Antonie van Leeuwenhoek (2012) 101:685–700
DOI 10.1007/s10482-012-9698-y

REVIEW PAPER

Microbiology and genetics of CO utilization in mycobacteria

Young Min Kim · Sae Woong Park

Received: 20 September 2011 / Accepted: 6 January 2012 / Published online: 26 January 2012

Abstract Although extensive studies on the oxidation of carbon monoxide (CO) in aerobic carboxydotrophic bacteria have been carried out for over 30 years, utilization of CO as a source of carbon and energy by mycobacteria was recognized only recently. Studies on pathogenic and nonpathogenic mycobacteria have revealed that the basis for CO utilization in these bacteria is different in many aspects from that of other aerobic carboxydobacteria. We review the basis for CO utilization in mycobacterial carboxydobacteria, which is unique from physiological, biochemical, molecular, genetic and phylogenetic points of view.

Keywords Carboxydotroph · Carboxydobacteria · Carbon monoxide · CO dehydrogenase · NO dehydrogenase · Mycobacteria · *Mycobacterium* sp. strain JC1

Y. M. Kim (✉) · S. W. Park
Molecular Microbiology Laboratory, Department of Systems Biology, Yonsei University, Seoul 120-749, Korea
e-mail: young547@yonsei.ac.kr

Present Address:
S. W. Park
Department of Microbiology and Immunology, Weill Cornell Medical College, New York, NY 10065, USA

Introduction

It has been established that a wide range of aerobic and anaerobic groups of bacteria are capable of growth with carbon monoxide (CO) as a sole source of carbon and energy (Kim and Hegeman 1983a; Meyer 1989; Oelgeschläger and Rother 2008). The term carboxydotrophic bacteria (carboxydobacteria) has been conventionally used for the aerobic CO-utilizing bacteria (Kim and Hegeman 1983a; Meyer 1989). However, it is reasonable to apply the term also for the anaerobic CO-utilizing bacteria, as suggested by Oelgeschläger and Rother (2008), because the anaerobic bacteria also grow on CO as sole carbon and energy sources.

Earlier studies on the aerobic carboxydobacteria have been focused mostly on Gram-negative bacteria. Therefore, the basis of CO utilization in aerobic carboxydobacteria has been elucidated mainly from studies on the Gram-negative carboxydobacteria. Recently, a large number of carboxydobacteria have been reported among Gram-positive actinomycetes (O'Donnell et al. 1993; Kim et al. 1998) in addition to the previously known species of Gram-positive genera (Meyer et al. 1986). This suggested that the carboxydotrophy may be widespread across Gram-positive bacteria. In fact, the full or partial genome sequences of several Gram-positive bacteria revealed that many of Gram-positive bacteria have a CO dehydrogenase (CO-DH) gene, a key enzyme for carboxydotrophic growth. One interesting thing for the Gram-positive carboxydobacteria is that many of mycobacteria have

a CO-DH gene and are able to grow aerobically with CO as a sole source of carbon and energy as well (King 2003; Park et al. 2003). Studies on the CO utilization in mycobacteria revealed that the basis for CO oxidation in this phylogenetic group is different in many aspects from the bases of other aerobic carboxydobacteria, including that of the more thoroughly studied Gram-negative carboxydobacteria.

Mycobacteria are a group of Actinobacteria that are rod-shaped, somewhat pleomorphic and exhibit acid-fastness. They are not readily stained by the Gram reaction due to the presence of high lipid content in the surface layer. The bacteria were first discovered by Robert Koch in 1882 and were found subsequently in a variety of environments, such as soils, waters, and various animals including humans. Some of them are pathogens known to cause serious diseases in mammals, such as tuberculosis and leprosy. Mycobacteria are aerobic and contain slow-growing and fast-growing representatives that utilize a diverse group of substrates under laboratory conditions. In some cases, some mycobacteria are also known to be obligate parasites.

Growth of mycobacteria on carbon monoxide

The possibility that mycobacteria grow aerobically on CO as a sole source of carbon and energy originated with an observation by Bartholomew and Alexander (1979). They reported that *Mycobacterium phlei* could oxidize CO to CO_2, but little of the CO was converted to cellular carbon. They suggested that the conversion of CO to CO_2 in *M. phlei* was not the result of autotrophic metabolism but of the cometabolic oxidation of CO.

The ability of mycobacteria to grow on CO as sole carbon and energy sources was first documented by Cho et al. (1985). They isolated a bacterium that grew CO-chemolithoautotrophically in a gas mixture of 30% CO–70% air in mineral medium and named it *Acinetobacter* sp. strain JC1. This organism was identified subsequently as a mycobacterium and renamed as *Mycobacterium* sp. strain JC1 (Song et al. 2002). The optimal conditions for autotrophic growth on CO of this isolate were determined to be a 30% CO mixing ratio in air, with incubation carried out at 30°C (t_d = 19 h; Cho et al. 1985). CO is also inhibitory to the Gram-positive *Mycobacterium* sp. strain JC1 as it does for Gram-negative carboxydobacteria (Kim and Hegeman 1981a, 1983b; Zavarzin and Nozhevnikova 1977), since the growth rate does not change significantly under 50% CO (t_d = 24 h) but decreases under a 70% CO mixing ratio (t_d = 74 h). The bacterium is able to grow with 0.01% CO (100 ppm; t_d = 180 h), but not in laboratory air (Cho et al. 1985). *Mycobacterium* sp. strain JC1 also grows mixotrophically under CO-containing heterotrophic conditions and neither CO nor organic substrate inhibits the utilization of the other source as a growth substrate (Kim and Kim 1989). CO-DH activity is present in cells grown under CO-containing heterotrophic conditions with glucose, succinate, fructose, and acetate, as in *Pseudomonas carboxydoflava* (currently *Hydrogenophaga pseudoflava* (Willems et al. 1989); Kiessling and Meyer 1982).

Subsequent studies demonstrated that several fast- and slow-growers of mycobacteria also were found to grow with a gas mixture of 30% CO–70% air in either mineral medium or Middlebrook 7H9 medium (Park et al. 2003). Examples include *M. smegmatis* MC2, *Mycobacterium gastri*, *Mycobacterium neoaurum*, *Mycobacterium flavescens*, *Mycobacterium parafortuitum*, *Mycobacterium peregrinum*, *M. phlei*, *Mycobacterium tuberculosis* H37Ra, and *Mycobacterium vaccae*. The ability of *M. phlei* to grow on CO was contradictory to the report of Bartholomew and Alexander (1979), possibly due to the difference in the concentration of CO provided in the two experiments; microorganisms in culture and natural waters may be unable to metabolize certain compounds at low concentrations, although able to do so at high levels (Boethling and Alexander, 1979). All the mycobacteria, except for *Mycobacterium* sp. strain JC1, exhibited a lag period of between 9 and 30 days when first subjected to growth with CO. The 7H9 medium, which is usually used as a basal medium for mycobacteria, is preferred as a mineral medium for CO-autotrophic growth of the mycobacteria, since the lag period and doubling time of each bacterium in 7H9 with CO (Table 1) are both a third of those times in mineral medium with CO. *M. flavescens* grows most rapidly (t_d = 3.7 h), while *M. tuberculosis* H37Ra grows most slowly (t_d = 24 h), in 7H9 medium with CO.

Growth of *M. smegmatis* on CO was also observed by King (2003), with 20% CO in 7H9 medium supplemented with oleic acid-albumin-dextrose-catalase (7H9-OADC). He also revealed a previously

Table 1 Mycobacteria reported to grow with or oxidize/uptake CO aerobically

Growth or oxidation	Species	Media	Incubation Temperature (°C)	CO concentration (%)	Doubling time (h)	References
	Mycobacterium sp. JC1	SMB	30	0.01%	180	Cho et al. (1985)
		SMB	30	1%	40	Cho et al. (1985)
		7H9	37	30%	6.4	Park et al. (2003)
	M. flavescens	7H9		30%	3.7	Park et al. (2003)
	M. gastri	7H9		30%	6.5	Park et al. (2003)
	M. neoaurum	7H9		30%	7.9	Park et al. (2003)
	M. parafortuitum	7H9		30%	4.4	Park et al. (2003)
Growth[a]	*M. peregrinum*	7H9		30%	6.0	Park et al. (2003)
	M. phlei	7H9		30%	7.6	Park et al. (2003)
	M. smegmatis mc^2	7H9		30%	6.2	Park et al. (2003)
		7H9-OADC	30	20%	~70.0[b]	King (2003)
	M. tuberculosis H37Ra	7H9	37	30%	23.9	Park et al. (2003)
	M. vaccae	7H9		30%	5.8	Park et al. (2003)
	M. bovis BCG	7H9-OADC	37	<50 ppm	NA	King (2003)
Oxidation[c]	*M. gordonae*	7H9-OADC	37	<50 ppm	NA	King (2003)
	M. marinum	7H9-OADC	30	<50 ppm	NA	King (2003)

NA not applicable

[a] Cells were cultivated under a gas mixture of appropriate concentration of CO in air

[b] The time is estimated by authors from a growth curve in King (2003)

[c] Cells were incubated aerobically in the presence of CO

unreported capacity for *Mycobacterium bovis* BCG, *Mycobacterium marinum*, and *Mycobacterium gordonae*, in addition to *M. tuberculosis* H37Ra and *M. smegmatis*, to oxidize (although not to grow on) <50 ppm CO in 7H9-OADC (Table 1). Because *M. phlei* oxidizes CO at 50 ppm concentration in addition to 30% CO, and that *M. tuberculosis* H37Ra and *M. smegmatis* grow on high (20–30%) and low (<50 ppm) CO, it is conceivable that *M. bovis* BCG, *M. marinum*, and *M. gordonae* may also grow on CO at higher concentrations. *M. smegmatis* and *M. gordonae* also oxidize CO under either suboxic (10 and 1% atmospheric oxygen) or anoxic conditions in the presence of nitrate, but not under anoxic conditions without nitrate. This indicates that oxidation of CO in these two mycobacteria, and possibly in all mycobacteria, is obligately linked to respiration. Among the mycobacteria tested, *M. avium* did not oxidize CO, as expected from the absence of CO-DH genes in its genome (GenBank accession no. CP000479). Finally, *Mycobacterium microti* does not have all the CO-DH genes and it does not oxidize CO.

All observations reported to date indicate that the ability to grow on CO as carbon and energy source is widely distributed among cultured mycobacteria, except for a couple of species, and suggest that this metabolic capacity may be an intrinsic property of this group of bacteria.

Physiology and biochemistry of CO utilization in mycobacteria

As described in the previous section and highlighted below, most mycobacteria studied to date have been identified to be carboxydobacteria that are able to grow aerobically with CO as a sole source of carbon and energy using CO-DH and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), with few exceptions, as key enzymes for the oxidation and assimilation of CO, respectively. However, the basis of CO utilization in mycobacterial carboxydobacteria has not been studied extensively and has only been elucidated in *Mycobacterium* sp. strain JC1.

Mechanism of CO oxidation

The presence of CO-DH (Cho and Kim 1986; Park et al. 2003) and RuBisCO (Kim et al. 1997; Park et al. 2003) in CO-autotrophically grown cells, and oxidation of CO coupled with the reduction of artificial electron acceptors in an anaerobic CO-DH assay system (Kim et al. 1997; Park et al. 2003) indicate that *Mycobacterium* sp. strain JC1 and other mycobacteria that grow with CO oxidize CO to CO_2. Further, like the Gram-negative carboxydobacteria (Cypionka et al. 1980), this process uses H_2O as the source of the second oxygen atom according to the following equation:

$$CO + H_2O \rightarrow CO_2 + 2H^+ + 2e^-.$$

With few exceptions, CO-DHs in several Gram-negative and non-mycobacterial Gram-positive carboxydobacteria share similar biochemical, molecular, and immunological properties (Bell et al. 1985; Cho and Kim 1986; Kim and Hegeman 1983a; Kim and Lee 1986; Meyer et al. 1986). The CO-DH enzyme in *Mycobacterium* sp. strain JC1 also shares many properties with those of Gram-negative carboxydobacteria. Nonetheless, the *Mycobacterium* sp. strain JC1 CO-DH also has several distinguishing properties from those of the Gram-negative CO-DHs (Table 2).

Mycobacterial CO dehydrogenase

CO-DHs of Gram-negative carboxydobacteria are inducible enzymes (Kim and Hegeman 1983a) except for that of *H. pseudoflava*, which is expressed constitutively in the absence of CO (Kiessling and Meyer 1982). The CO-DH in *Mycobacterium* sp. strain JC1 is also a constitutive enzyme (Ro and Kim 1993), as reported for other mycobacteria (Park et al. 2003). However, the enzyme is not present in cells grown on pyruvate and nutrient broth (Ro and Kim 1993). Pyruvate and nutrient broth themselves or certain metabolites of these substrates have been suspected to repress CO-DH gene expression in *Mycobacterium* sp. strain JC1. Ro and Kim (1993) speculated on the presence of CO-DH inhibitors or certain molecules that inhibit CO-DH assay systems in cells of *Mycobactetrium* sp. strain JC1 grown on most organic substrates, except ethanol and succinate, as described in *Pseudomonas carboxydohydrogena* (Do et al. 1990). The presence of such inhibitors also has been implied in several other mycobacteria grown in 7H9 medium with CO (Park et al. 2003).

CO-DH in *Oligotropha carboxidovorans* [formerly *Pseudomonas carboxydovorans* (Meyer et al. 1993)], a Gram-negative carboxydobacteria, is an enzyme bound loosely to the inner face of the cytoplasmic membrane (Meyer et al. 1986). The CO-DH of *Mycobacterium* sp. strain JC1 also has been identified to bind loosely to the identical face of the membrane (Kim and Kim 1986). This conclusion is based on the amount of distribution of CO-oxidizing activity in soluble and particulate fractions obtained after disruption by sonic oscillation of CO-grown cells and by osmotic shock of spheroplasts prepared from the cells (Kim and Kim 1986). The presence of particulate hydrogenase activity in the purified CO-DH also supported this observation (Kim et al. 1989).

CO-DHs from *Pseudomonas carboxydohydrogena* (Kim and Hegeman 1981a), a Gram-negative carboxydobacterium, and *O. carboxidovorans* (Meyer and Schlegel 1980) are quite stable under air. The CO-DH in *Mycobacterium* sp. strain JC1 however is unstable outside of cells (Cho and Kim 1986; Kim et al. 1989). As a result, the enzyme has been purified in the presence of stabilizing agents such as 1 mM iodoacetamide and 100 mM ammonium sulfate (Kim et al. 1989). Stabilization of the enzyme in the presence of iodoacetamide indicates that a sulfhydryl group of the enzyme is not involved in CO oxidation.

CO-DHs purified from *P. carboxydohydrogena* and *O. carboxidovorans* possesses particulate hydrogenase-like activity (2–8.5% of the CO-oxidizing activity) in addition to the CO-DH activity but no NAD-specific hydrogenase and formate dehydrogenase activities (Kim and Hegeman 1981a, Meyer and Schlegel 1980). The purified CO-DH of *Mycobacterium* sp. strain JC1 also exhibits particulate hydrogenase-like activities amounting to 10% of the CO-oxidizing activity, but no NAD-dependent hydrogenase and formate dehydrogenase activities (Kim et al. 1989). The presence of hydrogenase activity in CO-DH implies that *Mycobacterium* sp. strain JC1 is able to grow as a hydrogen bacterium, like most Gram-negative carboxydobacteria (Kim and Hegeman 1983a; Meyer and Rohde 1984; Zavarzin and Nozhevnikova 1977). This implication was suggested originally by Ro and Kim (1993).

The molecular weight of the native CO-DHs of Gram-negative carboxydobacteria are between 230,000 and 400,000 (Bell et al. 1985; Kim and

Table 2 Some properties of CO-DHs in *Mycobacterium* sp. strain JC1 and Gram-negative carboxydobacteria

Organism	Expression	Localization	Stability under air	Hydrogenase activity	MW (kDa)	Subunit structure
Gram-negative carboxydobacteria	Inducible by CO[a]	Inner face of CM[b]	Stable[c]	Particulate hydrogenase[c]	230–400[d]	$\alpha_2\beta_2\gamma_2^e$
Mycobacterium sp. strain JC1	Constitutive[i]	Inner face of CM[j]	Unstable[k]	Particulate hydrogenase[l]	300–380[l]	$\alpha_2\beta_2\gamma_2^l$

Organism	MW of subunits (kDa)	Cofactor composition	*K*m (μM)	pI[b]	Crystal structure	NO-DH activity
Gram-negative carboxydobacteria	α:β:γ = 14–17:25–33:70–85[e]	Mo:FAD: iron:labile sulfide (2:2:8:8)[f]	0.6–63[d]	4.5–4.6[g]	Butterfly-shape[h]	ND
Mycobacterium sp. strain JC1	α:β:γ = 16:34:85[l]	Mo:FAD: iron:labile sulfide (2:2:8:8)[l]	150[l]	5.8[l]	ND	Present[m]

MW molecular weight, *pI* Isoelectric point, *ND* not determined, *CM* cytoplasmic membrane

[a] Kim and Hegeman, 1983a; *P. hydrogenophaga* CO-DH is a constitutive enzyme (Kiessling and Meyer 1982)

[b] Meyer et al. 1986 and Kim and Kim 1986

[c] Kim and Hegeman 1981a and Meyer and Schlegel 1980

[d] Bell et al. 1985, Kim and Hegeman 1981a, and Meyer et al. 1986

[e] Kim and Hegeman 1981a and Meyer et al. 1986

[f] Kang and Kim 1999, Kim and Hegeman 1981a and Meyer 1982

[g] Kim and Hegeman 1981a and Meyer 1989

[h] Dobbek et al. 1999 and Hänzelmann et al. 2000

[i] Ro and Kim 1993

[j] Kim and Kim 1986

[k] Cho and Kim 1986 and Kim et al. 1989

[l] Kim et al. 1989

[m] Park et al. 2007

Hegeman 1981a; Meyer et al. 1986). The enzymes consist of three non-identical subunits of molecular weights 14,000–17,000 (α), 25,000–33,000 (β), and 70,000–85,000 (γ) in an $\alpha_2\beta_2\gamma_2$ structure (Kim and Hegeman 1981a; Meyer et al. 1986). The molecular weight (300,000–380,000) and subunit size [16,000 (α), 34,000 (β), and 85,000 (γ)] and composition ($\alpha_2\beta_2\gamma_2$) of *Mycobacterium* sp. strain JC1 (Kim et al. 1989) are identical or similar to those of the Gram-negative aerobic CO-DHs.

CO-DHs of Gram-negative carboxydobacteria are molybdenum-containing iron-sulfur flavoprotein (Kang and Kim 1999; Kim and Hegeman 1981a; Meyer 1982). The enzymes contain 2, 2, 8, and 8 mol of molybdenum, FAD, iron, and labile sulfide/mol of enzyme. Molybdopterin, FAD, and iron-sulfur centers are present in γ, β, and α subunits, respectively (Kang and Kim 1999; Dobbek et al. 1999; Hänzelmann et al. 2000). Like the Gram-negative CO-DHs, the CO-DH in *Mycobacterium* sp. strain JC1 also is a molybdo iron-sulfur flavoprotein (Kim et al. 1989). Localization of cofactors has not been performed for *Mycobacterium* sp. strain JC1 CO-DH. The cofactors of CO-DH in this bacterium may reside on the same subunits as in the Gram-negative CO-DHs since the mycobacterial and Gram-negative CO-DHs are identical in subunit and cofactor compositions. Methanol is an inhibitor of molybdoenzyme. CO-DH from *O. carboxidovorans* is completely inactivated in the presence of 125 mM methanol (Meyer 1982). Methanol at 75–100 mM completely inactivates *Mycobacterium* sp. strain JC1 CO-DH, which further supports that *Mycobacterium* sp. strain JC1 CO-DH is a molybdoenzyme.

The K_m for CO of the purified CO-DHs of Gram-negative carboxydobacteria is 0.6–63 μM (Bell et al. 1985; Kim and Hegeman 1981a; Meyer et al. 1986). The value for *Mycobacterium* sp. strain JC1 CO-DH is 150 μM (Kim et al. 1989), which is considerably higher than those for the Gram-negative aerobic CO-DHs. The high K_m of CO-DH raises an important question: can *Mycobacterium* sp. strain JC1 use atmospheric CO for growth? The concentration of CO in the atmosphere has been estimated to be less than 43.3 nmol/l [1.0 ppm (Khalil and Rasmussen 1984; Robbins et al. 1968; Seiler et al. 1984)].

The isoelectric points of the purified CO-DHs of *P. carboxydohydrogena* and *O. carboxidovorans* are 4.6 and 4.5, respectively (Kim and Hegeman 1981a; Meyer 1989). The isoelectric point of *Mycobacterium* sp. strain JC1 CO-DH is 5.8 (Kim et al. 1989), indicating that the amino acid sequence of CO-DH in *Mycobacterium* sp. strain JC1 is quite different from that of the Gram-negative aerobic carboxydobacteria.

CO-DHs in Gram-negative carboxydobacteria share common antigenic groups (Cho and Kim 1986; Park et al. 2003). The purified CO-DH of *Mycobacterium* sp. strain JC1, like that of *Bacillus schlegelii* (Krüger and Meyer 1984), has no antigenic groups in common with those of the Gram-negative CO-DHs. The enzyme, on the other hand, shares identical antigenic epitopes with those of other mycobacteria.

Analysis of crystal structure of CO-DHs from *O. carboxidovorans* and *H. pseudoflava* has been revealed that the two enzymes are in butterfly-shape with identical subunit arrangement (Dobbek et al. 1999; Hänzelmann et al. 2000). But crystal structure of CO-DH in *Mycobacterium* sp. strain JC1 as well as that of other mycobacterial CO-DHs has not been resolved yet. The structure of *Mycobacterium* sp. strain JC1 CO-DH may be similar to that of the Gram-negative CO-DHs since the subunit and cofactor compositions of *Mycobacterium* sp. strain JC1 CO-DH are identical to those of the Gram-negative CO-DHs.

Nitric oxide dehydrogenase activity in CO dehydrogenase

In addition to the CO-DH activity, mycobacterial CO-DH possesses a novel NO dehydrogenase (NO-DH) activity, which has not been reported for CO-DHs in other carboxydobacteria. The presence of putative CO-DH genes in several pathogenic mycobacteria [GenBank accession nos. AL123456 (*M. tuberculosis*), BX248333 (*M. bovis*), CP000854 (*M. marinum*), and CP000325 (*Mycobacterium ulcerans*)], the ability of *M. tuberculosis* to grow with CO, and the presence of CO-DH in the mycobacteria that grow on CO (Park et al. 2003), together with the structural similarity of CO and nitric oxide (NO) raised the hypothesis that CO-DH may assist pathogenic mycobacteria to evade host immune systems by acting as a NO dehydrogenase (NO-DH), a novel enzyme that had never been reported.

This hypothesis that the CO-DH enzyme of pathogenic mycobacteria acted as a NO-DH was recently confirmed. Park et al. (2007) demonstrated that CO-DHs in mycobacteria, including *M. tuberculosis*, possess NO-DH activity. Purified CO-DH from *Mycobacterium* sp. strain JC1 exhibits NO-DH activity,

which amounts to 23.4% of the reported CO-DH activity (Park et al. 2007). This indicates that NO is not the optimal substrate for mycobacterial CO-DH, but instead serves as an alternative substrate of the enzyme.

It is notable that sodium nitroprusside (SNP), which is known to produce NO, also induces the expression of CO-DH in cells of *Mycobacterium* sp. strain JC1 growing with either CO or glucose (Park et al. 2007). The CO-DH and NO-DH activities in cells of *Mycobacterium* sp. strain JC1 grown with CO in the presence of SNP increase with a concomitant increase in the amount of the CO-DH protein, which further supports the association of NO-DH activity with the CO-DH protein. This also supports the possibility that the NO-DH activity in the CO-DH plays a role in detoxification of NO, since all genes involved in the detoxification of reactive nitrogen species (RNS) in *M. tuberculosis* are inducible in the presence of RNS (Ehrt et al. 1997; Hu et al. 1999; Ruan et al. 1999). The presence of different regulatory systems for the induction of CO-DH, which are specific for CO and NO, has been implicated for *Mycobacterium* sp. strain JC1. This is because the expression of CO-DH is increased in the presence of SNP for cells growing with CO in which the synthesis of CO-DH was already induced (Park et al. 2007).

The purified CO-DH from *Mycobacterium* sp. strain JC1 protects *Escherichia coli* from SNP-induced killing in a dose–dependent manner (Park et al. 2007). This indicates that the NO-DH activity of CO-DH actually functions on NO, further supporting the notion that the NO-DH activity of CO-DH in mycobacteria plays a role in the detoxification of NO in pathogenic mycobacteria residing in macrophages. Park et al. (2007) claimed that the NO-DH activity of CO-DH in mycobacteria represents the third distinct group of enzymes involved in detoxifying RNS in addition to NO dioxygenases and peroxynitritases.

Carbon monoxide as carbon and energy source

Carbon assimilation

Autotrophic growth of mycobacteria was first reported in *Mycobacterium album* growing in the presence of H_2 and CO_2, but the enzymes involved in CO_2 fixation were not reported (Beijajewa 1954). Several mycobacteria, including *Mycobacterium fortuitum*, *M. marinum*, and *M. smegmatis*, have also been reported to grow autotrophically with H_2 (Dworkin and Foster 1958; Lukins and Foster 1963). The key enzyme for CO_2 fixation in mycobacteria grown with H_2 and CO_2 was identified later as a RuBisCO in *M. gordonae* and for two other unclassified mycobacteria (Park and DzCicco 1976).

Gram-negative carboxydobacteria operate the Calvin cycle during growth with CO (Meyer et al. 1986). The discovery of the RuBisCO enzyme involvement in CO_2 fixation for mycobacteria capable of growing chemolithoautotrophically with CO was first reported for *Mycobacterium* sp. strain JC1 (Kim et al. 1997), and later in *M. smegmatis* MC2, *M. gastri*, *M. neoaurum*, *M. flavescens*, *M. parafortuitum*, *M. peregrinum*, *M. phlei*, and *M. vaccae* (Park et al. 2003). These results indicate that CO is converted to cell biomass via the Calvin cycle in these mycobacterial carboxydobacteria. *M. tuberculosis* grown with CO, on the other hand, does not have RuBisCO (Park et al. 2003), which coincides with the absence of RuBisCO genes in this bacterium. It is possible for *M. tuberculosis* to use a reductive TCA cycle to fix CO_2, since the presence of genes for fumarate reductase, citrate lyase, and α-ketoglutarate:ferredoxin oxidoreductase (i.e. 2-oxoglutarate synthase) have been identified in this bacterium. *M. bovis* and *M. marinum*, which oxidize CO (King 2003), may also adopt the reductive TCA cycle to assimilate CO_2, if they grow on CO, since the bacteria also have been known to have genes encoding the key enzymes for the reductive TCA cycle, but not for those of the Calvin cycle. It is notable that *M. smegmatis* exhibits RuBisCO activity since the bacterium does not contain RuBisCO genes (GenBank accession no. CP000480). This activity may be a result of non-specific reaction of other unknown enzymes expressed in cells grown with CO.

The RuBisCO of *Mycobacterium* sp. strain JC1 is a typical form I RuBisCO consisting of two nonidentical subunits of molecular weights 53,500 and 15,000 (Kim et al. 1997). The K_m and V_{max} for CO_2 are 36.7 μM and 296.1 nmol/min/mg protein, respectively, and those of ribulose 1,5-bisphosphate were 3.7 μM and 770 nmol/min/mg protein, respectively. The enzyme does not have antigenic groups common with those of the RuBisCO enzymes of Gram-negative aerobic carboxydobacteria, but has identical antigenic sites to those of several other mycobacterial carboxydobacteria (Park et al. 2003). This suggests that the

ability to fix CO_2 using RuBisCO as a key enzyme has evolved independently in at least two different lines of descent.

Electron transport system

CO-DH in *Mycobacterium* sp. strain JC1 does not reduce coenzyme Q_{10} (UQ_{10}) with CO as a substrate (Kim and Cho 1986), indicating that UQ_{10} is not the physiological electron acceptor for oxidation of CO in this bacterium. The physiological electron acceptor may be similar to that of *O. carboxidovorans*, for which the redox potential is more positive than the UQ_{10}, because CO-DH of *P. carboxydohydrogena* uses UQ_{10} as an electron acceptor with CO as a substrate and does not reduce 2,6-dichlorophenol-indophenol (DCPIP; Kim and Hegeman 1981a, b). Also, the enzyme of *O. carboxidovorans* uses DCPIP, but not UQ_{10}, as an electron acceptor during oxidation of CO (Meyer 1982), similar to the *Mycobacterium* sp. strain JC1 enzyme (Kim and Cho 1986; Kim et al. 1989). *Mycobacterium* sp. strain JC1 grown with CO contains cytochromes of *a*, *b*, and *c* types and also a CO-insensitive *o* type cytochrome in the membrane fractions, indicating the presence of a branched respiratory chain in this bacterium, as the respiratory system in Gram-negative carboxydobacteria (Meyer et al. 1986). Among the cytochromes, cytochromes of *b* and *o* types, but not the *a* and *c* types, function during CO oxidation (Kim and Cho 1986). Therefore, the natural electron acceptor for CO oxidation in *Mycobacterium* sp. strain JC1 may be a quinone, other than UQ_{10}, or a *b*-type cytochrome. For unknown reasons, the *a* type cytochrome does not act as an oxidase (Cho et al. 1985). NAD(P) is not involved during oxidation of CO in *Mycobacterium* sp. strain JC1 (Kim and Cho 1986). The bacterium must generate reduced pyridine nucleotide through reverse electron transport, a process which is inefficient compared with the direct reduction of NAD(P). This explains the slow growth of the bacterium with CO as the sole source of carbon and energy (Cho et al. 1985).

Genetics of CO utilization

As in the case of the physiological and biochemical basis of CO utilization, the genetic background of CO oxidation and assimilation has been studied predominantly in *Mycobacterium* sp. strain JC1.

CO oxidation

Several pathogenic and nonpathogenic mycobacteria contain genes for putative CO-DH (see previous section) and other genes located upstream and downstream of the CO-DH genes (Fig. 1), which may be involved in the oxidation of CO and are similar in nucleotide sequences of several genes of Gram-negative aerobic carboxydobacteria, including CO-DH genes (Kang and Kim 1999; Fuhrmann et al. 2003; Pearson et al. 1994; Schübel et al. 1995).

Mycobacterium sp. strain JC1 contains two copies of CO-DH genes (*cutB1C1A1* and *cutB2C2A2*; *cutB*, *C*, and *A* denote genes for medium, small, and large subunits of CO-DH, respectively), with identical nucleotide sequences (Song et al. 2010). Both copies of the CO-DH genes are functional in the cell (Song et al. 2010). Several genes locating upstream and downstream of the CO-DH genes are also duplicated in *Mycobacterium* sp. strain JC1 and they also share deduced amino acid sequence similarity (but differ in organization) with homologues in *O. carboxidovorans* (Santiago et al. 1999; Fig. 1). All genes located upstream and downstream of CO-DH genes in *O. carboxidovorans* have been identified to be transcribed in the presence of CO (Santiago et al. 1999). The major difference in the organization of genes possibly involved in the oxidation of CO is the presence of *cutR* and *orf8* in *Mycobacterium* sp. strain JC1. The CutR enzyme is highly similar in amino acid sequence to the transcriptional regulators that belong to the LysR family. It also has a helix-turn-helix motif at the N-terminus and transcribed divergently from the two CO-DH genes, which is a general organization of the LysR-type regulators (Schell 1993). The function of Orf8 is unknown but is predicted to catalyze the cytidylation of the molybdenum cofactor after analysis of a hypothetical structure of the protein.

The CO-DH of *Mycobacterium* sp. strain JC1 is distinct in amino acid sequence from that of the Gram-negative aerobic CO-DHs; i.e. the amino acid sequences of large, medium, and small subunits of *Mycobacterium* sp. JC1 CO-DH are 55.7–57.8, 36.9–40.4, and 57.0–57.7% identical, respectively, to those of the Gram-negative CO-DHs (Kang and Kim

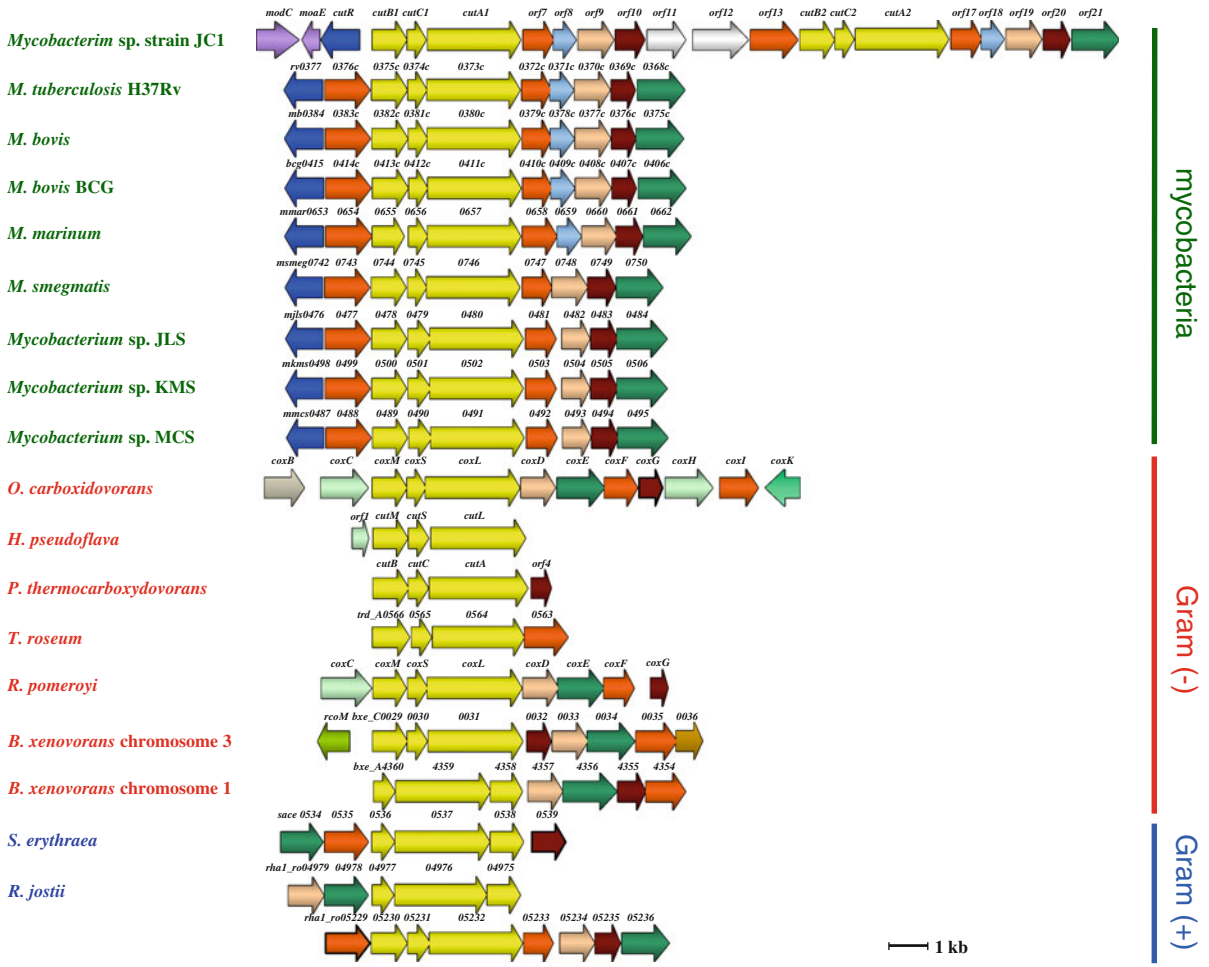

Fig. 1 Organization of CO-DH genes and conserved genes around CO-DH genes in several aerobic carboxydobacteria. *Mycobacterium* sp. strain JC1 and *R. jostii* have two copies of CO-DH genes, which are identical and different in the gene order, respectively. *Yellow arrows* represent the CO-DH structural genes. Same color refers genes similar in amino acid sequence. Genes colored in *purple* and *white* in *Mycobacterium* sp. strain JC1 denotes the genes related in the synthesis of molybdenum cofactor and those with no homology to other genes presented in the figure, respectively. The *orf1* in *H. pseudoflava* is a partial gene

1999; Pearson et al. 1994; Schübel et al. 1995). The low levels of identity in the deduced products of CO-DH genes may explain the difference in antigenic epitopes between mycobacterial and Gram-negative CO-DHs (Kim et al. 1989).

Sequence database searches show that genes specific for CO-DH and other genes possibly involved in the CO oxidation are also present in other mycobacteria (including *M. tuberculosis*) in identical organization (Fig. 1). An exception to this is that *M. smegmatis* and three unclassified strains have no *orf8* counterpart. Genes located upstream and downstream of CO-DH genes in mycobacteria are arranged in identical order, but are different from those of *O. carboxidovorans*. The identity in the deduced amino acid sequence of large, medium, and small subunits of CO-DHs in several mycobacteria are 87.6–89.9, 71.2–74.6, and 80.9–88.0%, respectively, to those of the *Mycobacterium* sp. strain JC1 enzyme. The high identity between the deduced amino acid sequences of CO-DH genes in mycobacteria coincides with the observations that mycobacterial CO-DHs share identical antigenic groups and are phylogenetically closely related (Park et al. 2003) (Fig. 2). It is interesting that the non-mycobacterial actinobacterium *Rhodococcus jostii* also has two copies of CO-DH

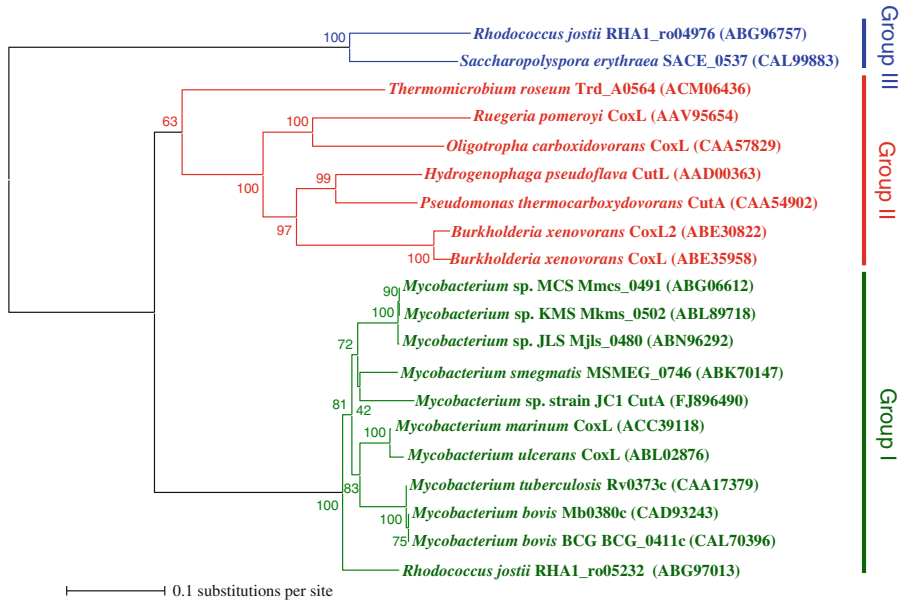

Fig. 2 Phylogenetic tree of deduced protein sequences of CO-DH large subunits. Amino acid sequence alignments were performed using CLUSTAL X (741 residues). The tree was generated by neighbor-joining (Poisson correction model) using the MEGA 3.1 program. The gaps in the alignment were completely deleted. The GenBank accession numbers for the sequences are given in *parentheses*. *Bootstrap values* were calculated from 1,000 replicates. Group I, II and III are for the CO-DH large subunits of mycobacteria, Gram-negative, and Gram-positive other than mycobacteria, respectively

genes, one of which is highly identical in the deduced amino acid sequence to that of the *Mycobacterium* sp. strain JC1 CO-DH (Fig. 2). Genes homologous to those located upstream and downstream of the CO-DH genes in *Mycobacterium* sp. strain JC1 are also organized in identical order upstream and downstream of one of the *R. jostii* CO-DH genes (Fig. 1), although *R. jostii* does not have the *cutR* and *orf8* homologs.

The duplicated CO-DH genes in *Mycobacterium* sp. strain JC1 are not only expressed during heterotrophic growth but are inducible in the presence of CO (Song et al. 2010). Two promoters are present upstream of each *cutB* gene of the two CO-DH genes and function separately in the presence or absence of CO (Song et al. 2010). Sequences similar to the mycobacterial −35 and −10 regions are identified from immediately upstream of the transcription initiation site that is transcribed in the presence of CO. Two inverted repeats are present upstream and downstream of the CO-inducible transcription start site. An inverted repeat, 5′-TGTGA-N6-TCACA-3′, located immediately upstream of and overlapping with the translational start codons of *cutB1* and *cutB2* is identical to the known binding site for Crp/Fnr proteins (Song et al. 2010; Oh et al. 2010). The palindrome sequence is identified to be involved in catabolite repression of the *cutBCA* operons (Oh et al. 2010), indicating that a Crp/Fnr protein may play a role in the regulation of CO-DH gene expression in *Mycobacterium* sp. strain JC1. Another protein CutR, a product of *cutR* gene which is located upstream of the divergently oriented *cutB1C1A1* (Fig. 1), is also identified as a transcriptional regulator for CO-DH genes in *Mycobacterium* sp. strain JC1 (Oh et al. 2010). CutR is a LysR family protein with a molecular mass of 34.1 kDa. Expression of *cutR* is higher in *Mycobacterium* sp. strain JC1 grown with glucose than that grown with CO. Using a *cutR* deletion mutant, it is demonstrated that CutR acts as a transcriptional activator for expression of the duplicated *cutBCA* operons in cells growing with glucose, is necessary for the efficient utilization of CO by the bacterium as the sole carbon and energy sources, and is involved in the induction of the *cutBCA* operons by CO (Oh et al. 2010). These reports indicate that the expression of CO-DH genes in *Mycobacterium* sp. strain JC1 is regulated in a quite complex way by at least two transcriptional regulators.

CO assimilation

Mycobacterium sp. strain JC1 contains two copies of form I RuBisCO genes (*cbbLS-1* and *cbbLS-2*; *cbbL* and *S* denote genes for large and small subunits of RuBisCO, respectively), as the CO-DH genes, with similar, but not identical, nucleotide sequences (Park et al. 2009). No sequences corresponding to the known mycobacterial promoters have been identified. However, several direct and inverted repeat sequences, including those with LysR motifs, are present upstream of the two *cbbLS* genes and these may be involved in binding of regulatory molecules. The *cbbX*- and *cbbR*-homologs are present downstream of *cbbS1* in the order of *cbbL-1-cbbS-1-cbbX-cbbR* and transcribed in the same orientation as the *cbbLS1*. The location and transcriptional orientation of *cbbR* is unique because all known *cbbR*s are located upstream of *cbbLS* and transcribed divergently from the RuBisCO genes (Kusian and Bowien 1997). The RuBisCO of *Mycobacterium* sp. strain JC1 contains antigenic epitopes identical to those of other mycobacterial RuBisCOs, but not to those of Gram-negative carboxydobacterial enzymes (Kim et al. 1997; Park et al. 2003). Unsurprisingly, the RuBisCO amino acid sequence of *Mycobacterium* sp. strain JC1 forms a discrete group separated from other form I subgroups of RuBisCO in the CbbL phylogenetic tree (Fig. 3). Based on these results, a new group in the form I RuBisCO subgroup, type IE, for mycobacterial RuBisCOs has been suggested (Park et al. 2009).

Mycobacterium sp. strain JC1 is a facultatively chemolithoautotrophic bacterium and employs three types of nutrition: chemoheterotrophy, chemolithotrophy, and methylotrophy (Cho et al. 1985). When growing with methanol, this bacterium employs both the xylulose monophosphate pathway and Calvin cycle to fix formaldehyde and CO_2, which are produced as an intermediate and end products of methanol oxidation, respectively (Park et al. 2003; Ro et al. 1997). The two sets of RuBisCO genes in *Mycobacterium* sp. strain JC1 are expressed simultaneously in cells grown either on CO or on methanol (Lee et al. 2009). However, the level of expression in cells growing with CO and methanol is higher for *cbbLS-2* than for *cbbLS-1*. The promoter activity of the two copies of RuBisCO genes in CO-grown cells is higher than that in methanol-grown cells. This indicates that the RuBisCO of *Mycobacterium* sp. strain JC1 is expressed more in cells growing on CO than in cells growing on methanol. This is understandable considering that methanol is assimilated through both the xylulose monophosphate pathway and the Calvin cycle (Ro et al. 1997), but CO is fixed to cellular material only via the Calvin cycle in this bacterium.

It is evident that the *cbbX* in *Mycobacterium* sp. strain JC1 is co-transcribed with the *cbbLS-1* (Lee et al. 2009), but *cbbR* is transcribed independently into a monocistronic mRNA. As for *cbbLS* genes, consensus sequences of mycobacterial promoters are not present in −10 and −35 regions of the *cbbR*. CbbR has been identified to bind specifically to two and one inverted repeats, of which one of each is a homologue of the LTTR binding motif (T-N_{11}-A; Schell 1993) in the promoter regions of *cbbLS-1* and *-2*, respectively. DNA footprinting analysis implied that the CbbR in *Mycobacterium* sp. strain JC1 regulates the expression of *cbbLS-1* similar to that of the CbbRs of other bacteria and *cbbLS-2* is regulated differently (Lee et al. 2009). The CbbR in *Mycobacterium* sp. strain JC1 regulates the RuBisCO genes positively (Lee et al. 2009), as in other organisms (Schell 1993), since RuBisCO was present in cells grown with either CO or methanol, but not in cells grown in nutrient broth (Kim et al. 1997; Lee et al. 2009). This was evident because the CbbR bound specifically to the promoter regions of RuBisCO genes (Lee et al. 2009), and because *Mycobacterium* sp. strain JC1 that contained overexpressed CbbR produced *cbbLS* transcripts during growth in nutrient broth (Lee et al. 2009).

Evolution

CO-DHs in mycobacteria, including that in *Mycobacterium* sp. strain JC1, share many biochemical and molecular properties in common with those of Gram-negative aerobic carboxydobacteria. However, the enzymes in mycobacteria are phylogenetically distinct from those of the Gram-negative carboxydobacteria. This means that the key enzyme for the oxidation of CO in mycobacterial carboxydobacteria has evolved separately from that in Gram-negative carboxydobacteria from an ancestral CO-DH. Considering the unique CO-DHs in thermophilic bacilli (Krüger and Meyer 1984), a *Streptomyces* strain (Bell et al. 1985), and homologues in *Saccharopolyspora erythraea* (GenBank accession no. AM420293) and *R. jostii*

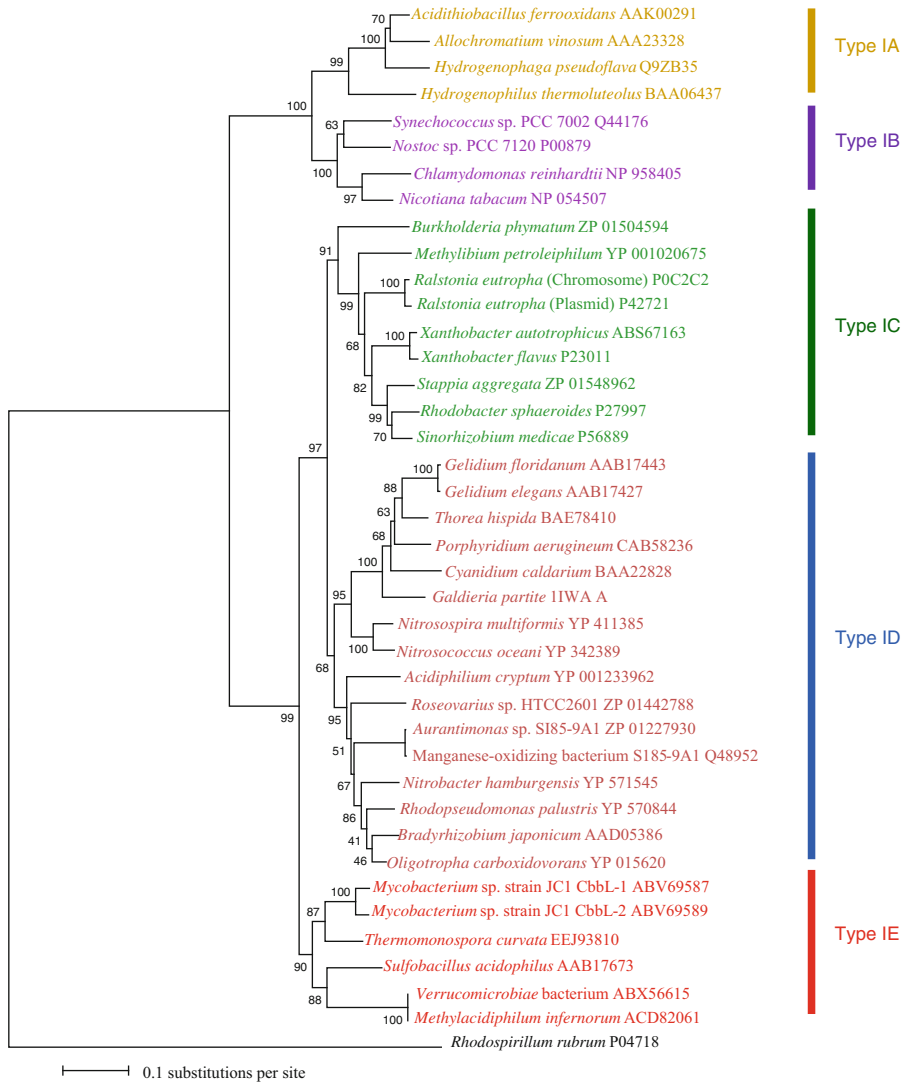

Fig. 3 Phylogenetic tree of deduced protein sequences of RubisCO large subunits from several bacteria. Amino acid sequence alignments were performed using CLUSTAL X (460 residues). The tree was generated by neighbor-joining (Poisson correction model) using the MEGA 3.1 program. Gaps in the alignment were completely deleted. The accession numbers for the sequences are given at the *right side* of species names. *Bootstrap values* were calculated from 1,000 replicates

(GenBank accession no. CP000431), it is evident that CO-DHs in aerobic bacteria cluster in three separate phylogenetic groups. These groups include group I enzymes of mycobacteria, group II enzymes of Gram-negative bacteria, and group III enzyme from Gram-positive bacteria other than mycobacteria (Fig. 2). Phlyogenetic analysis also indicates that slow-growers and rapid-growers of mycobacteria are also distinguishable in the phylogenetic properties of CO-DH.

The RuBisCO amino acid sequences in mycobacteria have similar evolutionary properties. Even though the enzyme playing a key role in the assimilation of CO in mycobacterial carboxydobacteria has many characteristics identical or similar to that of Gram-negative carboxydobacteria, there is also a phylogenetic evidence that distinguishes the mycobacterial enzymes from those of Gram-negative carboxydobacteria (Fig. 3). As discussed, several mycobacteria also have the ability to use the reductive TCA cycle instead of the Calvin cycle for carbon fixation.

These results indicate that the ability to use CO aerobically as a source of carbon and energy has

evolved at least in three different divergent lines of descent from an ancestral carboxydobacterium.

Environmental significance

As noted earlier, there is some doubt regarding whether *Mycobacterium* sp. strain JC1 might use atmospheric CO for growth because the K_m of CO-DH from *Mycobacterium* sp. strain JC1 is too high for efficient use of atmospheric CO (Kim et al. 1989). Although atmospheric CO mixing ratios have been estimated to range normally from 0.03 to 0.9 ppm, depending on the site of measurement (Robbins et al. 1968), urban areas may be associated with CO concentration that reach 50-100 ppm (Robinson and Robbins, 1970). Rainwater sometimes contains up to 200 times the concentration of CO present in the atmosphere (Swinnetrton et al. 1971) and certain microenvironments maintain high concentration of CO produced by continuous decomposition of flavonoids and porphyrin (Conrad and Seiler, 1980; Westlake et al. 1961). Considering the observations that *M. phlei*, *M. smegmatis*, and *M. tuberculosis* are able to oxidize < 50 ppm concentrations of CO and that *Mycobacterium* sp. strain JC1 could grow with 100 ppm CO (Cho et al. 1985), these results suggest that many or all mycobacteria that are able to oxidize < 50 ppm CO (Bartholomew and Alexander, 1979; King, 2003) may play a role in the removal (oxidation or assimilation) of CO in some circumstances. *Mycobacterium* sp. strain JC1 may be particularly active in some environmental circumstances because a) the bacterium is able to grow mixotrophically with CO and organic substrate (Kim and Kim, 1989), b) CO-DH is produced constitutively in cells even growing under heterotrophic conditions (Ro and Kim, 1993), and c) expression of CO-DH in cells growing heterotrophically increases in the presence of CO (Song et al. 2010).

Application

NO produced in macrophages exhibits cytostatic or cytotoxic activity against engulfed microorganisms (MacMicking et al. 1997). The presence of NO-DH activity associated with mycobacterial CO-DH, the protection by CO-DH of *E. coli* from bactericidal activity of SNP, the increase in the CO-DH activity in parallel with that of the amount of the CO-DH synthesized in response to the treatment of SNP, the presence of the genes for CO-DH in several pathogenic mycobacteria, and the presence of active CO-DH in *M. tuberculosis* grown even under heterotrophic conditions (see previous sections) all point to the important role of NO-DH activity of CO-DH playing a critical role in the detoxification of NO in pathogenic mycobacteria residing in phagosomes of macrophages. This implication may be assessed by observing the survival rate of wild-type and CO-DH-negative mutant strains of nonpathogenic and pathogenic mycobacteria in macrophages. The results will open a possibility that the mycobacterial CO-DH and proteins associated with its expression may be used as a novel target for the development of new antitubercular candidates.

Summary

Although the Gram-negative aerobic carboxydobacteria have been studied extensively for over thirty years, the ability of mycobacteria to grow with CO has been studied for only a short period. It is evident that, with few exceptions, both pathogenic and nonpathogenic mycobacteria are able to grow aerobically with CO as a sole source of carbon and energy. Among the mycobacterial carboxydobacteria, *Mycobacterium* sp. strain JC1 has been studied most extensively. Mycobacterial carboxydobacteria use CO-DH as a key enzyme for CO oxidation, which has many properties in common with those of Gram-negative aerobic carboxydobacterial CO-DHs but also has unique characteristics. For example, the mycobacterial CO-DH has NO-DH activity, has no antigenic epitopes in common with those of other CO-DHs, and has evolved separately from other CO-DHs. Many mycobacteria use the Calvin cycle to assimilate CO_2, but others may use the reductive TCA cycle. The RuBisCO in mycobacteria is a distinct phylogenetic type of form I RuBisCO, form IE. The genetic background of CO utilization in mycobacteria is only in the beginning stage of study. Nevertheless, there is a strong possibility that the genes for CO-DH in mycobacteria are regulated by a product of the Lys-R type regulatory gene located upstream of CO-DH genes, which has not been identified in other carboxydobacteria. Crp/Fnr

proteins may also be involved in the expression of CO-DH genes in mycobacteria. Aerobic carboxydobacterial CO-DHs may be classified into three phylogenetic groups including group I, II, and III enzymes in mycobacteria, Gram-negative bacteria, and Gram-positive bacteria other than mycobacteria, respectively. It is possible that mycobacterial carboxydobacteria in high-CO environments play a role in the oxidation or assimilation of CO but this remains to be assessed with cultivation-independent analyses. The mycobacterial CO-DH, which also exhibits NO-DH activity, may be used as a target for the development of novel anti-tubercular drugs.

Acknowledgments This work was supported by grants R01-2006-000-10846-0 and 2009-0093946 from the Korea Science and Engineering Foundation and National Research Foundation of Korea, respectively.

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