in the dark in a shaking incubator at 22°C and 100 rpm.

Headspace CO concentrations were monitored every 2–4 days using an Agilent 6890 N gas chromatograph (GC; Agilent Technologies, California, USA) equipped with a nickel catalyst methanizer (Agilent Technologies) allowing detection of CO after its reduction to methane using a flame ionization detector. The system was fitted with a 1.5 m long 80/100 Porapak Q column (inner diameter 2.1 mm; Sigma-Aldrich, USA) held at 250°C using nitrogen (50 ml/min) as the carrier gas. A 100 μl headspace sample was injected into the apparatus and peak readings for CO were taken. CO headspace concentrations in the headspace of enrichment cultures were calculated based on a calibration derived from injection of samples of known CO concentrations. CO concentrations between 0 (ambient air) and 50 ppm could not be differentiated and so <50 ppm was defined as the detectable limit.

Once CO concentrations were below the detection threshold, sub-cultures were prepared for both the enrichment sample and its corresponding control sample. Enrichment sub-cultures were prepared and monitored as above, using 2 ml of liquid culture from the corresponding sample as the microbial inoculum added to 23 ml of fresh mineral salts medium (plus yeast extract). This was repeated twice upon the degradation of CO for a total of three sub-culture cycles per sample. Once the final sub-culture had degraded the CO, 15 ml of liquid culture was extracted and centrifuged at 4500 × *g* for 20 min to form a pellet. The supernatant was discarded and the pellet was flash frozen in liquid nitrogen before storing at −80°C for future DNA extraction.

Once individual samples had degraded CO on their final sub-culture, they and their corresponding no-substrate control culture were also subcultured into mineral salts media without addition of yeast extract so that CO was the sole carbon source in order to investigate the occurrence of autotrophic degradation of CO. Inoculum for the 'autotrophic' subcultures was prepared by centrifugation of 15 ml of the final 'heterotrophic' subculture at 4500 × g for 10 min before discarding the supernatant and resuspending the pellet in 2 ml of autotrophic CO media. This was repeated three times before the washed pellet resuspension was added as an inoculum to 23 ml of mineral salts media without yeast extract. Degradation of CO was monitored and cultures were re-spiked with CO to bring the headspace concentration back to 800 ppm upon depletion. A total of three re-spikes were monitored and upon degradation of the final CO pulse, 15 ml of liquid culture was processed as described for heterotrophic CO enrichment cultures above for isolation of colonies and DNA extraction.

DNA extraction

DNA from CO enrichment cultures and no-substrate controls, as well as the orignal leaf wash filter samples, was

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extracted using the FastDNA™ Spin Kit for Soil (MP Bio Science, Derby, UK). The required amount of sodium phosphate buffer and MT buffer was added to the enrichment culture pellet in order to resuspend the pellet before being transferred to the Lysing Matrix E tube and proceeding with the protocol as per the manufacturer's instructions. At the final step, DNA was eluted using 50 μl of elution buffer. Aliquots of DNA extracts were assessed for quantity and quality by gel electrophoresis using 0.8% (w/v) agarose gels and a Qubit® 2.0 Fluorometer (Invitrogen, USA), using high-sensitivity reagents.

16S rRNA PCR and MiSeq library preparation

High-throughput sequencing was used to investigate the diversity of 16S rRNA genes in CO enrichment culture samples, corresponding no-substrate controls and original leaf wash samples. For each sample, 20 ng of DNA was used as template to amplify 292 bp of 16S rRNA genes using the 515f (5′-GTGCCAGCMGCCGCGGTAA-3′) and the 806r (5′-GGACTACHVGGGTATCTAAT-3′) primer pair (Caporaso *et al*., 2012) with Illumina Nextera Transposase Adapters attached to the 5′ end. PCRs were performed in 25 μl reaction volumes and contained 13 μl Q5® Hot Start High-Fidelity 2× Master Mix (New England Biolabs, USA), 0.5 μM of each primer and 0.4 μl of 25 mg ml^{-1} BSA. The reaction conditions were as follows: an initial denaturing step of 98°C for 30 s followed by 25 cycles of 98°C for 10 s, 50°C for 15 s and 72°C for 20 s and a final extension of 72°C for 10 min. PCR products were then purified using the AmpliClean™ Magnetic Bead PCR Clean-Up Kit (NIMAGEN, Nijmegen, the Netherlands), which was carried out as per the manufacturer's instructions.

Purified PCR products were given unique dual indexes at the 5′ end using the Nextera XT Index Kit v2 index primers (Illumina, USA). To attach the index primers, a PCR mixture was made consisting of 13 μl Q5® Hot Start High-Fidelity 2× Master Mix, 1 μM Index Primer 1, 1 μM Index Primer 2 and 4 μl of purified PCR product, with the addition of sterile, nuclease-free dH_2O for a final reaction volume of 26 μl per sample. The unique indexes were added by amplifying PCR products with the following reaction conditions: 95°C for 3 min, followed by 8 cycles of 98°C for 20 s, 55°C for 15 s, and 72°C for 15 s, then a final elongation step of 72°C for 5 min. Samples were normalized using the SequalPrep™ Normalization Plate Kit (Invitrogen) as per the manufacturer's instructions. All samples were pooled and adjusted to 4 nM before being submitted to the Genomics Facility at the University of Warwick, Coventry, United Kingdom for sequencing on the Illumina Miseq platform.

coxL *functional gene PCR amplification and construction of clone libraries*

Amplification of form I *coxL* genes using DNA from CO enrichment cultures was initially attempted using the OMPf and O/Br *coxL* primer pair (King, 2003). As this was not successful, this primer pair was reviewed and modified. Form I *coxL* sequences were obtained from the Integrated Microbial Genome and Metagenome (IMG) database (Chen *et al*., 2019) using the BLASTx function with a *Burkholderia fungorum* form I *coxL* sequence (AY307914.1) as a query. Five-hundred extra form I *coxL* genes were obtained in addition to the nine form I *coxL* genes used for the design of the original OMPf O/Br primer pair which were then aligned using ARB software (Ludwig *et al*., 2004) and a neighbour-joining tree was constructed. Primer sequences were reviewed with focus on *coxL* genes from terrestrial origins. An alignment was made with a total of 437 *coxL* sequences, the diversity of which is displayed in the Supporting Information Fig. S6. Mismatches were identified and modifications made to increase the redundancy of the primers in an attempt to improve upon the range of bacterial species targeted by the primers. Modifications made are shown in the Supporting Information Table S2.

The newly modified forward (5'-GGCGGNTTYGGNAA YAARGT) and reverse (5'-YTCDATDATCATNGGRTTD AT) primer pair was used to amplify definitive (form I) *coxL* genes from samples. PCRs were prepared containing 1x KAPA Taq Buffer A (includes 1.5 mM $MgCl_2$) (Kapa Biosystems, USA), dNTP nucleotide mix (0.2 mM each), DMSO (2% v/v), BSA (0.05% w/v), forward and reverse primer (0.4 μM each), KAPA Taq DNA polymerase (2 U) (Kapa Biosystems, USA) and DNA template (2–20 ng). The reaction was made up to a 50 μl reaction volume with sterile nuclease-free dH_2O. Positive controls were included which contained DNA template from *Ruegeria pomeroyi* DSS-3, a known CO oxidizer. Negative controls did not contain any DNA template. Reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of a 30 s denaturation at 94°C, 30 s annealing at 56°C and 1:15 min extension at 72°C, with a final extension of 7 min at 72°C. PCR products were visualized with GelRed (Biotium, USA) gel electrophoresis and gel-purified. PCR products were sequenced by Sanger sequencing either by GATC Biotech GmbH (Konstanz, Germany) or Eurofins Genomics (Ebersberg, Germany).

The diversity of *coxL* genes in CO enrichment culture samples was assessed by cloning purified PCR products using the TOPO TA cloning kit and TOP10 competent cells (Invitrogen) as per the manufacturer's instructions. Transformants were used as template for PCR

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amplification of *coxL* using the modified primer pair. Products were purified and sequenced as described previously.

Isolation, identification and genome sequencing of CO-oxidizing bacteria

Microbial culture (100 μl) sampled from heterotrophic or autotrophic enrichment vials after the final sub-culture was serially diluted with mineral salts media (Meyer and Schlegel, 1983). Dilutions were spread plated onto mineral salts plates (containing 0.25 g l^{-1} yeast extract) and LB plates and incubated at 22°C in the dark. Single colonies were selected at random and screened by PCR for presence of *coxL* gene as described previously. If products of the correct size were amplified, the corresponding colony was then re-suspended into mineral salts medium with 800 ppm CO and monitored for CO degradation by GC.

For taxonomic identification of isolates, the 16S rRNA gene was amplified as described above for *coxL* PCR but using the 27f (5′-AGAGTTTGATCMTGGCTCAG) and 1492r (5′-TACGGYTACCTTGTTACGACTT) primer pair (Weisburg *et al*., 1991) and using 1 μl of cell suspension from isolates that had first been heated at 100°C for 5 min as the template. Reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of a 30 s denaturation at 94°C, 30 s annealing at 55.5°C and 1:15 min extension at 72°C, with a final extension of 7 min at 72°C. PCR products were visualized, purified and sequenced as described previously.

For whole genome sequencing of the CO-oxidizing isolate, a lawn of cells was grown on an LB plate before harvesting of the cells which were sent to MicrobesNG (Birmingham, UK) where DNA was extracted and sequenced using the Illumina HiSeq platform (250 bp paired-end protocol). Adapters were trimmed and assembled by MicrobesNG using Trimmomatic (Bolger *et al*., 2014) and SPAdes version 3.7 (Bankevich *et al*., 2012), respectively. Contigs were then submitted to RAST (Aziz *et al*., 2008) for annotation and analysis of CO oxidation gene clusters.

Bioinformatic and statistical analyses

For the processing of MiSeq data, low-quality sequences were removed from the ends of the pre-demultiplexed reads using Trimmomatic version 0.36 (Bolger *et al*., 2014). USEARCH (Edgar, 2010) was then used for the majority of downstream analyses. Paired ends were joined, quality filtering was applied to a 280 bp minimum, primers were removed, and samples were dereplicated to remove any singletons. OTUs were clustered to a 97% identity threshold which also removed the majority of chimeras. Remaining chimeras were removed using the GOLD database (Edgar *et al*., 2011) for 16S rRNA amplicons. Reads were then mapped back to the OTU database for generation of OTU tables.

QIIME (Caporaso *et al*., 2010) was used along with the Greengenes (McDonald *et al*., 2012) reference database to assign taxonomy to 16S rRNA gene amplicons. OTU tables were generated and any OTUs identified as mitochondria or chloroplasts were removed. Number of reads ranged between 9017 and 236 540 and so samples were rarefied to 9000 reads per sample. Rarefaction curves were constructed (Supporting Information Fig. S7) in order to confirm that rarefaction to 9000 reads yielded sufficient saturation of diversity.

Non-metric multi-dimensional scaling (NMDS) analysis of Bray Curtis similarity of bacterial community structure were conducted using the VEGAN package in R (Dixon, 2003). ANOSIM tests were used to determine how dissimilar bacterial communities were between groups using PRIMER v6 software (Clarke and Gorley, 2005). Candidate CO-oxidizing OTUs were identified by a combination of Similarity Percentages (SIMPER) analysis using PRIMER v6 software (Clarke and Gorley, 2005), to find those which drove the most dissimilarity between CO enrichment cultures and controls, and identification of OTUs which showed a significant log2fold increase in CO enrichment cultures compared to controls, which was done using the DESeq2 package (Love *et al*., 2014) and visualized using the EnhancedVolcano package (Blighe, 2019) in RStudio.

Significant differences in CO degradation times between sample types were calculated using Wilcoxon tests in R studio (version 1.1.447, RStudio, USA) to calculate *P* values.

All sequence traces were checked for quality using 4Peaks (Nucleobytes.com, Aalsmeer, The Netherlands) and high-quality sequence was used as a query for BLASTn (nucleotide BLAST for 16S rRNA sequences) or BLASTx (translated nucleotides protein BLAST for functional genes) searches (Johnson *et al*., 2008). Sequences were aligned with their top hits from the BLAST database using MUSCLE (Edgar, 2004) in MEGA7 (Kumar *et al*., 2016). Phylogenetic trees were constructed using the neighbour-joining algorithm (Saitou and Nei, 1987) with a bootstrap test applied (Felsenstein, 1985) with 500 replicates in MEGA7.

The PICRUSt2 (Douglas *et al*., 2019) package was used to predict the functional capability of the original leaf wash community to oxidize CO, based on 16S rRNA amplicon data. A NSTI 0.05 (branch length of 0.05, approximately 95% identity) was used in order to discard any OTUs for which their closest relative was unlikely to be in the same genus from the downstream analyses. A total of 10 649 OTUs were used in the analysis, of which

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2896 (27%) had a genome of approximately 95% identity available on the IMG database. The relative abundance of OTUs predicted to contain CoxL (KEGG K03520) was then compared between leaf wash sample types.

Mining of CODH homologues from phyllosphere metagenomes

In addition to predictions made by PICRUSt2 of the abundance of *coxL* genes from samples in this study, the metagenomics-rapid annotation using subsystems technology (MG-RAST) database (Keegan *et al*., 2016) was used to determine how abundant CODH genes are in available phyllosphere metagenomes. Abundance data for gene homologues (identified by the MG-RAST pipeline) of CODH subunits (*coxL*, *coxM* and *coxS*), methanol dehydrogenase subunits (*mxaF* and *mxaI*) and three housekeeping genes (*recA*, *rho* and *rpoA*) were extracted from all publicly available phyllosphere metagenomes which included clover, soybean, *Arabidopsis thaliana* (Delmotte *et al*., 2009), rice (Knief *et al*., 2012) and 48 neotropical tree samples (Kembel *et al*., 2014). The abundances of *cox* and methanol dehydrogenase homologues were then normalized against the three housekeeping genes to determine the relative abundance of the target functional genes in the bacterial communities. Attempts to align a sub-set (77 AA sequences) of clover metagenome *coxL* amino acid sequences in order to confirm that sequences were *coxL* homologues was unsuccessful due to the short length (~60–90 AA) of partial sequences of *coxL* homologues. However, BLASTp searches indicated that the majority had high (>70%) AA sequence identity to other *coxL* or xanthine dehydrogenase (XDH) homologues. In addition, when the same subset (77 AA sequences) of *coxL* homologues were aligned with both characterized *coxL* (King, 2003) and xanthine dehydrogenase large subunits (Dwivedi *et al*., 2013), the majority (68/77) had a higher sequence identity with *coxL* than with xanthine dehydrogenase sequences.

Data Availability

The raw 16S rRNA gene sequences from Illumina MiSeq amplicon sequencing were deposited at NCBI Sequence Read Archive (SRA) under the BioProject accession ID PRJNA699530. The 16S rRNA gene sequences of all CO-oxidizing candidate species were deposited at NCBI Genbank under the accession numbers MW596379-MW596407. All *coxL* genes from clone libraries were deposited at NCBI Genbank under the accession numbers MW596683-MW596710. The 16S rRNA gene of CO-oxidizing isolate CO17 was submitted at NCBI Genbank under the accession MW592845, whereas the assembled genome sequence was submitted under the accession of JAFNIO000000000.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information

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