JOURNAL OF BACTERIOLOGY, Jan. 2003, p. 142–147 Vol. 185, No. 1
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.1.142–147.2003
Copyright © 2003, American Society for Microbiology. All Rights Reserved.

Growth of Mycobacteria on Carbon Monoxide and Methanol

Sae W. Park,[1] Eun H. Hwang,[1] Hyuck Park,[1] Jeong A. Kim,[1] Jinho Heo,[1] Key H. Lee,[1] Taeksun Song,[1]† Eungbin Kim,[1] Young T. Ro,[2] Si W. Kim,[3] and Young M. Kim[1]*

Department of Biology, Yonsei University, Seoul 120-749,[1] Laboratory of Biochemistry, Konkuk College of Medicine, Chungju 380-701,[2] and Department of Environmental Engineering, Chosun University, Kwangju 501-759,[3] Korea

Received 8 August 2002/Accepted 8 October 2002

Several mycobacterial strains, such as *Mycobacterium flavescens*, *Mycobacterium gastri*, *Mycobacterium neoaurum*, *Mycobacterium parafortuitum*, *Mycobacterium peregrinum*, *Mycobacterium phlei*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and *Mycobacterium vaccae*, were found to grow on carbon monoxide (CO) as the sole source of carbon and energy. These bacteria, except for *M. tuberculosis*, also utilized methanol as the sole carbon and energy source. A CO dehydrogenase (CO-DH) assay, staining by activity of CO-DH, and Western blot analysis using an antibody raised against CO-DH of *Mycobacterium* sp. strain JC1 (formerly *Acinetobacter* sp. strain JC1 [J. W. Cho, H. S. Yim, and Y. M. Kim, Kor. J. Microbiol. 23:1-8, 1985]) revealed that CO-DH is present in extracts of the bacteria prepared from cells grown on CO. Ribulose bisphosphate carboxylase/oxygenase (RubisCO) activity was also detected in extracts prepared from all cells, except *M. tuberculosis*, grown on CO. The mycobacteria grown on methanol, except for *M. gastri*, which showed hexulose phosphate synthase activity, did not exhibit activities of classic methanol dehydrogenase, hydroxypyruvate reductase, or hexulose phosphate synthase but exhibited *N,N*-dimethyl-4-nitrosoaniline-dependent methanol dehydrogenase and RuBisCO activities. Cells grown on methanol were also found to have dihydroxyacetone synthase. Double immunodiffusion revealed that the antigenic sites of CO-DHs, RuBisCOs, and dihydroxyacetone synthases in all mycobacteria tested are identical with those of the *Mycobacterium* sp. strain JC1 enzymes.

Carboxydobacteria are a group of bacteria which are able to grow chemolithotrophically on carbon monoxide (CO) as the sole carbon and energy source under aerobic conditions (22, 30). Most of the carboxydobacteria are gram negative (16, 22, 27, 31; G. King and H. Cosby, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. I-4, 2002), but several gram-positive carboxydobacteria, such as species of *Arthrobacter* (31), *Bacillus* (31), *Streptomyces* (5, 14, 31, 34), *Sarcina*, *Nocardia*, and *Corynebacterium* (P. Hirsh, Abstr. 65th Annu. Meet. Am. Soc. Microbiol. 1965, abstr. P108, 1965), and *Actinoplanes*, *Microbispora*, and *Mycobacterium* (4), have also been described.

The facultatively chemolithotrophic bacterium *Mycobacterium* sp. strain JC1 (originally *Acinetobacter* sp. strain JC1 DSM 3803; reclassified by Song et al. [41]), is capable of growing aerobically not only on CO but also on methanol as a sole source of carbon and energy (8, 39). This means that the bacterium is able to employ three distinct types of nutrition, chemoheterotrophy, chemolithotrophy, and methylotrophy, depending on substrate availability.

Combined with these results, the facts that many mycobacterial species including *Mycobacterium tuberculosis* (10; GenBank accession no. AL123456), *Mycobacterium avium* (NCBI reference sequence [RefSeq] NC-002943), *Mycobacterium bovis* (NCBI RefSeq NC-002945), *Mycobacterium leprae* (9; GenBank accession no. AL450380), and *Mycobacterium smegmatis* (NCBI RefSeq NC-002974) have genes encoding amino acid sequences similar to those of *Mycobacterium* sp. strain JC1 CO dehydrogenase (CO-DH) (T. Song and Y. M. Kim, unpublished data), that *Mycobacterium phlei* is able to oxidize CO (4), and that *Mycobacterium cuneatum* (40), *Mycobaterium gastri* (18), and *Mycobacterium* ID-Y (36) are capable of growing on methanol raise the possibility that all known mycobacteria have an intrinsic ability to grow on CO and/or methanol as the sole carbon and energy source.

In order to address this question, we examined several well-known mycobacteria for the ability to grow on CO and/or methanol, and we found that all the mycobacteria tested grew well on each of these substrates as the sole source of carbon and energy, except that *M. tuberculosis* did not grow on methanol. We also present several enzymological backgrounds for the growth of the mycobacteria on CO and methanol.

MATERIALS AND METHODS

Strains and cultivation conditions. *Mycobacterium* sp. strain JC1 (DSM 3803) (3, 41), *Mycobacterium flavescens* (ATCC 14474), *M. gastri* (ATCC 15754), *Mycobacterium neoaurum* (ATCC 25795), *Mycobacterium parafortuitum* (ATCC 19686), *Mycobacterium peregrinum* (ATCC 14467), *M. phlei* (ATCC 11758), *M. smegmatis* mc^2 (ATCC 700084), *M. tuberculosis* H37Ra (ATCC 35835), and *Mycobacterium vaccae* (ATCC 15483) were used throughout this study. Cells were cultivated at 37°C under CO chemolithoautotrophy with a gas mixture of 30% CO–70% air in either standard mineral base (SMB) medium (SMB-CO) (21) or 0.47% (wt/vol) Middlebrook 7H9 medium (7H9-CO; Becton Dickinson, Cockeysville, Md.). For methylotrophic growth, cells were grown at 37°C in SMB medium supplemented with 1% (vol/vol) methanol (SMB-MeOH). For the methanol assimilation enzyme assay, *Methylobacterium extorquens* AM1 (NCIB 9133) and *Methylobacillus* sp. strain SK1 (DSM 8269) grown at 30°C in SMB-MeOH were used as controls. Growth was measured with a spectrophotometer by determination of turbidity at 436 nm.

* Corresponding author. Mailing address: Molecular Microbiology Laboratory, Department of Biology, Yonsei University, Seoul 120-749, Korea. Phone: 82-2-2123-2658. Fax: 82-2-312-5657. E-mail: young547@yonsei.ac.kr.

† Present address: The Genome Research Center for Respiratory Pathogens, Yonsei University College of Medicine, Seoul 12-752, Korea.

Cell extract preparation. All preparation steps were carried out at 4°C. Cells were harvested at the late-exponential-growth phase, washed once with 0.05 M Tris hydrochloride buffer (pH 7.5) (except that 0.05 M phosphate buffer [pH 7.0] was used to prepare cell extracts for the *N,N*-dimethyl-4-nitrosoaniline [DMNA]-dependent methanol dehydrogenase [MDH] assay), and resuspended in the same buffer. Cells in the suspension were then disrupted by sonic treatment (10 s/ml) in 20-ml portions and centrifuged at 15,000 × *g* for 30 min, and the resulting supernatant was used as the cell extract.

Protein determination. Protein quantities were determined by the method described by Bradford (6), with bovine serum albumin as a standard.

Enzyme assays. All assays were carried out at 30°C unless otherwise described. CO-DH activity was assayed photometrically by measuring CO-dependent reduction of 2-(4-indophenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride (INT; ε_{496} = 17.981 mM^{-1} cm^{-1}) by the method of Kraut et al. (23).

Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO) activity was assayed by the method of McFadden and Tu (28). One unit of enzyme activity was defined as the amount of enzyme required to incorporate 1 μmol of CO_2 per min.

Hydroxypyruvate reductase (HPR) activity was assayed by measuring the hydroxypyruvate-dependent oxidation of NADH (ε_{340} = 6.22 mM^{-1} cm^{-1}) by the method of Large and Quayle (26). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 μmol of NADH per min.

Hexulose 6-phosphate synthase (HPS) activity was assayed at 37°C according to the method of Ferenci et al. (13) by measuring the decrease in the amount of formaldehyde after the reaction of the added formaldehyde with the ribulose 5-phosphate formed in the reaction mixture. Formaldehyde levels were determined by the method of Nash (32). One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of formaldehyde per min.

DMNA-dependent MDH activity was assayed by measuring the methanol-dependent reduction of DMNA (ε_{440} = 35,400 M^{-1} cm^{-1}) by the method of van Ophem et al. (43). One unit of enzyme activity was defined as the amount of protein required to reduce 1 μmol of DMNA per min.

Pyrroloquinoline quinone (PQQ)-containing MDH activity was assayed by measuring the methanol-dependent decrease in the absorbancy of 2,6-dichlorophenol indophenol (DCPIP; ε_{600} = 22,000 M^{-1} cm^{-1}) by the method of Anthony and Zatman (2). NAD-dependent PQQ-containing MDH activity was assayed by measuring the methanol-dependent decrease in the extinction of DCPIP by the method of Duine et al. (12). NAD-dependent MDH activity was assayed by measuring methanol-dependent NADH (ε_{340} = 6,220 M^{-1} cm^{-1}) production by the method of Arfman et al. (3). NAD-dependent glutathione (GSH)-requiring MDH activity was assayed by measuring methanol-dependent NADH production at 340 nm by the method of Mehta (29). Cytochrome *c*-dependent MDH activity was assayed by determining the methanol-dependent increase in the extinction at 550 nm by the method of Kato et al. (17). Methanol oxidase was assayed by measuring the amount of formaldehyde or H_2O_2 produced during enzyme reaction by the method of Tani et al. (42).

Electrophoresis. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed by a procedure described by Laemmli (25), but without sodium dodecyl sulfate.

Activity staining. Staining by activity of CO-DH was carried out in standard buffer flushed with CO, by using a nondenaturing gel in the presence of 0.05% phenazine methosulfate and 0.25% nitroblue tetrazolium as described previously (21).

Immunological test. Antisera against *Mycobacterium* sp. strain JC1 CO-DH (20), RuBisCO from cells grown on methanol (MeOH-RuBisCO) (19), and dihydroxyacetone synthase (DHAS) (38) were raised in New Zealand White rabbits as described previously (21). Double immunodiffusion assays were performed in a 1.2% agarose gel by a modification (24) of the method of Ouchterlony and Nilson (35). Immunoblot analyses were carried out as described in the ECL (enhanced chemiluminescence) Western blot protocols (Amersham, Little Chalfont, Buckinghamshire, England) after transfer of the proteins in the nondenaturing gel to a nitrocellulose membrane (Hybond-ECL; Amersham).

RESULTS AND DISCUSSION

CO- and methanol-dependent growth of mycobacteria. All mycobacteria tested, including the nonpathogenic strain of *M. tuberculosis*, were found to grow in 7H9-CO and SMB-CO (Table 1). The abilities of *M. tuberculosis* and *M. smegmatis* to grow on CO were expected, since genome databases revealed that CO-DH structural genes are present in both *M. tuberculosis* (10; GenBank accession no. AL123456) and *M. smegmatis* (NCBI RefSeq NC-002974). However, except for *Mycobacterium* sp. strain JC1, which was originally isolated as a carboxydobacterium (8), the mycobacteria required a lag period of 9 to 10 days after they were first subjected to growth in 7H9-CO. The average lag period was even longer, 28 to 30 days, when cells were grown in SMB-CO. Once the cells were adapted to growth on CO, no lag period was observed in either medium.

TABLE 1. Rates of growth of several mycobacteria on CO and methanol[a]

Species	Doubling time (h)		Source
	7H9-CO	SMB-MeOH	
Mycobacterium sp. strain JC1	6.4	7.8	DSM 3803
M. flavescens	3.7	10.2	ATCC 14474
M. gastri	6.5	9.2	ATCC 15754
M. neoaurum	7.9	9.0	ATCC 25795
M. parafortuitum	4.4	5.9	ATCC 19686
M. peregrinum	6.0	6.9	ATCC 14467
M. phlei	7.6	9.5	ATCC 11758
M. smegmatis mc^2	6.2	7.7	ATCC 700084
M. tuberculosis H37Ra	23.9	NG[b]	ATCC 35835
M. vaccae	5.8	9.0	ATCC 15483

[a] Cells were cultivated at 37°C in 0.47% (wt/vol) 7H9 medium under a gas mixture of 30% CO–70% air (7H9-CO) and in SMB medium containing 1% (vol/vol) methanol (SMB-MeOH).
[b] NG, no observable growth.

It was observed that the 7H9 medium, which is usually used as a basal medium for mycobacteria, was better than the SMB medium for CO-autotrophic growth of the mycobacteria, since the rate of growth of each bacterium in 7H9-CO (Table 1) was on average three times higher than that in SMB-CO (data not shown). Among the cells tested, *M. flavescens* grew most rapidly (doubling time [t_d] = 3.7 h), while *M. tuberculosis* grew most slowly (t_d = 24 h), in 7H9-CO. During growth in 7H9-CO for 50 h, cultures of all mycobacteria reached optical densities at 436 nm (OD_{436}) of 0.40 (*M. neoaurum*) to 0.54 (*Mycobacterium* sp. strain JC1 and *M. gastri*), except for *M. tuberculosis* (OD_{436} = 0.02). All cultures reached their maximum OD_{436} (0.45 to 0.55) in 60 h of cultivation, except the *M. tuberculosis* culture, which reached its maximum value of 0.2 at 140 h after inoculation. No growth of the cells was observable under a CO-free atmosphere in either the SMB or the 7H9 medium, indicating that sodium and ferric ammonium citrates present in the 7H9 medium (total citrate concentration, ca. 0.56 mM) did not affect the growth of the cells.

It was found that all the mycobacteria tested, except for *M. tuberculosis*, were also able to grow on SMB-MeOH (Table 1). The mycobacteria, except for *Mycobacterium* sp. strain JC1, which has already been adjusted to grow on methanol (39), also required a lag period of 7 to 8 days after they were first subjected to SMB-MeOH. Since the bacteria grew well in SMB-MeOH (t_d = 5.9 to 10.2 h [Table 1]), growth was not tested with 7H9 medium supplemented with methanol. During growth in SMB-MeOH, cultures of all mycobacteria reached maximum OD_{436} of 0.55 (*M. flavescens*) to 0.68 (*Mycobacterium* sp. strain JC1) in 50 h after inoculation.

The present results indicate that the ability to use both CO and methanol as carbon and energy sources is widely distributed among mycobacteria and suggest that this metabolic ca-

TABLE 2. Activities of key enzymes involved in the utilization of CO and methanol in several mycobacteria grown on CO and methanol[a]

Species	Sp act					
	CO-DH[b]	CO-RuBisCO[c]	DMNA-dependent MDH[d]	MeOH-RuBisCO[e]	HPS[f]	HPR[g]
Mycobacterium sp. strain JC1	33.7	17.6	10.2	25.6	—[h]	—
M. flavescens	18.1	14.8	6.2	16.5	—	—
M. gastri	8.5	39.7	7.3	23.4	2.3	—
M. neoaurum	13.2	17.8	4.5	27.2	—	—
M. parafortuitum	10.4	12.2	6.2	15.2	—	—
M. peregrinum	15.6	18.7	7.9	20.5	—	—
M. phlei	7.7	22.6	6.2	15.9	—	—
M. smegmatis mc^2	14.3	10.0	6.8	17.4	—	—
M. tuberculosis H37Ra	20.5	—	NA[i]	NA	NA	NA
M. vaccae	16.2	14.4	6.2	16.3	—	—

[a] Activity was determined with extracts prepared from cells grown in SMB-CO (CO-DH and CO-RuBisCO) or SMB-MeOH (DMNA-dependent MDH, MeOH-RuBisCO, HPS, and HPR) medium.
[b] Nanomoles of INT reduced per milligram of protein per minute.
[c] Nanomoles of CO_2 incorporated per milligram of protein per minute.
[d] Nanomoles of DMNA reduced per milligram of protein per minute.
[e] Nanomoles of CO_2 incorporated per milligram of protein per minute.
[f] Micromoles of formaldehyde consumed per milligram of protein per minute.
[g] Micromoles of NADH oxidized per milligram of protein per minute.
[h] —, no activity.
[i] NA, not applicable, since the bacterium did not grow on methanol.

pability may be an intrinsic property of this group of bacteria. The presence of CO-DH genes in *M. avium* (NCBI RefSeq NC-002943), *M. bovis* (NCBI RefSeq NC-002945), and *M. leprae* (9), the growth on methanol of *M. cuneatum* (40) and *Mycobacterium* ID-Y (36), and the oxidation of CO by *M. phlei* (4) support this hypothesis.

The ability of all mycobacteria tested, except *M. tuberculosis*, to grow on both CO and methanol is interesting, because no other taxonomic group of bacteria is known to grow on both substrates as the sole source of carbon and energy.

Key enzymes for CO utilization. An enzyme assay (Table 2) and analysis by activity staining (Fig. 1) and immunoblotting (Fig. 2A) of gels after nondenaturing PAGE of cell extracts revealed that all mycobacteria grown in SMB-CO contained CO-DH, as did *Mycobacterium* sp. strain JC1 (20). Identical results were obtained with extracts prepared from cells grown in 7H9-CO, except that the CO-DH activity was not detectable by enzyme assay (data not shown), possibly due to the presence of a certain molecule(s) inhibiting the CO-DH assay when extracts prepared from cells grown in the presence of organic material were used, as suggested previously (11, 37). CO-DHs in the mycobacteria tested were also found to be expressed in cells grown in SMB-MeOH or 7H9 medium supplemented with 0.2% (wt/vol) glucose (data not shown), indicating that the enzymes in mycobacteria may be constitutively expressed, like that of *Mycobacterium* sp. strain JC1 (37). Double immunodiffusion revealed that the extracts prepared from cells grown in SMB-CO (Fig. 3A) or 7H9-CO (data not shown) cross-react with an antiserum raised against the purified CO-DH of *Mycobacterium* sp. strain JC1 and that all of the immunogenic epitopes on the *Mycobacterium* sp. strain JC1 enzyme used to generate the antiserum are also present on all the mycobacterial CO-DHs.

It has been reported that the CO_2 resulting from oxidation of CO in carboxydobacteria is converted to cellular material via the Calvin cycle (22, 30). An enzyme assay revealed that, except for *M. tuberculosis*, all mycobacteria tested exhibited

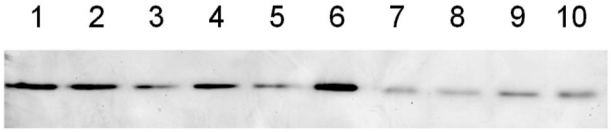

FIG. 1. Activity staining of CO-DHs in mycobacteria. After extracts (20 μg each) prepared from cells grown in 7H9-CO medium were subjected to nondenaturing PAGE (7.5% acrylamide), CO-DHs in these extracts were stained by activity by using CO as a substrate as described in Materials and Methods. Lanes: 1, *Mycobacterium* sp. strain JC1; 2, *M. flavescens*; 3, *M. gastri*; 4, *M. neoaurum*; 5, *M. parafortuitum*; 6, *M. peregrinum*; 7, *M. phlei*; 8, *M. smegmatis*; 9, *M. tuberculosis*; 10, *M. vaccae*.

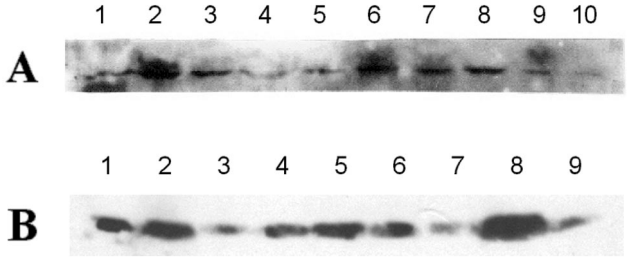

FIG. 2. Immunoblotting of CO-DHs and RuBisCOs in mycobacteria. The presence of CO-DH or MeOH-RuBisCO in cells of mycobacteria was analyzed by ECL Western blotting protocols after nondenaturing PAGE (7.5% acrylamide) of extracts (20 μg each) prepared from cells grown in 7H9-CO or SMB-MeOH medium, respectively, as described in Materials and Methods. (A) Immunoblotting of CO-DHs. Blots were incubated with an antibody raised against *Mycobacterium* sp. strain JC1 CO-DH. Lanes: 1, *Mycobacterium* sp. strain JC1; 2, *M. flavescens*; 3, *M. gastri*; 4, *M. neoaurum*; 5, *M. parafortuitum*; 6, *M. peregrinum*; 7, *M. phlei*; 8, *M. smegmatis*; 9, *M. tuberculosis*; 10, *M. vaccae*. (B) Immunoblotting of MeOH-RuBisCOs. Blots were incubated with an antibody raised against *Mycobacterium* sp. strain JC1 MeOH-RubisCO. Lanes: 1 to 8, same as in panel A; 9, *M. vaccae*. Extracts prepared from cells grown in 7H9-CO medium produced CO-RuBisCO immunoblot patterns identical to those of the MeOH-RuBisCOs.

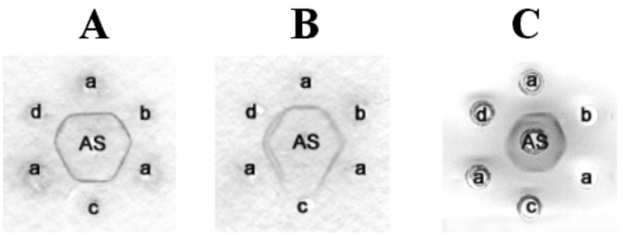

FIG. 3. Double-immunodiffusion patterns for CO-DHs, RuBisCOs, and DHASs in mycobacteria. Immunodiffusion assays were performed in a 1.2% agarose gel for 24 h at 30°C, followed by staining with Coomassie brilliant blue R-250 as described in Materials and Methods. Extracts were prepared from cells grown in 7H9-CO (A and B) or SMB-MeOH (C) medium. (A) Immunodiffusion patterns of CO-DHs. Wells: AS, antiserum raised against purified CO-DH of *Mycobacterium* sp. strain JC1 (7 μl); a, *Mycobacterium* sp. strain JC1 (41 μg); b, *M. flavescens* (83 μg); c, *M. gastri* (23 μg); d, *M. neoaurum* (22 μg). Cell extracts of *M. parafortuitum*, *M. peregrinum*, *M. phlei*, *M. smegmatis*, *M. tuberculosis*, and *M. vaccae* also showed the presence of antigenic groups in their CO-DHs identical to those of the *Mycobacterium* sp. strain JC1 CO-DH. (B) Immunodiffusion patterns of CO-RuBisCOs. Wells: AS, antiserum raised against *Mycobacterium* sp. strain JC1 MeOH-RubisCO (3 μl); a, *Mycobacterium* sp. strain JC1 (45 μg); b, *M. smegmatis* (88 μg); c, *M. tuberculosis* (45 μg); d, *M. vaccae* (43 μg). Cell extracts of *M. flavescens*, *M. gastri*, *M. neoaurum, M. parafortuitum, M. peregrinum,* and *M. phlei* also exhibited the presence of antigenic sites in their RuBisCOs identical to those of the *Mycobacterium* sp. strain JC1 MeOH-RuBisCO. Extracts prepared from cells grown in SMB-MeOH medium gave MeOH-RuBisCO immunodiffusion patterns identical to those of the CO-RuBisCOs. (C) Immunodiffusion patterns of DHASs. Wells: AS, antiserum raised against *Mycobacterium* sp. strain JC1 DHAS (10 μl); a, *Mycobacterium* sp. strain JC1 (68 μg); b, *M. parafortuitum* (78 μg); c, *M. peregrinum* (67 μg); d, *M. phlei* (79 μg). Cell extracts of *M. flavescens*, *M. gastri*, *M. neoaurum*, *M. smegmatis*, and *M. vaccae* also were found to have antigenic groups in their DHASs identical to those of the *Mycobacterium* sp. strain JC1 DHAS.

RuBisCO activity when grown in SMB-CO (Table 2), as *Mycobacterium* sp. strain JC1 did (19). Identical results were obtained with cells grown in 7H9-CO (data not shown). The present finding that *M. tuberculosis* does not have RuBisCO is supported by a previous report that RuBisCO genes are absent in this bacterium (10) and indicates that *M. tuberculosis* adopts a metabolic pathway other than the Calvin cycle to convert CO_2 to organic material. The presence of genes homologous to those encoding fumarate reductase (*frdA* [Rv2241], *frdB* [Rv1553], *frdC* [Rv1554], and *frdD* [Rv1555]), the ATP-citrate lyase β-chain (*citE* [Rv2498c]), and α-ketoglutarate: ferredoxin oxidoreductase (α-subunit [Rv2455C] and β-subunit [Rv2454C]) in *M. tuberculosis* (10) suggests that a reductive tricarboxylic acid cycle may function to fix CO_2 in this bacterium. The presence of RuBisCO in all mycobacteria tested, except *M. tuberculosis*, when they were grown in SMB-CO was further supported by immunoblot analysis of cell extracts (data not shown).

Mycobacterium sp. strain JC1 contains two copies of highly homologous RuBisCO genes (H. S. Jang, E. H. Hwang, and Y. M. Kim, unpublished data). The MeOH-RuBisCO of this bacterium was found to contain antigenic groups identical to those of the RubisCO in CO-grown cells (CO-RuBisCO) but not to those of the enzymes in gram-negative carboxydobacteria (19). Double immunodiffusion revealed that the CO-RuBisCOs of the mycobacteria tested shared antigenic sites identical to those of the *Mycobacterium* sp. strain JC1 MeOH-RuBisCO (Fig. 3B).

Key enzymes for methanol utilization. It has been reported (39) that cells of *Mycobacterium* sp. strain JC1 grown on methanol do not exhibit activities of the classical PQQ-containing MDH in gram-negative bacteria (2), the NAD-dependent PQQ-containing MDH (12) and cytochrome *c*-dependent MDH (17) of *Amycolatopsis methanolica*, the NAD-dependent MDH of methylotrophic *Bacillus* (3), or the NAD-dependent glutathionine-requiring MDH (29) and methanol oxidase (42) of methylotrophic yeast.

It was found that all mycobacteria tested here, when grown on methanol, also failed to show activities of the enzymes listed above. However, when grown on methanol, the bacteria, including *Mycobacterium* sp. strain JC1, were found to exhibit DMNA-dependent MDH activity (Table 2), which had previously been reported for *M. gastri* (7), suggesting that the DMNA-dependent MDH may be responsible for the oxidation of methanol in these bacteria.

It has been reported that *Mycobacterium* sp. strain JC1 grown on methanol exhibited neither HPS nor HPR activity, but contained RuBisCO and DHAS, indicating that the bacterium employs a novel way of C_1 assimilation (39); i.e., the Calvin cycle and the eukaryote-specific xylulose monophosphate (XuMP) pathway, instead of the prokaryote-specific ribulose monophosphate (RuMP) and serine pathways, are functioning in the cells growing on methanol.

None of the methanol-grown mycobacteria exhibited HPS and HPR activities, except that *M. gastri* showed HPS activity as previously reported (18). *Methylobacterium extorquens* AM1 and *Methylobacillus* sp. strain SK1, containing serine and RuMP pathways, showed activities of HPR (2.0 U per mg of protein) and HPS (0.8 U per mg of protein), respectively. It was found that all cells grown on methanol exhibited RuBisCO activity (Table 2). The presence of RuBisCO in all methanol-grown mycobacteria was identified by immunoblot analysis of cell extracts (Fig. 2B). Double immunodiffusion revealed that the antigenic sites of the MeOH-RuBisCOs in the mycobacteria were identical to those of the *Mycobacterium* sp. strain JC1 MeOH-RubisCO (data not shown), like those of the CO-RuBisCOs (Fig. 3B). Immunoblot analysis revealed that all mycobacteria grown on methanol also contained the yeast enzyme DHAS (data not shown). The DHASs in the mycobacteria were found to share antigenic groups common to the *Mycobacterium* sp. strain JC1 enzyme (38) (Fig. 3C).

The absence of HPR and HPS, and the presence of RuBisCO and DHAS, in all mycobacteria (except *M. gastri*, which also has HPS in addition to RuBisCO and DHAS) indicates that the mycobacteria do not synthesize cellular materials from methanol through the RuMP and serine pathways, the most common routes for C_1 assimilation in methylotrophic bacteria (1), but synthesize through the Calvin and XuMP cycles, as does *Mycobacterium* sp. strain JC1 (38).

It has been generally known that several methylotrophic bacteria contain key enzymes of more than one C_1 assimilation pathway, though not the XuMP cycle, and no methylotrophic yeasts possess key enzymes for prokaryotic C_1 assimilation pathways such as the RuMP and serine pathways and Calvin cycle (1). *Mycobacterium* sp. strain JC1, however, was found to possess the key enzymes for both the XuMP and Calvin cycles

(38) and was considered the first exception to the general concept. The present result that all mycobacteria grown on methanol contain RuBisCO and DHAS, therefore, is very interesting, since the mycobacteria may be the first prokaryotic group possessing the eukaryotic C_1 assimilation pathway and also the first taxonomic group possessing both the prokaryotic and eukaryotic C_1 assimilation enzymes. It is even more interesting that *M. gastri* contains HPS in addition to DHAS and RuBisCO, indicating that *M. gastri* may be the first bacterium possessing the eukaryotic C_1 assimilation pathway in addition to the two prokaryotic routes.

Until now, *Mycobacterium* sp. strain JC1 (39) and *Pseudomonas gazotropha* (33) were the only carboxydobacteria which were known to be able to grow on methanol (22, 30), and no methylotrophic bacteria were known to be able to utilize CO (1, 15) as the sole carbon and energy source. It was also known that the CO-DH (20) and RuBisCO (19) of *Mycobacterium* sp. strain JC1 had no immunological relationship with those of gram-negative carboxydobacteria. The present work clearly shows that all mycobacteria tested, except *M. tuberculosis*, grow not only on CO but also on methanol, that all the bacteria grown on methanol possess the novel DMNA-dependent MDH as a key enzyme for methanol oxidation and the eukaryotic DHAS in addition to RuBisCO for C_1 assimilation, that all adopt common pathways for CO and methanol utilization, and that several key enzymes for the pathways share common antigenic groups. Considering the fact that bacteria from many diverse biological groups utilize CO or methanol as a growth substrate, these results suggest that the ability to utilize CO and methanol in mycobacterial carboxydobacteria has been acquired primarily by conservative independent evolution of an ancestral mycobacterium able to grow on both CO and methanol at a very early time before divergence occurred, rather than by genetic exchange through mechanisms dispersing the respective common ancestral genes for CO and methanol utilization to each mycobacterium at some remote time.

ACKNOWLEDGMENT

This work was supported by a research grant (KRF-2000-015-DP0382) from the Korea Research Foundation.

REFERENCES

1. **Anthony, C.** 1982. The biochemistry of methylotrophs. Academic Press, New York, N.Y.
2. **Anthony, C., and L. J. Zatman.** 1967. The microbial oxidation of methanol. Purification and properties of the alcohol dehydrogenase of *Pseudomonas* sp. M27. Biochem. J. **104:**953–959.
3. **Arfman, N., E. M. Watling, W. Clement, R. J. van Oosterwijk, G. E. de Vries, W. Harder, M. M. Attwood, and L. Dijkhuizen.** 1989. Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. Arch. Microbiol. **152:**280–288.
4. **Bartholomew, G. W., and M. Alexander.** 1979. Microbial metabolism of carbon monoxide in culture and in soil. Appl. Environ. Microbiol. **37:**932–937.
5. **Bell, J. M., C. Falconer, J. Colby, and E. Williams.** 1987. CO metabolism by a thermophilic actinomycete, *Streptomyces* strain G26. J. Gen. Microbiol. **133:**3445–3456.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Bystrykh, L. V., N. I. Govorukhina, P. W. van Ophem, H. J. Hektor, L. Dijkhuizen, and J. A. Duine.** 1993. Formaldehyde dismutase activities in Gram-positive bacteria oxidizing methanol. J. Gen. Microbiol. **139:**1979–1985.
8. **Cho, J. W., H. S. Yim, and Y. M. Kim.** 1985. *Acinetobacter* isolate growing with carbon monoxide. Kor. J. Microbiol. **23:**1–8.
9. **Cole, S. T., K. Eiglmeier, J. Parkhill, K. D. James, N. R. Thomson, P. R. Wheeler, N. Honore, T. Garnier, C. Churcher, D. Harris, K. Mungall, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. M. Davies, K. Devlin, S. Duthoy, T. Feltwell, A. Fraser, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, C. Lacroix, J. Maclean, S. Moule, L. Murphy, K. Oliver, M. A. Quail, M. A. Rajandream, K. M. Rutherford, S. Rutter, K. Seeger, S. Simon, M. Simmonds, J. Skelton, R. Squares, S. Squares, K. Stevens, K. Taylor, S. Whitehead, J. R. Woodward, and B. G. Barrell.** 2001. Massive gene decay in the leprosy bacillus. Nature **409:**1007–1011.
10. **Cole, S. T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S. V. Gordon, K. Eiglmeier, S. Gas, C. E. Barry III, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M. A. Quail, M.-A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J. E. Sulston, K. Taylor, S. Whitehead, and B. G. Barrell.** 1998. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. Nature **393:**537–544.
11. **Do, Y. S., E. Kim, and Y. M. Kim.** 1990. Carbon monoxide dehydrogenase inhibitor in cell extracts of *Pseudomonas carboxydovorans*. J. Bacteriol. **172:**1267–1270.
12. **Duine, J. A., J. Frank, and M. P. J. Berkhout.** 1984. NAD-dependent, PQQ-containing methanol dehydrogenase: a bacterial dehydrogenase in a multienzyme complex. FEBS Lett. **168:**217–221.
13. **Ferenci, T., T. Strom, and J. R. Quayle.** 1974. Purification and properties of 3-hexulose phosphate synthase and phospho-3-hexuloisomerase from *Methylococcus capsulatus*. Biochem. J. **144:**477–486.
14. **Gadkari, D., K. Schricker, G. Acker, R. M. Kroppenstedt, and O. Meyer.** 1990. *Streptomyces thermoautotrophicus* sp. nov., a thermophilic CO- and H_2-oxidizing obligate chemolithoautotroph. Appl. Environ. Microbiol. **56:**3727–3734.
15. **Green, P. N.** 1992. Taxonomy of methylotrophic bacteria, p. 23–84. *In* J. C. Merrell and H. Dalton (ed.), Methane and methanol utilizers. Plenum Press, New York, N.Y.
16. **Jones, R. D., and R. Y. Morita.** 1983. Carbon monoxide oxidation by chemolithotrophic ammonium oxidizers. Can. J. Microbiol. **29:**1545–1551.
17. **Kato, N., K. Tsuji, Y. Tani, and K. Ogata.** 1975. Utilization of methanol by an actinomycete, p. 91–98. *In* Microbial growth on C_1 compounds. Proceedings of the 1st International Symposium. The Society of Fermentation Technology, Tokyo, Japan.
18. **Kato, N., N. Miyamoto, M. Shimao, and C. Sakazawa.** 1988. 3-Hexulose phosphate synthase from a new facultative methylotroph, *Mycobacterium gastri* MB19. Agric. Biol. Chem. **52:**2659–2661.
19. **Kim, E. Y., Y. T. Ro, and Y. M. Kim.** 1997. Purification and some properties of ribulose 1,5-bisphosphate carboxylases/oxygenases from *Acinetobacter* sp. strain JC1 and *Hydrogenophaga pseudoflava*. Mol. Cells **7:**380–388.
20. **Kim, K. S., Y. T. Ro, and Y. M. Kim.** 1989. Purification and some properties of carbon monoxide dehydrogenase from *Acinetobacter* sp. strain JC1 DSM 3803. J. Bacteriol. **171:**958–964.
21. **Kim, Y. M., and G. D. Hegeman.** 1981. Purification and some properties of carbon monoxide dehydrogenase from *Pseudomonas carboxydohydrogena*. J. Bacteriol. **148:**904–911.
22. **Kim, Y. M., and G. D. Hegeman.** 1983. Oxidation of carbon monoxide by bacteria. Int. Rev. Cytol. **81:**1–32.
23. **Kraut, M., I. Hugendieck, S. Herwig., and O. Meyer.** 1989. Homology and distribution of CO dehydrogenase structural genes in carboxydotrophic bacteria. Arch. Microbiol. **152:**335–341.
24. **Kwon, M. O., and Y. M. Kim.** 1985. Relationship between carbon monoxide dehydrogenase and a small plasmid in *Pseudomonas carboxydovorans*. FEMS Microbiol. Lett. **29:**155–159.
25. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
26. **Large, P. J., and J. R. Quayle.** 1963. Microbial growth on C_1 compounds. 5. Enzyme activities in extracts of *Pseudomonas* AM1. Biochem. J. **87:**386–396.
27. **Lorite, M. J., J. Tachil, J. Sanjun, O. Meyer, and E. J. Bedmar.** 2000. Carbon monoxide dehydrogenase activity in *Bradyrhizobium japonicum*. Appl. Environ. Microbiol. **66:**1871–1876.
28. **McFadden, B. A., and C. C. L. Tu.** 1967. Regulation of autotrophic and heterotrophic carbon dioxide fixation in *Hydrogenomonas facilis*. J. Bacteriol. **93:**886–893.
29. **Mehta, R. J.** 1975. Pyridine nucleotide-linked oxidation of methanol in methanol-assimilating yeast. J. Bacteriol. **124:**1165–1167.
30. **Meyer, O., and H. G. Schlegel.** 1983. Biology of aerobic carbon monoxide-oxidizing bacteria. Annu. Rev. Microbiol. **37:**277–310.
31. **Meyer, O., S. Jacobitz, and B. Krüger.** 1986. Biochemistry and physiology of aerobic carbon monoxide-utilizing bacteria. FEMS Microbiol. Rev. **39:**161–179.
32. **Nash, T.** 1953. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochemistry **55:**416–421.

33. **Nozhevnikova, A. N., and G. A. Zavarzin.** 1974. On the taxonomy of CO-oxidizing Gram-negative bacteria. Izv. Akad. Nauk. SSSR Ser. Biol. **3:**436–440.
34. **O'Donnell, A. G., C. Falconer, M. Goodfellow, A. C. Ward, and E. Williams.** 1993. Biosystematics and diversity amongst novel carboxydotrophic actinomycetes. Antonie Leeuwenhoek **64:**325–340.
35. **Ouchterlony, O. J., and L. A. Nilson.** 1978. Immunodiffusion and immunoelectrophoresis, p. 19.1–19.44. *In* D. M. Wier (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford, England.
36. **Reed, W. M., and P. R. Dugan.** 1987. Isolation and characterization of the facultative methylotroph *Mycobacterium* ID-Y. J. Gen. Microbiol. **133:**1389–1395.
37. **Ro, Y. T., and Y. M. Kim.** 1993. Constitutive expression of carbon monoxide dehydrogenase in *Acinetobacter* sp. strain JC1 DSM 3803. Kor. J. Microbiol. **31:**214–217.
38. **Ro, Y. T., C. Y. Eom, T. Song, J. W. Cho, and Y. M. Kim.** 1997. Dihydroxyacetone synthase from a methanol-utilizing carboxydobacterium, *Acinetobacter* sp. strain JC1 DSM 3803. J. Bacteriol. **179:**6041–6047.
39. **Ro, Y. T., J. G. Seo, J. Lee, D. Kim, I. K. Chung, T. U. Kim, and Y. M. Kim.** 1997. Growth on methanol of a carboxydobacterium, *Acinetobacter* sp. strain JC1 DSM 3803. J. Microbiol. **35:**30–39.
40. **Seto, N., S. Sakayanagi, and H. Iizuka.** 1975. C_1-compound utilizers isolated from the oil and natural gas fields in Japan, p. 35–44. *In* Microbial growth on C_1 compounds. Proceedings of the 1st International Symposium. The Society of Fermentation Technology, Tokyo, Japan.
41. **Song, T., H. Lee, Y.-H. Park, E. Kim, Y. T. Ro, S. W. Kim, and Y. M. Kim.** 2002. Reclassification of a carboxydobacterium, *Acinetobacter* sp. strain JC1 DSM 3803, as *Mycobacterium* sp. strain JC1 DSM 3803. J. Microbiol. **40:**237–240.
42. **Tani, Y., T. Miya, and K. Ogata.** 1972. The microbial metabolism of methanol. Part II. Properties of crystalline alcohol oxidase from *Kloeckera* sp. no. 2201. Agric. Biol. Chem. **36:**76–83.
43. **van Ophem, P. W., J. van Beeumen, and J. A. Duine.** 1993. Nicotinoprotein [NAD(P)-containing] alcohol/aldehyde oxidoreductases. Purification and characterization of a novel type from *Amycolatopsis methanolica*. Eur. J. Biochem. **212:**819–826.