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Molecular and Culture-Based Analyses of Aerobic Carbon Monoxide Oxidizer Diversity†

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Isolates belonging to six genera not previously known to oxidize CO were obtained from enrichments with aquatic and terrestrial plants. DNA from these and other isolates was used in PCR assays of the gene for the large subunit of carbon monoxide dehydrogenase (coxL). CoxL and putative coxL fragments were amplified from known CO oxidizers (e.g., Oligotropha carboxidivorans and Bradyrhizobium japonicum), from novel CO-oxidizing isolates (e.g., Aminobacter sp. strain COX, Burkholderia sp. strain LUP, Mesorhizobium sp. strain NMB1, Stappia strains M4 and M8, Stenotrophomonas sp. strain LUP, and Xanthobacter sp. strain COX), and from several well-known isolates for which the capacity to oxidize CO is reported here for the first time (e.g., Burkholderia fungorum LB400, Mesorhizobium loti, Stappia stellulata, and Stappia aggregata). PCR products from several taxa, e.g., O. carboxidivorans, B. japonicum, and B. fungorum, yielded sequences with a high degree (>99.6%) of identity to those in GenBank or genome databases. Aligned sequences formed two phylogenetically distinct groups. Group OMP contained sequences from previously known CO oxidizers, including O. carboxidivorans and Pseudomonas thermocarboxidivorans, plus a number of closely related sequences. Group BMS was dominated by putative coxL sequences from genera in the Rhizobiaceae and other α-Proteobacteria. PCR analyses revealed that many CO oxidizers contained two coxL sequences, one from each group. CO oxidation by M. loti, for which whole-genome sequencing has revealed a single BMS-group putative coxL gene, strongly supports the notion that BMS sequences represent functional CO dehydrogenase proteins that are related to but distinct from previously characterized aerobic CO dehydrogenases.

Carbon monoxide occurs in nonurban atmospheres at about 60 to 300 ppb (11, 33, 43). In spite of these low concentrations, CO participates in a number of chemical reactions that determine the oxidative state of the troposphere as well as the residence times of many organic species, including greenhouse gases such as methane (11, 12, 20, 34). Atmospheric CO also serves as a substrate for soil microbes, which remove as much as 15% of the annual CO flux to the atmosphere (7, 21). Although soil microbes play an important role in the global CO budget, the populations involved remain poorly known (7, 8). Limited evidence indicates that fungi, actinomycetes, and bacteria actively consume CO but that “classic” carboxydotrophs serve as a substrate for soil microbes, which remove as much as 15% of a 1,260- to 1,290-bp region of the coxL gene. These primers were aligned and used to design primers for PCR amplification of a 1,260- to 1,290-bp region of the coxL gene. These primers were evaluated using CO oxidizers enriched from terrestrial and marine sources (G. M. King and H. Crosby, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. I-4, p. 247, 2002), including a previously described high-affinity soil isolate (Aminobacter sp. strain COX) (14). The results significantly expand the known diversity of CO-oxidizing bacteria and provide a basis for new ecological studies.

MATERIALS AND METHODS

Culture sources, isolation, and characterization. Oligotropha carboxidivorans OMS and M. tuberculosis H37Ra were obtained from the American Type Culture Collection. Bradyrhizobium japonicum USDA 6, M. loti USDA 3471, and Sinorhizobium fredii USDA 205 were obtained from P. van Berkum (U.S. Department of Agriculture, Beltsville, Md.); Bradyrhizobium sp. strain CPP was obtained from T. Wacek (Urbana Laboratories, Urbana, Ill.); Burkholderia fungorum LB400 was obtained from J. Haddick (Southern Indiana University, Carbondale, Ill.); Burkholderia sp. strain JS-150 was obtained from J. Spain (Tndall Air Force Base, Fla.); Burkholderia cepacia and Burkholderia multivorans were obtained from T. Lesie (University of Massachusetts, Amherst); M. avium and M. smegmatis were obtained from M. Glickman (Sloan-Kettering Cancer Memorial Center, New York, N.Y.); Stappia sp. strain CV812-530, Stappia sp. strain CV902-700, S. aggregata, S. stellulata, and Vibrio fischeri were obtained from K. Boettcher (University of Maine, Orono, Maine); and Silicibacter laccucaenuensis and S. pomeroyi were obtained from M. A. Moran (University of Georgia, Athens, Ga.).

† Contribution 384 from the Darling Marine Center.

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CO-oxidizing bacteria were also isolated from terrestrial and aquatic plant roots and macroalgal tissue. Isolates were obtained by incubating dried peat, rinsed macroalgae (*Ulva lactuca* or *Ascophyllum nodosum*), or soil-free roots of *Lupinus perennis* (lupine), or *Hydrogenophaga pseudoalga* (pickrelweed) in basal salts medium containing 0.01 to 0.05% yeast extract (14). All of these materials supported CO-oxidizing microbes (24, 36; King and Crosby, 2001). DNA was extracted and purified DNA was sequenced bidirectionally (with an ABI model 377 sequencer) by the University of Maine Sequencing Facility. Nearest phylogenetic neighbors for the isolates were determined using BLAST analysis of the GenBank database or Sequence Aligner software from the Ribosomal Database Project (rdp.cme.msu.edu).

Selected biochemical, physiological, and diagnostic traits for new isolates were determined using API 20 NE test strips (BioMerieux Inc., Marcy l’Etoile, France), GN2 substrate plates (Biolog Inc, Hayward, Calif.), and standard microbiological methods for Gram staining, motility, and oxidase reactions (41).

phase at 30°C in stoppered 160-ml serum bottles containing 10 ml of basal salts medium with 0.05% yeast extract and 25 mM pyruvate (14) or Middlebrook 7H9 medium with OADC supplement at 37°C (for *M. avium* and *M. tuberculosis* H37Rv). CO was added to bottle headspaces at final concentrations up to 1%. Headspace subsamples were obtained (using a needle and syringe) at intervals; CO concentrations were determined by gas chromatography as previously described (22, 23).

**PCR amplification and analysis of coxl gene fragments.** Sequences for the large subunit of authentic CO dehydrogenase genes were obtained from GenBank; putative CO dehydrogenase sequences were obtained from the genome databases for several species (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). *M. loti*, *M. tuberculosis* H37Rv, *Pyrobaculum aerophilum*, *Ralstonia solanacearum*, and *Sinorhizobium meliloti*. Sequences for *B. fungorum* LB400 and *Ralstonia eutropha* CH34 were obtained from the Joint Genome Institute (spider.jgi-psf.org/JGI_microbial/html). On the basis of several conserved motifs, inferred amino acid sequences (including that of the active site) were aligned using Clustal X, with manual adjustments as necessary (Fig. 1). Corresponding nucleic acid sequences were used to design two forward primers, OMPf (5'GGCGGTTGCGAAGGT-3') and a reverse primer, O/Br (5'GGCGGCTCTTGGCGTCC/ GGAAGAT-3'), and a reverse primer, O/Br (5'GGCGGCTCTTGGCGTCC/GGAAGAT-3'). Each amplification cycle consisted of a 45-s denaturation step at 94°C, a 60-s annealing step with the temperature profile described below, and a 90-s extension at 72°C. Annealing temperatures differed according to a “touchdown” protocol, which was initiated at 62°C for two amplification cycles followed by 1°C stepwise decreases in annealing temperature (two amplification cycles for each step) to a final value of 58°C, which was used for 30 amplification cycles. A final 20-min extension at 72°C completed amplification. PCR products were visualized with ethidium bromide gel electrophoresis and then purified with MoBio PCR cleanup kits. Purified DNA was sequenced bidirectionally (using the PCR primers as ABI model 377 sequencer) by the University of Maine Sequencing Facility.

Inferred amino acid sequences were derived from partial coxl sequences and aligned (using Clustal X as described above) with corresponding sequences from GenBank and genome databases, with manual adjustments as necessary. Sequences were analyzed using PAUP+ 4.0b software (Sinauer Associates, Inc., Sunderland, Mass.) to determine phylogenetic relationships among taxa. After excluding gapped positions, 348 residues were subjected to a neighbor-joining algorithm (1,000 bootstrap replicates) for tree construction. A putative coxl sequence from *P. aerophilum* was used as an outgroup. Several additional MCD-binding enzymes with sequences similar to that of CO dehydrogenase (e.g., quinoline oxidase, nicotine dehydrogenase) were included in the analysis.

**Nucleotide sequence accession number.** The sequences described in this work have been deposited in GenBank (see Table 1 for accession numbers).

**RESULTS**

**Pure culture CO oxidation.** Newly isolated CO oxidizers from marine and terrestrial sources consumed CO at rates from about 6 to 101 µg of CO mg⁻¹ of protein h⁻¹ for initial concentrations of about 1,000 ppm (Table 2). Similar CO consumption rates were also observed for a number of strains for which a CO oxidation capacity had not been previously reported (rate values represent means ± 1 standard error [in micrograms]: *Bradyrhizobium* sp. strain CPP, 9 ± 2; *B. fungorum* LB400, 15 ± 1; *M. loti*, 17 (n = 2); *S. fredii*, 21 (n = 2); *S. pomeroyi*, 17 (n = 2); *Stappia* sp. strain CV902-700, 21 ± 3; and *S. stellulata*, 32 ± 7). CO was not oxidized by *Burkholderia* sp. strain JS-150, *B. cepacia*, *B. multivorans*, *M. avium*, *S. luciscuadensis*, or three marine isolates, *C. spirillinus*, *L. anuloeedans*, and *V. fischeri*. Isolate characterization. CO-oxidizing bacteria were isolated from terrestrial sources (peat and plant roots) and marine macroagal enrichments. Isolates were identified to the genus level primarily on the basis of 16S rDNA sequence results. Nearest phylogenetic neighbors and percent similarities (in parentheses) for the isolates were as follows: *B. fungorum* LB400 for *Burkholderia* sp. strain LUP (99.3%); *Mesorhizobium* sp. strain SH15003 for *Mesorhizobium* sp. strain LMB1 (99.9%); *Stappia* sp. strain CV902-700 for *Stappia* sp. strain M4 (97.9%); *Stappia* sp. strain CV812-530 for *Stappia* sp. strain M8 (99.9%); *Stenotrophomonas maltophilia* sp. strain LMG-957 for *Stenotrophomonas* sp. strain LUP (99.1%); and *Xanthobacter* sp. strain INA43/2-2 for *Xanthobacter* sp. strain COX (98.2%).
Genus assignments were supported by the results for Gram reactions (all negative), oxidase reaction, morphology, motility, pigmentation, ability to reduce nitrate, ability to denitrify, sodium requirement, and patterns of heterotrophic substrate utilization (Table 2). In addition, Burkholderia sp. strain LUP oxidized biphenyl, which was consistent with the characteristics of its nearest phylogenetic neighbor, B. fungorum LB400, and Xanthobacter sp. strain COX produced a yellow pigment characteristic of the genus. Stenotrophomonas sp. strain LUP and Xanthobacter sp. strain COX also grew with methanol as a sole carbon and energy source.

All isolates oxidized CO during stationary phase when incubated with headspace CO concentrations from 1% to <10 ppm (data not shown). However, only one isolate, Xanthobacter sp. strain COX, grew with elevated CO (20%) as a sole carbon and energy source; this isolate also grew with 20% H₂–5% CO₂ (Fig. 2). The remaining isolates did not grow appreciably with elevated CO under oxic conditions even during extended (several-month) incubations. Further, all isolates except Xanthobacter sp. strain COX appeared to be at least partially inhibited by high CO concentrations, since CO at levels >1% was consumed slowly if at all.

**coxL PCR and sequence analysis.** PCR products of the expected size (about 1,260 to 1,290 bp) were obtained from all taxa that oxidized CO (Fig. 3). For certain taxa (e.g., *O. carboxidovorans* and Xanthobacter sp. strain COX) a single coxL or putative coxL product was obtained from reactions with one or the other of the two forward primers (e.g., OMPf and BMSf), while for other taxa (e.g., *B. japonicum* and *S. stellulata*) distinct products were obtained from reactions with each of the two primer sets. PCR results were negative for taxa that did not oxidize CO (e.g., *Burkholderia* sp. strain JS-150, *B. cepacia*, *M. multivorans*, *M. avium*, *C. spirillensus*, and *L. anuloeaters*).

Nucleic acid sequences for PCR products from *B. japonicum*, *B. fungorum* LB400, *M. tuberculosis* H37Ra, *M. smegmatis*, and *O. carboxidovorans* were identical or nearly identical (<5 bases differing in about 1,200 bp) to coxL or putative coxL sequences deposited in GenBank or genome databases. For *B. japonicum* and *B. fungorum* LB400, coxL sequences from reactions with each of the forward primers matched distinct sequences annotated as putative CO dehydrogenases in the respective genome databases.

**Phylogenetic analysis of inferred amino acid sequences revealed two clades (Fig. 4).** One clade, OMP, contained sequences similar to that of *O. carboxidovorans* coxL; these sequences characteristically contained the active site motif, AYRCFSR (see references 30 and 41). A second clade, BMS, contained putative coxL sequences from several taxa that were not represented in OMP (e.g., *M. lotii* and *S. melliloti*). Otherwise, the BMS clade was dominated by taxa containing both OMP and BMS coxL sequences. BMS sequences characteristically contained the active site motif, AYRGAGR. Within each clade, taxa tended to form clusters congruent with 16S rRNA phylogeny (Fig. 5). Thus, α-proteobacterial taxa formed a group distinct from those of the β/γ-Proteobacteria and *Mycobacteria*. Nicotine dehydrogenase and quinoline oxidase se-

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**TABLE 2. Selected characteristics of root and macroalgal isolates**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Result for isolate:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>strain LUP</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase reaction</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>–</td>
</tr>
<tr>
<td>Denitrification</td>
<td>–</td>
</tr>
<tr>
<td>CO uptake rate&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 ± 1</td>
</tr>
<tr>
<td>Hydrogen oxidation</td>
<td>+</td>
</tr>
<tr>
<td>Growth substrates&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Sugars</td>
<td>5/9</td>
</tr>
<tr>
<td>Amino acids</td>
<td>7/8</td>
</tr>
<tr>
<td>Organic acids</td>
<td>15/15</td>
</tr>
<tr>
<td>Methanol</td>
<td>–</td>
</tr>
<tr>
<td>Sodium required</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> CO uptake rates are means ± 1 standard error for triplicate determinations.

<sup>b</sup> Numbers for growth substrates represent positive substrate responses out of total examined.
sequences were distinct from those of both the OMP and BMS clades. The general topology of the coxL tree was supported strongly by bootstrap analysis and was comparable for both maximum parsimony and maximum likelihood analyses (data not shown). In addition, the coxL tree topology was retained for analyses of full amino acid sequences from select taxa and for analyses of a smaller region (about 150 residues) encompassing the active site and only one of the MCD-binding motifs (data not shown).

**DISCUSSION**

Results from enrichment cultures using macroalgal tissues and terrestrial and aquatic plant roots provisionally expand the range of known CO-oxidizing genera to include *Burkholderia, Mesorhizobium, Stenotrophomonas, Xanthobacter,* and *Stappia,* a strictly marine genus (Table 1 and Table 2). Genus assignments for the isolates are made on the basis of similarities between isolate 16S rDNA sequences and sequences of the nearest neighbors in GenBank. The biochemical and physiological characteristics of the various isolates (Table 2) are also consistent with published reports for the respective genera (see, e.g., references 1, 6, 13, 26, 31, 34, 36, 37, 41, and 42), none of which has been recognized before as carboxydrotrophic.

Although the genomes of *B. fungorum* LB400, *M. loti,* *S. meliloti,* and *S. pomeroyi* contain sequences annotated as putative CO dehydrogenases, results presented here are the first to document that these and (or) congeneric taxa actually oxidize CO. These include *P. aerophilum* (www.ncbi.nlm.nih.gov/PMGifs/Genomes/micr.html) and *Rhodospirillum rubrum* (spider.jgi-psf.org/JGI_microbial.html).

With the exception of *Xanthobacter* sp. strain COX, neither root nor marine isolates grow with elevated CO (i.e., >1% headspace concentration). Although all isolates oxidize CO at levels <1%, high concentrations inhibit CO utilization for most of the strains used in this study. This is consistent with previous reports for a high-affinity soil isolate (14) and intact soil (22, 27). Thus, it appears that two groups of CO oxidizers may be distinguished: a CO-tolerant group consisting of isolates that grow with high CO and a CO-sensitive group inhibited by concentrations higher than about 1%. The physiological basis for the distinction between these groups is not yet apparent. However, the latter may be more significant in situ, judging on the basis of responses of soil to elevated CO levels (22, 27).

CO consumption by marine heterotrophs, e.g., *Stappia* isolates and *S. pomeroyi,* is significant, because microbial activity represents an important CO sink in the oceans (9, 15, 44) and because carbon may represent a significant source of metabolizable carbon. Ammonia oxidizers have been considered dominant CO oxidizers in at least some systems (16–18), but more conventional carboxydrotrophs such as those documented here may contribute substantially, as occurs in soils where the role of ammonia oxidizers appears minimal (see, e.g., references 22 and 27).

Thus far, all known *Stappia* isolates, including two strains from oysters (4), oxidize CO and possess genes for ribulose-1,5-bisphosphate (G. M. King, unpublished results), which plays a central role in lithotrophic carbon fixation. This suggests that the genus *Stappia* may be carboxydrotrophic and function lithotrophically, mixotrophically, or heterotrophically. CO utilization appears more variable in the genus *Silicibacter,*
with a marine isolate, S. pomeroyi, oxidizing CO while a non-marine isolate, S. lacuscaerulensis, shows no uptake. Genome sequence and physiological results further indicate that S. pomeroyi does not contain rubisco or fix CO₂ into biomass (M. A. Moran, personal communication). This suggests the possibility that CO can serve as an energy source for S. pomeroyi and perhaps other strains. However, since three other marine isolates assayed in this study, C. spirodatum, L. anuloderans, and V. fischeri, do not oxidize CO, the distribution and functional roles of CO oxidation obviously differ markedly.

Although a prior survey indicated that Xanthobacter does not oxidize CO (35), results presented here suggest otherwise. However, it is unclear whether CO oxidation occurs rarely within the genus or whether it is associated with hydrogen

![Phylogenetic tree showing the distribution of CO oxidation genes among various bacterial species.](https://example.com/phylogenetic-tree.png)
to α-Proteobacteria, with only one example of a β-proteobacterial legume symbiont (32).

Primers developed from known and putative coxL sequences may prove useful in developing new insights about CO dehydrogenase distribution and evolution. PCR products obtained from CO-oxidizing strains in this study yield sequences that form two closely related but phylogenetically distinct clades. The OMP clade contains several well-known carboxydotrophs (e.g., O. carboxidovorans and H. pseudoalga) plus newly identified CO oxidizers.

In contrast, newly documented CO oxidizers dominate the BMS clade, which is characterized by putative coxL for which inferred amino acid sequences are about 70% similar to OMP sequences. The active site of BMS sequences, ARGAGGR, differs distinctly from the OMP active site, AYRCSFR, but is similar to that of several other molybdenum hydroxylases, a family of diverse enzymes that includes CO dehydrogenases, quinoline oxidase, aldehyde oxidoreductase, and nicotine dehydrogenases among others (19, 40). Nonetheless, both OMP coxL and BMS putative coxL are phylogenetically distinct from other molybdenum hydroxylases (Fig. 5).

Although the M. loti genome sequence indicates that BMS putative cox genes differ from OMP cox genes in their structural arrangements (subunit orders of MLS [medium, large, small] with no flanking accessory genes versus MSL with flanking accessory genes, respectively) (19, 39, 40), three lines of evidence suggest that the BMS genes code for functional CO dehydrogenases. First, the M. loti genome appears to contain only putative BMS cox genes; no OMP gene sequences have been documented. Thus, CO oxidation by M. loti can be reasonably ascribed to BMS genes. Second, some of the isolates described here, e.g., Mesorhizobium sp. strain NMB1 and Xanthobacter sp. strain COX, oxidize CO (Table 2; Fig. 2) but yielded PCR products only from the BMS primer (Fig. 3). This observation is also consistent with BMS genes coding for functional CO dehydrogenases. Third, BMS putative coxL in the genomes of B. fungorum LB400, M. loti, S. mellioli, and S. pomeroyi is associated with putative coxM and coxS genes, which provide for the synthesis of functional CO dehydrogenase.

While some isolates contain only BMS or OMP genes, several contain both, including all Bradyrhizobium strains, B. fungorum LB400, S. pomeroyi, and all Stappia strains (Fig. 3 and 4). The co-occurrence of BMS and OMP coxL sequences raises questions about the expression and physiological and ecological roles of CO dehydrogenases. Whether both are expressed in the same organism and under what conditions are not known. However, BMS and OMP coxL may affect whole-cell affinities for CO, since Aminobacter sp. strain COX, a high-affinity CO oxidizer (14), may contain only BMS coxL, while O. carboxidovorans, a low-affinity CO oxidizer (10), contains only the OMP sequence. In strains that possess both sequence types, expression may be related to available CO concentrations, with OMP expressed when concentrations are relatively high. Thus, differences among strains in affinities for CO may reflect which coxL sequence types are present. The availability of primers for each offers options for exploring this question and for addressing responses of CO oxidizers to both ambient and elevated CO levels.

Finally, the topologies of OMP coxL and BMS putative coxL...
sequences parallel the topology of 16S rRNA, with two exceptions (Fig. 5). First, *S. pomeroyi* BMS codL groups with *β*-proteobacterial *coxL* sequences and its 16S rRNA groups with sequences of *α*-Proteobacteria. Second, OMP codL from *Stenotrophomonas* sp. strain LUP, a *γ*-proteobacterium, falls within a group of *α*-proteobacterial sequences (Fig. 4). These exceptions notwithstanding, it does not appear that lateral gene transfer plays a prominent role in the distribution of the trait for CO oxidation among major bacterial lineages. Obviously, isolation and analysis of additional cultures will help clarify the evolution of the *cox* system.

In summary, the phylogenetic and taxonomic diversity of aerobic CO-oxidizing bacteria has been significantly expanded through enrichment and isolation of CO oxidizers and through culture-based assays of taxa containing putative *cox* but not previously known to oxidize CO. Previous reports have identified 12 genera with approximately as many species that oxidize CO (2, 29, 30). Direct assays of CO uptake indicate that seven additional genera contain CO oxidizers. Results from molecular studies presented here and from genome sequencing indicate that other taxa, including certain archaea (e.g., *Pyrolobaculum* and *Sulfologus*), likely oxidize CO aerobically as well. Collectively, these results suggest that the ability to utilize CO occurs widely among aerobic bacteria, perhaps because CO can serve as an energy supplement when organic substrates are limiting. This notion is consistent with recent observations that CO utilization is associated with microbial community development on volcanic deposits (25) and that populations of CO oxidizers in these systems appear to be very diverse (K. E. Dunfield and G. M. King, Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., N-250, p. 443, 2003).

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