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 carboxidovorans* 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. carboxidovorans*, *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. carboxidovorans* and *Pseudomonas thermocarboxydovorans*, 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 play a minimal role in situ (3, 10). However, the role of such carboxydotrophs has only been inferred from laboratory studies that may not accurately reflect capabilities expressed in situ.

Carboxydotrophic bacteria constitute a small but diverse group of aerobes (31), primarily within the α-*Proteobacteria* and the *Firmicutes*, that utilize CO as sole carbon and energy sources at high (much higher than 1%) concentrations while expressing relatively low-affinity CO uptake systems (apparent K_m, ≥500 ppm). While the ecological roles of these organisms need clarification, several carboxydotrophs have provided important insights about the biology, biochemistry, and molecular biology of CO oxidation (30, 31).

In particular, similarities to authentic CO dehydrogenase have provided a basis for identifying putative CO dehydrogenases in genomes of bacteria not previously reported to oxidize

CO, including *Burkholderia fungorum* LB400, *Mesorhizobium loti*, *Mycobacterium smegmatis*, *M. tuberculosis*, and the marine roseobacterium, *Silicibacter pomeroyi*, among others. Since genomic data have indicated that the trait for CO oxidation occurs more commonly than previously recognized, a survey of selected taxa was undertaken to establish whether putative CO dehydrogenases were associated with CO oxidation in vivo. In addition, known and putative CO dehydrogenase sequences 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 carboxidovorans* OM5 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.); *B. fungorum* LB400 was obtained from J. Haddock (Southern Indiana University, Carbondale, Ind.); *Burkholderia* sp. strain JS-150 was obtained from J. Spain (Tyndall Air Force Base, Fla.); *Burkholderia cepacia* and *Burkholderia multivorans* were obtained from T. Lessie (University of Massachusetts, Amherst); *M. avium* and *M. smegmatis* were obtained from M. Glickman (Sloan-Kettering Memorial Cancer 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 lacuscaerulensis* and *S. pomeroyi* were obtained from M. A. Moran (University of Georgia, Athens, Ga.).

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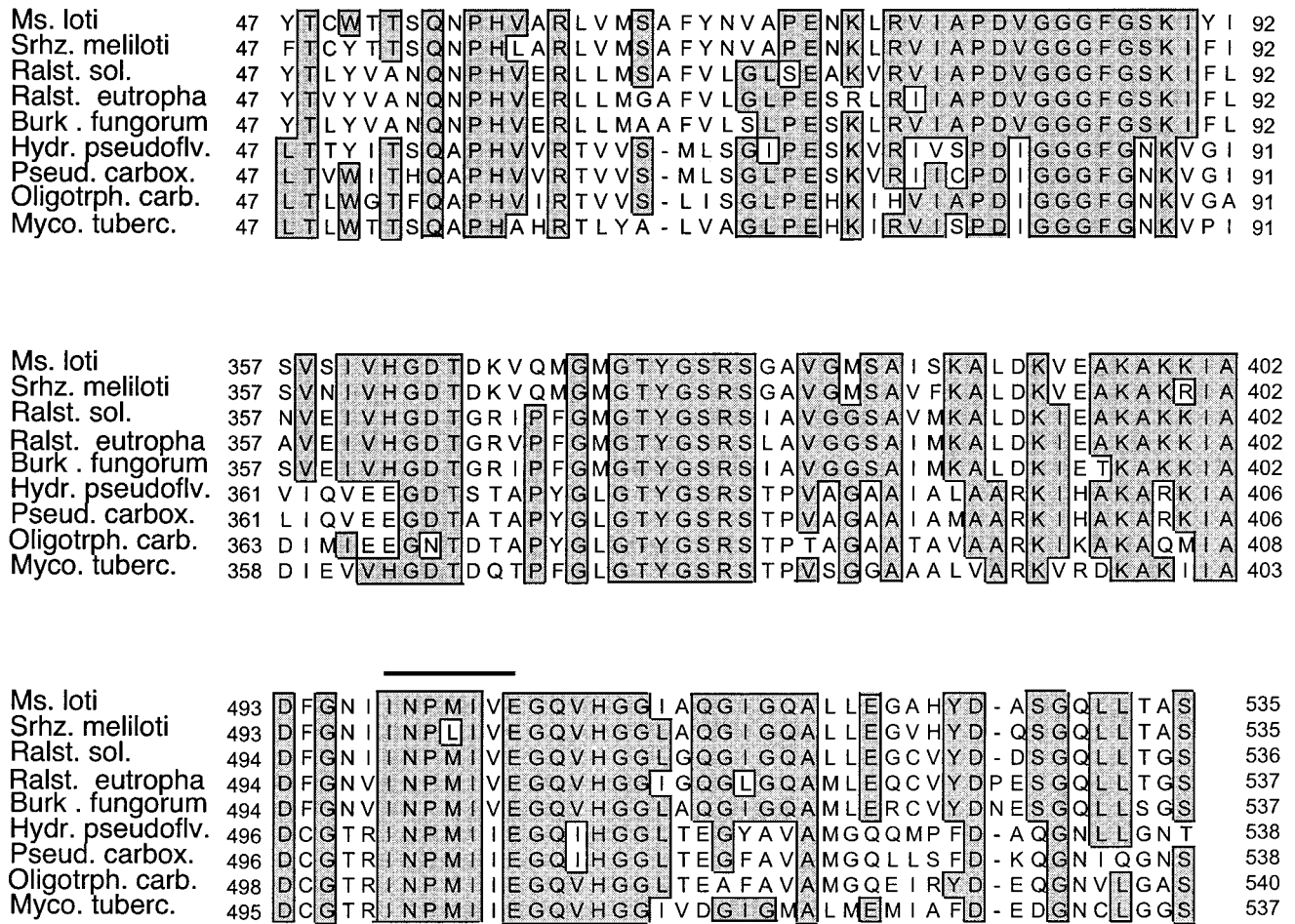
FIG. 1. Partial alignments of inferred amino acid sequences of the large subunit of CO dehydrogenase (*coxL*) and putative *coxL*. Regions targeted for PCR primer design are indicated by horizontal bars. Regions with similar sequences are indicated with shaded boxes. Abbreviations: Ms. loti, *Mesorhizobium loti*; Srhz. meliloti, *Sinorhizobium meliloti*; Ralst. sol., *Ralstonia solanacearum*; Ralst. eutropha, *Ralstonia eutropha*; Burk. fungorum, *Burkholderia fungorum*; Hydr. pseudoflv., *Hydrogenophaga pseudoflava*; Pseud. carbox., *Pseudomonas thermocarboxydovorans*; Oligotrph. carb., *Oligotropha carboxidovorans*; Myco. tuberc., *Mycobacterium tuberculosis*.

CO-oxidizing bacteria were also isolated from terrestrial and aquatic plant roots and macroalgal tissue. Isolates were obtained by incubating air dried peat, rinsed macroalgae (*Ulva lactuca* or *Ascophyllum nodosum*), or soil-free roots of *Phaseolus vulgaris* (bean), *Lupinus perennis* (lupine), or *Pontederia cordata* (pickerelweed) 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, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol.). The headspace of enrichment flasks contained 1,000 ppm of CO in air. CO was replenished through several cycles of depletion and addition. Subsamples of medium from flasks that tested positive for CO consumption were transferred to fresh medium and subjected to additional cycles of CO addition and depletion. Positive enrichments were diluted serially and plated onto basal salts agar medium with or without 25 mM pyruvate and incubated in the presence or absence of CO at concentrations up to 20%. Colonies were transferred to liquid medium, grown to stationary phase, and incubated with CO to determine oxidation potentials. Colonies that proved positive for CO oxidation were purified by repeated serial dilution with liquid and solid medium and CO at concentrations of 1,000 ppm. Headspace CO and time courses of CO utilization were assayed by gas chromatography as reported previously (14).

Identification of isolates to the genus level was accomplished by amplifying and sequencing 16S rRNA genes and by using various biochemical and physiological tests. For 16S rRNA analyses, cultures were harvested from late log to stationary phase, resuspended in phosphate buffer, and then subjected to three cycles of heating at 65°C and freezing at −80°C. DNA was extracted and purified using MoBio spin kits (MoBio Inc.; Solana Beach, Calif.). 16S ribosomal DNA (rDNA) was amplified with primers 27f and 1492r in 50-μl reaction mixtures in an Eppendorf Mastercycler (Brinkmann Inc., Westbury, N.Y.) with Master*Taq* DNA polymerase (Brinkmann Inc.) and standard amplification conditions (28, 38). PCR products were visualized with ethidium bromide gel electrophoresis and then purified with MoBio PCR cleanup kits. 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). Substrates that supported growth were assessed using basal salts supplemented with 0.05% yeast extract and various carbon and energy sources, including CO (20%)–CO_2 (5%) or H_2 (20%)–CO_2 (5%). Growth was measured spectrophotometrically (600 nm) and compared to that of controls containing yeast extract only. Cultures for these analyses were grown at 30°C with rotary shaking.

Cultures not previously known to oxidize CO (*Bradyrhizobium* sp. strain CPP, *Burkholderia* sp. strain JS-150, *B. fungorum* LB400, *B. cepacia*, *B. multivorans*, *Cycloclasticus spirillensus*, *Lutibacterium anuloederans*, *M. loti*, *Mycobacterium avium*, *M. smegmatis*, *M. tuberculosis* H37Ra, *S. fredii*, *S. lacuscaerulensis*, *S. pomeroyi*, *Stappia aggregata*, *S. stellulata*, and *V. fischeri*) were grown to stationary

TABLE 1. Accession numbers for 16S rDNA and *coxL* gene sequences obtained from isolates analyzed in this study[a]

Isolate	16S accession no.	BMS *coxL* accession no.	OMP *coxL* accession no.
Aminobacter sp. strain COX	ND[b]	AY307908	—[c]
Bradyrhizobium japonicum USDA 6	ND	AY307910	AY307921
Bradyrhizobium sp. strain CPP	ND	AY307900	AY307913
Burholderia fungorum LB400	ND	AY307901	AY307916
Burkholderia sp. strain LUP	AY307923	AY307907	—
Mesorhizobium sp. strain NMB1	AY307926	AY307906	—
Mycobacterium smegmatis	ND	—	AY307918
Stappia sp. strain M4	AY307928	AY307902	AY307916
Stappia sp. strain M8	AY307927	AY307903	AY307917
Stappia sp. strain CV812-530	ND	AY307898	AY307193
Stappia sp. strain CV902-700	ND	AY307899	AY307914
Stappia aggregata	ND	AY307904	AY307918
Stappia stellulata	ND	AY307905	AY307919
Stenotrophomonas sp. strain LUP	AY307922	AY307920	—
Xanthobacter sp. strain COX	AY307925	AY307911	—

[a] See text for methods used.
[b] ND, not determined.
[c] Dashes indicate that no *coxL* product was detected by PCR analysis.

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* H37Ra). 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) (19, 39) 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′-GGCGGCTTT[C/T]GG[C/G]AA[C/G]AAGGT-3′) and BMSf (5′-GGCGGCTTT[C/T]GG[C/G]TC[C/G]AAGAT-3′), and a reverse primer, O/Br (5′-[C/T]TCGA[T/C]GATCATCGG[A/G]TTGA-3′). Both forward primers targeted GGFGS(N)K, while the reverse primer targeted INPMIV; these motifs contribute to molybdopterin cytosine dinucleotide (MCD) binding (19, 39). The BMSf-plus-O/Br primer pair was used to amplify putative *coxL* fragments.

PCRs typically used 50-μl volumes in 200-μl tubes with standard final concentrations for buffer, deoxynucleoside triphosphates, and primers (0.1 μM each); magnesium ion was present at a final concentration of 1.5 mM. DNA extracts from cultures were used at final concentrations of approximately 1 to 10 ng, with separate reactions for each of the two forward primers. Positive controls included DNA extracts from known CO oxidizers (e.g., *O. carboxidovorans* and *B. japonicum*) (28); negative controls included extracts from organisms for which no CO oxidation has been observed (*Burkholderia* sp. strain JS-150, *B. cepacia*, *B. multivorans*, *M. avium*, and two recently described marine species, *C. spirillensus* and *L. anuloedorans* (5). Templates were denatured at 94°C in an Eppendorf Mastercycler thermocycler (Brinkmann Inc.) for 3 min prior to the addition of 0.5 U of Master*Taq* DNA polymerase (Brinkmann Inc.) at 80°C. 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 with an 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 CV812-530, 13 ± 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. lacuscaerulensis*, or three marine isolates, *C. spirillensus*, *L. anuloederans*, and *V. fischeri*.

Isolate characterization. CO-oxidizing bacteria were obtained from terrestrial sources (peat and plant roots) and marine macroalgal 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 SH 15003 for *Mesorhizobium* sp. strain NMB1 (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 maltophila* 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).

TABLE 2. Selected characteristics of root and macroalgal isolates

Characteristic	Result for isolate:					
	Burkholderia sp. strain LUP	*Mesorhizobium* sp. strain NMB1	*Stappia* sp. strain M4	*Stappia* sp. strain M8	*Stenotrophomonas* sp. strain LUP	*Xanthobacter* sp. strain COX
Motility	+	+	+	+	−	−
Oxidase reaction	+	+	+	+	−	+
Nitrate reduction	−	+	+	+	−	+
Denitrification	−	−	+	+	−	−
CO uptake rate[a]	34 ± 1	25 ± 3	10 ± 1	6 ± 1	101 ± 14	17 ± 0.3
Hydrogen oxidation	+	−	+	+	−	+
Growth substrates[b]						
Sugars	5/9	7/8	9/9	9/9	2/9	0/8
Amino acids	7/8	4/8	4/8	5/8	2/8	1/4
Organic acids	15/15	6/15	13/15	11/15	6/15	10/12
Methanol	−	−	−	−	+	+
Sodium required	−	−	+	+	−	−

[a] CO uptake rates are means ± 1 standard error for triplicate determinations.
[b] Numbers for growth substrates represent positive substrate responses out of total examined.

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% H_2[5% CO_2] (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*, *B. multivorans*, *M. avium*, *C. spirillensus*, and *L. anuloederans*).

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, AYRCSFR (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. loti* and *S. meliloti*). 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-
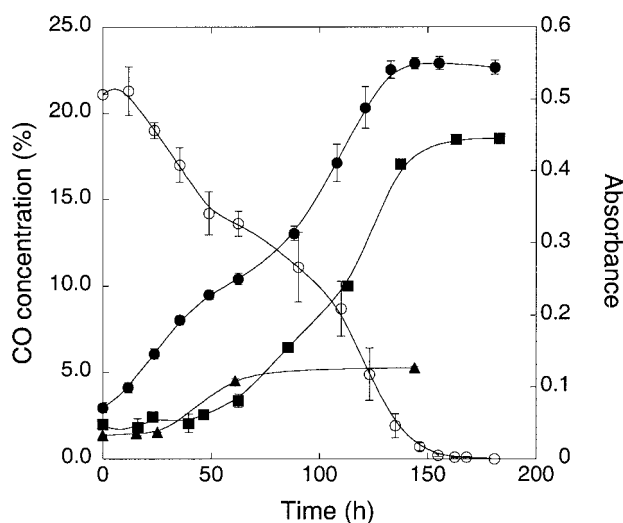

FIG. 2. Growth of *Xanthobacter* sp. strain COX on mineral salts with 0.05% yeast extract–20% CO (●), 20% H2–5% CO2 (■), or 0.05% yeast extract only (▲) over the time course (in hours) of the experiment. Values represent means of triplicate determinations ± 1 standard error.
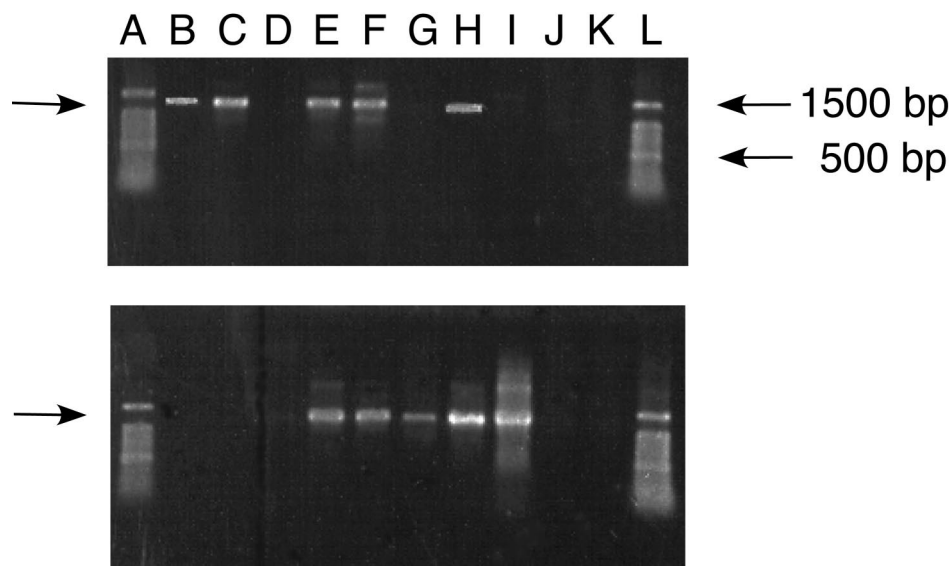
FIG. 3. PCR products from amplification with OMPf (upper panel) and BMSf (lower panel) primers of genomic extracts of CO-oxidizing cultures. Products were visualized with ethidium bromide after gel electrophoresis using 1% agarose. Lanes A and L contain a 100-bp ladder (Promega, Inc.); 500- and 1,500-bp markers are indicated by arrows on the right side of the upper panel. Lane B, *Oligotropha carboxidotropha*; lane C, *M. smegmatis*; lane D, *M. avium*; lane E, *B. fungorum* LB400; lane F, *Stappia stellulata*; lane G, *Mesorhizobium* sp. strain NMB1; lane H, *Bradyrhizobium* sp. strain CPP; lane I, *Aminobacter* sp. strain COX; lane J, *L. anuloederans*; lane K, negative control.

quences 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 a high (>97%) degree of similarity 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 carboxydotrophic.

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 observations and documentation of CO oxidation by *M. smegmatis* and *M. tuberculosis* H37Ra (26, 33), for which genome sequences first suggested the possibility of CO oxidation (see, e.g., reference 41), indicate that a number of additional taxa containing putative CO dehydrogenases might

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 CO 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 carboxydotrophs 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 carboxydotrophic and function lithotrophically, mixotrophically, or heterotrophically. CO utilization appears more variable in the genus *Silicibacter*,
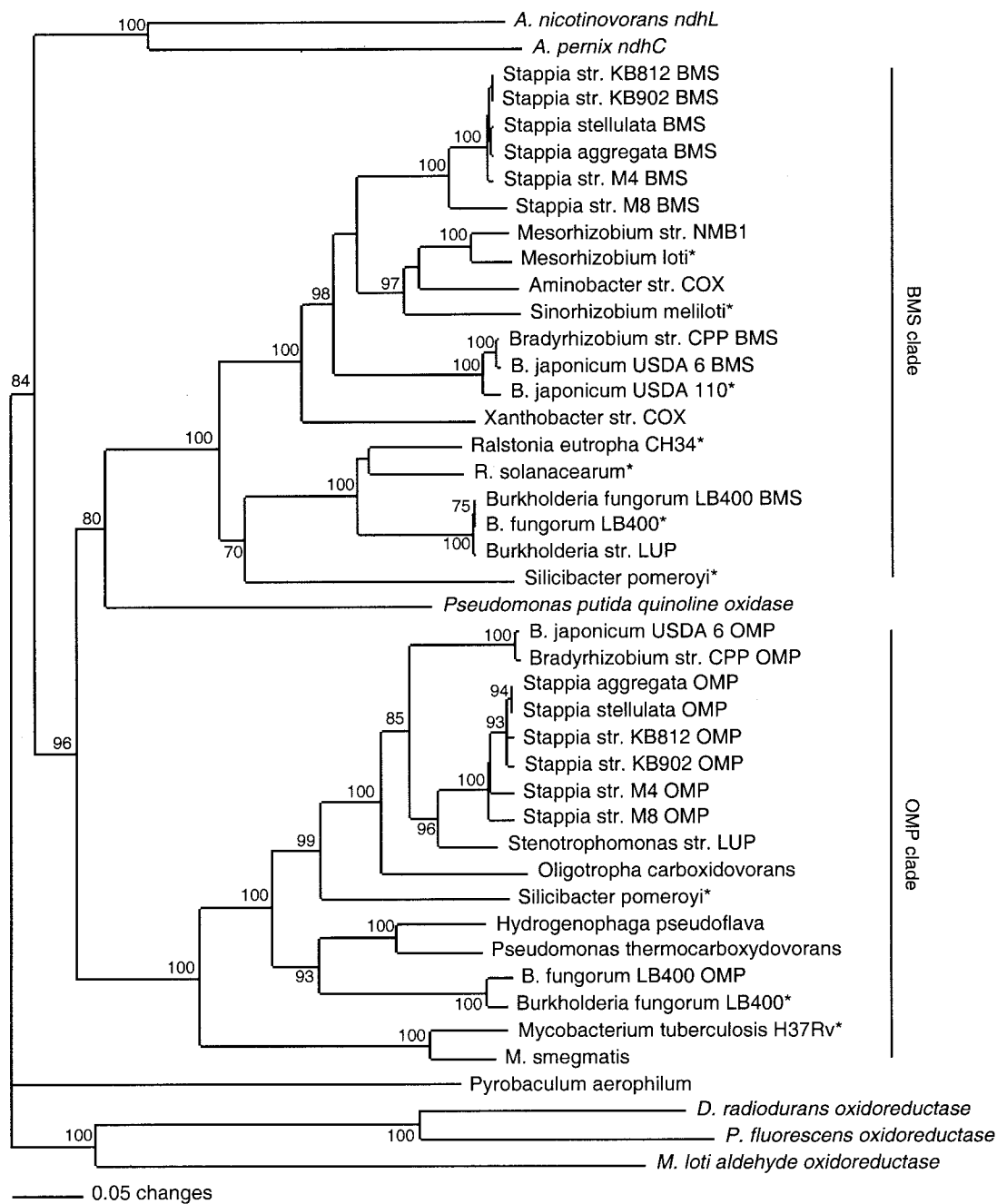
FIG. 4. Neighbor-joining analysis (1,000 bootstrap replicates) of partial inferred amino acid sequences for authentic and putative *coxL* and inferred amino acid sequences of selected molybdenum hydroxylases (indicated in italic characters in the figure): *Aeropyrum pernix* nicotine dehydrogenase, chain C, accession number NP148464; *Arthrobacter nicotinovorans* nicotine dehydrogenase, large subunit, AAK6423; *Deinococcus radiodurans* oxidoreductase, 285554; *M. loti* aldehyde oxidoreductase, NP105651; and *Pseudomonas putida* quinoline 2-oxidoreductase, CAA66830. Sequences derived from genome databases are indicated with asterisks. Bootstrap values < 70% are not shown. A *P. aerophilum* putative *coxL* sequence was used as an outgroup.

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 CO_2 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. spirillensus*, *L. anuloederans*, 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
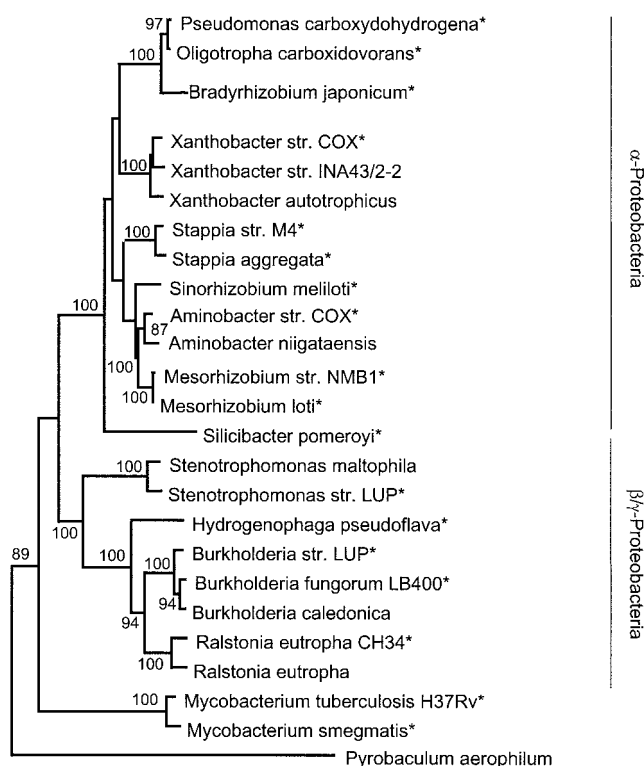
FIG. 5. Neighbor-joining analysis (1,000 bootstrap replicates, Jukes-Cantor correction) of 1,100 bp of an aligned 16S rDNA sequence for selected CO-oxidizing isolates (indicated by asterisks) and related taxa. Accession numbers for taxa not listed in Table 1: *Aminobacter niigataensis*, AJ011761; *B. japonicum* USDA 110, AF363150; *B. caledonica*, AF215704; *B. fungorum* LB400, U86373; *Hydrogenophaga pseudoflava*, AF078770; *M. loti*, AY278875; *M. smegmatis*, AJ131761; *M. tuberculosis*, AJ536031; *Oligotropha carboxidotropha*, AB099660; *R. eutropha* CH34, Y10824; *R. eutropha*, M32021; *S. pomeroyi*, AF098491; *S. aggregata*, D88520; *Xanthobacter autotrophicus*, X94201; and *Xanthobacter* sp. strain INA43/2-2, AJ306541. Bootstrap values < 50% are not shown. An *P. aerophilum* sequence was used as an outgroup.

lithotrophy or methylotrophy, traits expressed by *Xanthobacter* sp. strain COX (Table 2). CO oxidation by *Burkholderia* was also previously unknown, but results for *Burkholderia* sp. strain LUP and *B. fungorum* LB400 clearly expand the physiological diversity of the genus. Isolation from Hawaiian volcanic deposits of a CO-oxidizing strain similar to *Burkholderia caledonica* (King, unpublished) suggests that the trait may be relatively common within the *Burkholderia graminis* clade defined by Coeyne et al. (6). Nonetheless, the negative results for *B. cepacia*, *B. multivorans*, and *Burkholderia* sp. strain JS-150 obtained in this study indicate that the capacity for CO oxidation differs considerably within the genus.

Physiological and molecular data indicate that members of the genus *Stenotrophomonas* oxidize CO. This represents only the second documentation of CO oxidation by a γ-proteobacterium. Thus far, α-*Proteobacteria* dominate the known carboxydotrophs. This may reflect the limited nature of surveys to date or more fundamental aspects of the evolution and ecology of CO oxidation (30, 31). A comparable situation exists for symbiotic nitrogen fixation, which is limited almost exclusively

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. pseudoflava*) 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, AYRGAGR, 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. meliloti*, 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 *coxL* groups with β-proteobacterial *coxL* sequences and its 16S rRNA groups with sequences of α-*Proteobacteria*. Second, OMP *coxL* 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 *coxL* 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., *Pyrobaculum* and *Sulfolobus*), 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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