Cloning and expression analysis of the duplicated genes for carbon monoxide dehydrogenase of *Mycobacterium* sp. strain JC1 DSM 3803

Taeksun Song,^{1,2†} Sae Woong Park,^{1†‡} Su-Jeong Park,³ Ji Hyang Kim,¹ Ji Young Yu,² Jeong-Il Oh³ and Young M. Kim¹

Correspondence

Young M. Kim
young547@yonsei.ac.kr

¹Department of Biology, Yonsei University, Seoul 120-749, Republic of Korea

²Genome Research Center for Respiratory Pathogens, Yonsei University College of Medicine, Seoul 120-752, Republic of Korea

³Department of Microbiology, Pusan National University, Busan 609-735, Republic of Korea

Carbon monoxide dehydrogenase (CO-DH) is an enzyme catalysing the oxidation of CO to carbon dioxide in *Mycobacterium* sp. strain JC1 DSM 3803. Cloning of the genes encoding CO-DH from the bacterium and sequencing of overlapping clones revealed the presence of duplicated sets of genes for three subunits of the enzyme, *cutB1C1A1* and *cutB2C2A2*, in operons, and a cluster of genes encoding proteins that may be involved in CO metabolism, including a possible transcriptional regulator. Phylogenetic analysis based on the amino acid sequences of large subunits of CO-DH suggested that the CO-DHs of *Mycobacterium* sp. JC1 and other mycobacteria are distinct from those of other types of bacteria. The growth phenotype of mutant strains lacking *cutA* genes and of a corresponding complemented strain showed that both of the duplicated sets of CO-DH genes were functional in this bacterium. Transcriptional fusions of the *cutB* genes with *lacZ* revealed that the *cutBCA* operons were expressed regardless of the presence of CO and were further inducible by CO. Primer extension analysis indicated two promoters, one expressed in the absence of CO and the other induced in the presence of CO. This is believed to be the first report to show the presence of multiple copies of CO-DH genes with identical sequences and in close proximity in carboxydobacteria, and to present the genetic evidence for the function of the genes in mycobacteria.

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INTRODUCTION

Carbon monoxide dehydrogenase (CO-DH) is the key enzyme for the oxidation of CO in carboxydobacteria, which grow aerobically with CO as the sole source of carbon and energy (Kim & Hegeman, 1983; Meyer *et al.*, 1993). The genes encoding CO-DH have been cloned from the Gram-negative carboxydobacteria *Pseudomonas*

thermocarboxydovorans, *Oligotropha carboxidovorans* and *Hydrogenophaga pseudoflava*, and functionally characterized. The CO-DH genes are conserved in these organisms and form the same operonic structure, with the ORFs for three subunits of the enzyme in the transcriptional order *cutBCA(MSL)* or *coxMSL* (Kang & Kim, 1999; Pearson *et al.*, 1994; Schübel *et al.*, 1995). However, the CO-DH genes have not yet been cloned from Gram-positive bacteria, although the presence of genes homologous to the CO-DH genes of Gram-negative carboxydobacteria has been reported in the genomes of *Rhodococcus jostii* (GenBank accession no. CP000431), *Saccharopolyspora erythraea* (GenBank accession no. AM420293) and several mycobacterial species (GenBank accession nos AL123456, AM408590, BX248333, CP000325, CP000384, CP000480, CP000518, CP000580, CP000854). Furthermore, little is known about the physiological role and expression of the CO-DH genes under various nutritional conditions, except that CO-dependent transcription of *coxL* in *O. carboxidovorans* has been demonstrated by slot-blot analysis (Santiago *et al.*, 1999).

†These authors contributed equally to this work.

‡Present address: Department of Microbiology and Immunology, Weill Cornell Medical College, NY 10065, USA.

Abbreviations: CO-DH, carbon monoxide dehydrogenase; INT, 2-(4-indophenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride; NO-DH, nitric oxide dehydrogenase.

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Two supplementary figures, showing genomic co-localization of the sequenced regions of λ EMBL3-2 and λ EMBL3-4 as revealed by PCR analysis, and Southern blot analysis of λ EMBL3-5H2 carrying the duplicated copies of *cutBCA* operons, are available with the online version of this paper.

Mycobacterium sp. strain JC1 DSM 3803 is a Gram-positive carboxydobacterium isolated from soil in Seoul, Korea (Cho *et al.*, 1985; Song *et al.*, 2002). CO-DH from the bacterium is similar in molecular mass, subunit structure, cofactor composition and other properties to the enzymes of Gram-negative carboxydobacteria (Gram-negative CO-DH), although not immunologically related to them (Kim *et al.*, 1989). We have also shown that several mycobacterial species are capable of carboxydotrophic growth and possess CO-DH enzymes that share common antigenic epitopes (Park *et al.*, 2003). In addition, we have recently demonstrated that the CO-DHs from several mycobacteria, including *Mycobacterium* sp. JC1 and *Mycobacterium tuberculosis* H37Ra, possess a nitric oxide dehydrogenase (NO-DH) activity (Park *et al.*, 2007). The Gram-negative CO-DHs, however, do not exhibit NO-DH activity (S. W. Park & Y. M. Kim, unpublished results). These results suggest that mycobacterial CO-DHs have divergently evolved from the Gram-negative CO-DHs.

In this study, we cloned and characterized for what is believed to be the first time the genes for mycobacterial CO-DH from *Mycobacterium* sp. JC1 to identify the function of CO-DH genes for growth with CO in

mycobacteria and to explore further the diversity of CO-DH genes in carboxydobacteria, including mycobacteria.

We describe here the presence of duplicated genes in *Mycobacterium* sp. JC1 for CO-DH, both of which encode functional enzymes that are considerably different in amino acid sequence from the Gram-negative CO-DHs, as well as their expression under different nutritional conditions at the transcriptional level.

METHODS

Bacterial strains, phages and cultivation. Details of the bacterial strains, phages and plasmids used in this work are given in Table 1. *Mycobacterium* sp. JC1 was grown at 37 °C in standard mineral base (SMB) medium or Middlebrook 7H9 medium (Becton Dickinson) containing 0.05 % Tween 80 with a gas mixture of 30 % CO/70 % air (SMB-CO and 7H9-CO, respectively), as described elsewhere (Kim & Hegeman, 1981), or in SMB and 7H9 medium supplemented with 0.2 % glucose (SMB-glucose and 7H9-glucose, respectively). Cultivation of *Escherichia coli* and phage infection were performed under the conditions described in Sambrook & Russell (2001).

DNA manipulation and transformation. Genomic DNA of *Mycobacterium* sp. JC1 was isolated from cells grown in 7H9-glucose

Table 1. Bacterial strains, phages and plasmids used in this study

Abbreviations: Ap^r, ampicillin resistant; Hy^r, hygromycin resistant; Km^r, kanamycin resistant.

Strain, phage or plasmid	Description	Reference or source
<i>Mycobacterium</i> strains		
<i>Mycobacterium</i> sp. JC1	Wild-type	Cho <i>et al.</i> (1985), Song <i>et al.</i> (2002)
YK001	<i>Mycobacterium</i> sp. JC1 <i>cutA1</i> ⁻	This study
YK002	<i>Mycobacterium</i> sp. JC1 <i>cutA1</i> ⁻ <i>cutA2</i> ⁻	This study
YK003	YK002 transformed with pSW104	This study
<i>E. coli</i> strains		
DH5α	<i>supE44 lac16 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Gibco
XL1-Blue MRA (P2)	Δ(<i>mcrA</i>)183 Δ(<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 gyrA96 relA1 lac</i> (P2 lysogen)	Stratagene
Phages		
λ EMBL3-2	λ EMBL3 clone carrying <i>cutA1cutB1cutC1</i> and flanking sequences	This study
λ EMBL3-4	λ EMBL3 clone carrying <i>cutA2cutB2cutC2</i> and flanking sequences	This study
λ EMBL3-34	λ EMBL3 clone carrying <i>cutA2cutB2cutC2</i> and flanking sequences	This study
Plasmids		
pBluescript II SK(+)	<i>E. coli</i> cloning vector; Ap ^r	Stratagene
pCUTB1LACZ2	pNC carrying <i>cutB1::lacZ</i> transcriptional fusion	This study
pCUTB2LACZ2	pNC carrying <i>cutB2::lacZ</i> transcriptional fusion	This study
pGEM-7Zf	<i>E. coli</i> cloning vector; Ap ^r	Promega
pKO	Mycobacterial suicide vector with <i>sacB</i> ; Km ^r , Hy ^r	Sherman <i>et al.</i> (2001)
pNBV1	<i>E. coli</i> -mycobacterial shuttle vector with ColE1 and pAL5000 replication origins; Hy ^r	Howard <i>et al.</i> (1995)
pNC	pNBV1 with promoterless <i>lacZ</i> ; Hy ^r	This study
pSW84	pKO carrying a 1401 bp in-frame deletion in <i>cutA1</i> and flanking sequences	This study
pSW104	pNBV1 carrying the intact <i>cutA1</i> gene on the 4424 bp <i>PvuII</i> fragment from pTS16	This study
pTS8	pBluescript II SK(+) carrying the 3242 bp <i>BamHI</i> fragment from λ EMBL3-2	This study
pTS16	pBluescript II SK(+) carrying the 4685 bp <i>EcoRI</i> fragment from λ EMBL3-2	This study
pTS19	pBluescript II SK(+) carrying the 2343 bp <i>EcoRI</i> fragment from λ EMBL3-2	This study
pTS49	pBluescript II SK(+) carrying the 3166 bp <i>BamHI</i> fragment from λ EMBL3-4	This study

medium by the CTAB method (Wilson, 1989) for a large-scale preparation, or using the Genomic DNA Prep kit (Solgent) for a rapid small-scale preparation. *Mycobacterium* sp. JC1 was transformed by electroporation of a 100 µl portion of competent cells prepared according to Parish & Stoker (1998) in a 0.2 cm cuvette using an ECM 630 apparatus (Harvard Apparatus) set to 2.5 kV, 25 µF and 1,000 Ω.

Cloning and DNA sequencing. Genomic DNA from *Mycobacterium* sp. JC1 was partially digested with *Sau3A*I, ligated to lambda EMBL3 DNA that had been digested with *Bam*HI, packaged using Gigapack III Gold Packaging Extracts (Stratagene), and transduced to *E. coli* XL1-blue MRA (P2) cells following the manufacturer's instructions. Subclones of selected lambda clones were constructed, and the sequences of the subclones and nested deletions were determined using the Sequenase Version 2.0 DNA Sequencing kit (United States Biochemical Corp.).

Mutagenesis and complementation. A DNA fragment carrying the complete sequence of the *cutB1C1A1* genes and their flanking regions was cloned by ligation of a 2543 bp *Eco*RV/*Pvu*II fragment from pTS8, a subclone of λ EMBL3-2, into the pBluescript II SK(+) vector digested with *Pvu*II, followed by insertion of a 4424 bp *Pvu*II fragment from pTS16, a subclone of λ EMBL3-2, into the unique *Pvu*II site. After digestion of the resulting clone with *Eco*RV, 6889 and 1190 bp DNA fragments were purified and ligated to yield pSW79, in which the 1401 bp internal sequence of the *cutA1* gene was deleted. The resulting plasmid pSW79 was then digested with *Sac*I, and after filling in the ends using Klenow enzyme (New England Biolabs), a 3218 bp DNA fragment carrying the impaired *cutA1* gene and its flanking sequences was ligated into the pKO vector (Sherman *et al.*, 2001), which had been digested with *Sma*I, to generate pSW84. *Mycobacterium* sp. JC1 was transformed with pSW84 by electroporation, as described by Parish & Stoker (1998), and candidate clones were selected on 7H9 agar plates containing hygromycin (100 µg ml⁻¹). Transformants were grown in 7H9-glucose medium in the absence of hygromycin, and plated on 7H9-glucose agar plates containing 10% (w/v) sucrose. Sucrose-resistant and hygromycin-sensitive colonies were selected and analysed for the deletion of a portion of *cutA1* by PCR employing the oligonucleotide primers 5'-GCATGACGACTGCAGACGTTA-3' and 5'-GTCACCTCGTGACCGCAGCAT-3'. The resulting *cutA1*-defective mutant strain (*cutA1*⁻; strain YK001) was transformed again with pSW84 and screened for sucrose-resistant and hygromycin-sensitive colonies, as described above, to obtain the mutant strain with a double deletion of both *cutA1* and *cutA2* (*cutA1*⁻*cutA2*⁻; strain YK002). Deletion of a portion of the *cutA2* gene was verified in candidate clones by PCR using the same set of primers. Because the primers hybridized with the common flanking sequences upstream and downstream of both *cutA1* and *cutA2*, PCR performed with the double mutant alone resulted in the amplification of a single 1784 bp DNA fragment from both the mutated sequences, instead of simultaneous production of the 3185 bp DNA fragment from the wild-type *cutA2* sequence and the 1784 bp DNA fragment from *cutA1* deletion. For complementation of the *cutA1*⁻*cutA2*⁻ mutant, YK002 was transformed with pSW104, which was constructed by ligation of a 4424 bp *Pvu*II fragment from pTS16 carrying the intact *cutA1* gene to *Pvu*II-digested pNBV1, resulting in strain YK003.

RNA isolation and primer extension. *Mycobacterium* sp. JC1 was grown to mid-exponential phase in 7H9-CO or 7H9-glucose. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen) and Lysing Matrix B (Qbiogene) according to the manufacturer's instructions, with a FastPrep FP120 homogenizer (Thermo Electron Corp.). Primer extension experiments were performed as described elsewhere (Sambrook & Russell, 2001) with an oligonucleotide

primer, 5'-CTCGTACTCGAATGGTCCGG-3', complementary to the nucleotide position 10–29 bp downstream of the *cutB* start codon.

Construction of *lacZ* transcriptional fusion plasmids

pCUTB1LACZ2. To construct the *cutB1::lacZ* transcriptional fusion, the *cutB1* promoter region was amplified using the primers 5'-TGCAAGGATCCGCCGCACCGTTATCGATTTCG-3' (*Bam*HI site underlined) and 5'-CGTCAAGCTTTCGGAGGGATCTGCCTGGC-3' (*Hind*III site underlined). Plasmid pTS19 was used as a template to generate a 1166 bp DNA fragment containing a 478 bp 5' portion of *cutR*, an intergenic region of 314 bp between *cutR* and *cutB1*, and a 374 bp 5' portion of *cutB1*. The PCR product was digested with *Bam*HI and *Hind*III, and cloned into pCV77, yielding the plasmid pCUTB1LACZ1. pCUTB1LACZ1 was then digested with *Cl*aI and *Xba*I, and the resulting 1.2 kb *Cl*aI/*Xba*I fragment was cloned into the promoterless *lacZ* vector pNC, resulting in the plasmid pCUTB1LACZ2.

pCUTB2LACZ2. The *cutB2::lacZ* transcriptional fusion was constructed by amplification of the *cutB2* promoter region using pTS49 as the template, and the primers 5'-GCCGGGATCCTCGACGGGCGCACGGTTCAT-3' (*Bam*HI site underlined) and 5'-CTCGAAGC-TTGTGCTCAAGTCCCTCGGAGG-3' (*Hind*III site underlined). This amplification generated an 847 bp DNA fragment encompassing a 401 bp 3' portion of *orf13*, an intergenic region of 60 bp between *orf13* and *cutB2*, and a 386 bp 5' portion of *cutB2*. The PCR product was cloned into pCV77 and then into pNC, as described above for the construction of pCUTB1LACZ2, resulting in the *cutB2::lacZ* transcriptional fusion plasmid pCUTB2LACZ2.

Enzyme assays and Western blot analysis. Cell-free extract preparation and protein concentration determination were performed as described by Park *et al.* (2007). CO-DH activity was assayed spectrophotometrically by measuring the CO-dependent reduction of 2-(4-indophenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride (INT; $\epsilon_{496}=17.981 \text{ mM}^{-1} \text{ cm}^{-1}$) by the method of Kraut *et al.* (1989). Staining by CO-DH activity in a non-denaturing polyacrylamide gel was carried out as described by Kim & Hegeman (1981). β -Galactosidase activity was measured by the method of Miller (1972).

RESULTS AND DISCUSSION

Cloning of duplicated genes for CO-DH

Subunits of CO-DH, purified from *Mycobacterium* sp. JC1 as described by Kim *et al.* (1989), were separated by SDS-PAGE and transferred onto a PVDF membrane (Millipore), and the amino acid sequence of the N-terminal region of the medium-sized subunit was determined using a protein sequencer (476A-01-120, Applied Biosystems). Based on the sequence MQVPGPFEEYEXAT-SVAHAVSLLDXLGDSAMMVATXXSLLXM (X=unidentified residue), two oligonucleotide primers, BF and BR, were synthesized to amplify the region covering the 5'-end region of the CO-DH medium-sized subunit gene. Primer BF was a 1024-fold degenerate 23-mer (5'-ATGCARGTN-CCNGGNCNTTYYGA-3') derived from the N-terminal sequence MQVPGPFE (underlined in the protein sequence above), and primer BR was a 128-fold degenerate 20-mer (5'-ACCATCATNGCNGARTCNCC-3') designed based on the sequence GDSAMMV (underlined in the protein sequence above). A 0.1 kb DNA fragment was amplified by

Fig. 1. Southern blot hybridization of *Mycobacterium* sp. JC1 genomic DNA and DNAs of selected clones. Genomic DNA (a) and DNAs isolated from four clones (b), λ EMBL3-2, -3, -4 and -6, were digested with *Bam*HI (Bm) and *Eco*RI (Ec) separately, and following agarose gel electrophoresis, were subjected to Southern hybridization with a random-primed DNA probe synthesized based on the 95 bp PCR product corresponding to the 5' end of the medium-sized subunit gene of CO-DH. Phage λ DNA digested with *Pst*I was used as a size marker (M).

PCR using *Mycobacterium* sp. JC1 genomic DNA as a template (data not shown). The DNA was found to be a 95 bp fragment, and its sequence exactly matched the sequence encoding the N-terminal amino acids of the medium-sized subunit of *Mycobacterium* sp. JC1 CO-DH, which had been determined by peptide sequencing. Southern blot hybridization of genomic DNA with a random-primed probe synthesized from the 95 bp PCR product revealed that the probe hybridized to two *Eco*RI fragments (2.3 and 5.2 kb), while the *Bam*HI-digested

DNA revealed a thick band of 3.2 kb, possibly a doublet of two fragments of similar size (Fig. 1a). These results suggested that the gene encoding the medium-sized subunit was duplicated in the genome.

Twenty-four positive clones were obtained by plaque hybridization of the lambda library with the probes prepared using the 95 bp PCR product. Four of these clones, λ EMBL3-2, -3, -4 and -6, were subjected to Southern blot hybridization using the DNA probe after digestion with *Eco*RI (Fig. 1b). The hybridization revealed that λ EMBL3-2, -3 and -6 carried a 2.3 kb *Eco*RI fragment, while λ EMBL3-4 harboured the 5.2 kb *Eco*RI fragment. These results indicated that the gene for the medium-sized subunit of CO-DH was duplicated in the genome of *Mycobacterium* sp. JC1, and that each copy of the gene was cloned separately in the lambda clones. This conclusion was supported by the observation that the 3.2 kb *Bam*HI fragment hybridized to the probe in λ EMBL3-2, -3 and -6 was slightly larger than the fragment in λ EMBL3-4 (Fig. 1b). The nucleotide sequences determined for the insert DNAs in λ EMBL3-2 and -4 revealed that the larger *Bam*HI fragment in λ EMBL3-2 was 3242 bp long and the smaller one in λ EMBL3-4 was 3166 bp long (Fig. 2).

Two positive lambda clones were identified using plaque hybridization of the lambda library, employing another DNA probe prepared using the 707 bp *Pst*I fragment from pTS49, a subclone of λ EMBL3-4, as a template. Restriction analysis of the clones revealed that one of the clones, λ EMBL3-34, carried additional DNA fragments, which were adjacent to those in λ EMBL3-4 (data not shown).

Determination of nucleotide sequences of the lambda clones

Subclones of λ EMBL3-2 and -4 separately digested with *Eco*RI and *Bam*HI were constructed in pBluescript II

Fig. 2. Organization of the genes in the sequenced region of *Mycobacterium* sp. JC1. At the top are 22 genes, including two partial ORFs, *modC* and *orf22*, displayed as arrows indicating the direction of transcription. Genes encoding subunits of CO-DH are shown as solid arrows in the duplicated regions, which are represented by the hatched bars below. Vertical lines below the scale of 21 721 bp are restriction sites for *Bam*HI and *Eco*RI. Solid lines designate the *Bam*HI and *Eco*RI fragments used to determine the nucleotide sequences, the lengths of which are noted above the lines. Dotted lines denote the *Bam*HI and *Eco*RI fragments revealed by Southern blot hybridization shown in Fig. 1. The probe binding sites are marked with asterisks. Regions covered by each lambda clone are indicated at the bottom.

SK(+), and the sequences of the inserts in the subclones were determined. The sequences were compared with the known sequences of CO-DH genes from Gram-negative carboxydobacteria (Kang & Kim, 1999; Pearson *et al.*, 1994; Schübel *et al.*, 1995), and restriction maps of the insert DNA in the lambda clones were generated, based on the assumption that the transcriptional order of CO-DH genes in *Mycobacterium* sp. JC1 is the same as that in the Gram-negative carboxydobacteria.

Sequencing of two overlapping fragments in each of the clones λ EMBL3-2 and λ EMBL3-4 (a 3242 bp *Bam*HI and a 4685 bp *Eco*RI fragment in λ EMBL3-2, and a 3166 bp *Bam*HI and a 4685 bp *Eco*RI fragment in λ EMBL3-4; Fig. 2) revealed the presence, in each clone, of three complete ORFs of 2394, 873 and 486 bp. The ORFs encoded proteins with calculated molecular masses of 85 967, 30 620 and 17 389 Da, respectively, comparable with those obtained by denaturing PAGE of the purified CO-DH (85 000, 34 000 and 16 000 Da, respectively; Kim *et al.*, 1989). The genes were homologous to the genes encoding the three subunits of CO-DH in Gram-negative carboxydobacteria and organized in the same transcriptional order as that of the Gram-negative CO-DH genes. We therefore named the genes in λ EMBL3-2 and λ EMBL3-4 *cutB1C1A1* and *cutB2C2A2*, respectively.

Targeted deletion of the *cutA* genes as described in Methods often resulted in simultaneous deletions of both *cutA1* and *cutA2* along with flanking genes, providing a clue that both copies of the genes might be localized in close proximity. Therefore, the outward primers for both ends of the sequences designed for λ EMBL3-2 and λ EMBL3-4 as described above were employed in combinations for PCR analysis to verify the relative location of the sequences in the genome of *Mycobacterium* sp. JC1 (Supplementary Fig. S1). Only the combination of the primers 243F1 at the 3' end of the 4685 bp *Eco*RI fragment, which is common to λ EMBL3-2 and λ EMBL3-4, and 45R1 at the 5' end of the 3166 bp *Bam*HI fragment in λ EMBL3-4 resulted in amplification of an approximately 3.2 kb DNA fragment, indicating that the sequenced region in λ EMBL3-4 was located downstream of that in λ EMBL3-2 in the genome. PCR amplification of the portions of chromosomal DNA with additional pairs of primers and restriction digestion of the PCR products further supported the idea that the sequenced regions are in close proximity in the genome (Supplementary Fig. S1). Subsequently, the nucleotide sequence of the *Bam*HI fragment located upstream of the 3166 bp *Bam*HI fragment in λ EMBL3-4 was determined, and the resulting sequence of 3726 bp was found to overlap, on one end, with the 3' portion of the sequence from λ EMBL3-2 (Fig. 2). The sequences were verified by determining the sequence of the 3.2 kb DNA fragment amplified by PCR, using genomic DNA as a template and the pair of the outward primers. The co-localization of *cutB1C1A1* and *cutB2C2A2* operons was also confirmed by Southern blot

analysis of another λ EMBL3 clone carrying both copies of the operons (Supplementary Fig. S2).

Additional sequencing of a *Bam*HI fragment from λ EMBL3-34, which is 3765 bp in length, resulted in a single sequence of 21 721 bp (Fig. 2).

Characteristics of the cloned gene cluster

A total of 22 ORFs, 20 complete and two partial, were found in the 21 721 bp sequence. Seven of these, including the three ORFs encoding CO-DH subunits, were duplicated in two nearly identical sequences of 6845 and 6859 bp (Table 2, Fig. 2). The only difference between the two sequences was the presence of a 14 bp direct repeat (5'-CCTGATCTGAAGAA-3') between *cutB2* and *cutC2*, which did not seem to affect translation of the genes.

It has been shown that several α -proteobacteria [*Mesorhizobium plurifarium*, *Stappia aggregate* (King & Weber, 2007) and *Silicibacter pomeroyi* (GenBank accession nos CP000031, CP000032)], β -proteobacteria [*Burkholderia xenovorans* (GenBank accession nos CP000270–000272)] and actinobacteria [*R. jostii* (GenBank accession no. CP000431)] also have multiple copies of CO-DH genes. The sequences of multiple copies of CO-DH genes in these bacteria are not identical to each other, however, unlike the CO-DH genes in *Mycobacterium* sp. JC1. It has also not been shown that the CO-DH genes in any of these bacteria are in close proximity in the chromosome, as is the case with *Mycobacterium* sp. JC1.

Comparison of the deduced amino acid sequences of CO-DH in *Mycobacterium* sp. JC1 with those in other bacteria indicated that the CO-DH of *Mycobacterium* sp. JC1 was distinct from the non-mycobacterial CO-DHs, except that of *R. jostii* RHA1, which contains two non-identical CO-DH gene homologues. The amino acid sequences of large, medium and small subunits, respectively, of *Mycobacterium* sp. JC1 CO-DH were 42.6–60.7, 34.6–44.7 and 52.0–65.8 % identical to those of the non-mycobacterial CO-DHs and one of the two *R. jostii* RHA1 CO-DHs (GenBank accession nos ABG96757, AM420293, CP000031, CP000270, CP000272, CP001275, U80806, X77931 and X82447), but 87.6–89.9, 68.6–74.6 and 79.7–88.0 % identical to those of mycobacterial CO-DHs and the other *R. jostii* RHA1 CO-DHs (GenBank accession nos ABG97013, AL123456, AM408590, BX248333, CP000325, CP000384, CP000480, CP000518, CP000580 and CP000854). These differences may explain the previous finding that CO-DH of *Mycobacterium* sp. JC1 has no epitopes identical to those of the non-mycobacterial CO-DHs (Kim *et al.*, 1989).

Several mycobacterial species have been shown to consume CO (King, 2003; Park *et al.*, 2003) and to have nucleotide sequences corresponding to a part of the large subunit of CO-DH (King, 2003). Phylogenetic analyses with a part of the deduced amino acid sequences of the tentative large subunit genes of CO-DHs in other mycobacteria have also suggested that mycobacterial CO-DHs form a subclade

Table 2. ORFs identified in the sequenced region of *Mycobacterium* sp. JC1

ORF	Alias	Homologue (gene or CDS, percentage identity*)	
		<i>M. tuberculosis</i> H37Rv†	<i>O. carboxidovorans</i> OM5‡
<i>orf1</i>	<i>modC</i>	Molybdenum ABC transporter ATP-binding protein (<i>modC</i> , 61)	No significant match found
<i>orf2</i>	<i>moaE2</i>	Molybdenum cofactor biosynthesis protein E2 (<i>moaE2</i> , 74)	Molybdopterin converting factor, subunit 2 (<i>moaE</i> , 30)
<i>orf3</i>	<i>cutR</i>	LysR-family transcriptional regulator (Rv0377, 73)	Transcriptional regulator, LysR family (OCAR5079, 32)
<i>orf4</i> , <i>orf14</i>	<i>cutB1</i> , <i>cutB2</i>	CO-DH medium subunit (Rv0375c, 71)	CO-DH medium chain (<i>coxM</i> , 38)
<i>orf5</i> , <i>orf15</i>	<i>cutC1</i> , <i>cutC2</i>	CO-DH small subunit (Rv0374c, 81)	CO-DH small chain (<i>coxS</i> , 58)
<i>orf6</i> , <i>orf16</i>	<i>cutA1</i> , <i>cutA2</i>	CO-DH large subunit (Rv0373c, 87)	CO-DH large chain (<i>coxL</i> , 55)
<i>orf7</i> , <i>orf17</i>		Hypothetical protein (Rv0372c, 68)	Biosynthesis of the [CuSMoO ₂] cluster (<i>coxF</i> , 31)
<i>orf8</i> , <i>orf18</i>		Hypothetical protein (Rv0371c, 62)	4-Diphosphocytidyl-2C-methyl-D-erythritol synthase (OCAR5486, 30§)
<i>orf9</i> , <i>orf19</i>		Oxidoreductase (Rv0370c, 78)	Biosynthesis of the [CuSMoO ₂] cluster (<i>coxD</i> , 49)
<i>orf10</i> , <i>orf20</i>		Membrane oxidoreductase (Rv0369c, 55)	Membrane anchor of CO-DH (<i>coxG</i> , 25§)
<i>orf11</i>		No significant match found	No significant match found
<i>orf12</i>		Hypothetical protein (Rv1592c, 26§)	No significant match found
<i>orf13</i>		Hypothetical protein (Rv0376c, 73)	Biosynthesis of the [CuSMoO ₂] cluster (<i>coxF</i> , 28)
<i>orf21</i>		Hypothetical protein (Rv0368c, 62)	Biosynthesis of the [CuSMoO ₂] cluster (<i>coxE</i> , 30§)
<i>orf22</i>		No significant match found	No significant match found

*The percentage identity was obtained by aligning the deduced amino acid sequences using BLASTP.
†GenBank accession number AL123456.
‡GenBank accession number X82447.
§Only a portion of the amino acid sequence was aligned. The percentage identity was calculated from the homologous region.

distinct from other CO-DHs in other bacteria (King, 2003). In fact, phylogenetic analysis revealed that CO-DHs are divided into three groups: Gram-negative CO-DHs, Gram-positive CO-DHs and mycobacterial CO-DHs (Fig. 3). The mycobacterial CO-DHs were also divided into two subgroups: those of fast-growing (*Mycobacterium* sp. JLS, *Mycobacterium* sp. JC1 and *Mycobacterium smegmatis*) and slow-growing mycobacteria (*Mycobacterium bovis* and *M. tuberculosis*). Considering the finding that NO-DH activity is inherent in mycobacterial CO-DH, but not in non-mycobacterial CO-DH (Park *et al.*, 2007), further studies

on the evolution of mycobacterial CO-DHs might be worthwhile.

An ORF in the 5' upstream region of *cutB1C1A1* was noted as a possible regulatory element for the expression of *cutB1C1A1*. The deduced amino acid sequence of the ORF was similar to transcriptional regulators that belong to the LysR family (Schell, 1993). Genes for LysR-type regulators are generally transcribed divergently from the genes that are under their transcriptional control, and LysR-type regulators bind to the intergenic region, which is consistent

Fig. 3. Phylogenetic analysis of CO-DH large subunits. Deduced amino acid sequences from bacterial genes encoding the CO-DH large subunit were aligned using CLUSTAL_X (Larkin *et al.*, 2007), and the tree was generated by neighbour-joining (Poisson correction model) using MEGA 3.1 (Kumar *et al.*, 2004). The GenBank accession numbers for the sequences are given in parentheses. Bootstrap values were calculated from 1000 replicates.

with the organization of the ORF and *cutB1C1A1*. The helix–turn–helix motif that is crucial for the ability of LysR-type regulators to bind DNA was also well conserved at the N terminus of the presumed ORF protein. The ORF was named *cutR*, as a possible transcriptional regulator of CO-DH genes.

Further analysis of the upstream region of *cutB1C1A1* resulted in the identification of another ORF, *moaE2*, similar to genes of the MoaE superfamily, which are involved in the metabolism of molybdopterin (Marchler-Bauer *et al.*, 2007). A partial ORF was also identified in the 5' upstream region of the tentative *moaE2* gene that is homologous to *modC*, which encodes a component of the ATP-binding cassette (ABC) transporter for molybdate in bacteria (Walkenhorst *et al.*, 1995). Considering that molybdopterin is an essential cofactor for CO-DH activity and contains molybdate as a functional component (Dobbek *et al.*, 1999), it is plausible that genes involved in the biosynthesis of CO-DH may be clustered in this region.

Downstream of the *cutB1C1A1* and *cutB2C2A2* genes were other ORFs that are conserved in several mycobacteria in terms of both their genomic organization and their

sequence. Among them were several ORFs with homology to the ORF found downstream of the CO-DH structural genes in the Gram-negative carboxydobacterium *O. carboxidovorans*, but in a different genomic organization (Fuhrmann *et al.*, 2003; Table 2). The cluster of genes in *O. carboxidovorans* is induced in the presence of CO, implying that these genes are related to CO oxidation (Santiago *et al.*, 1999). In fact, CoxD in *O. carboxidovorans*, which is homologous to the protein products deduced from *orf9* and *orf19* of *Mycobacterium* sp. JC1, has recently been shown to function in the introduction of sulfur and copper into the [MoO₃] centre of *O. carboxidovorans* CO-DH (Pelzmann *et al.*, 2009). Functional studies of protein products of *Mycobacterium* sp. JC1 ORFs identified in this study might reveal the detailed mechanism of CO-DH assembly and the role of these proteins in CO utilization in carboxydobacteria.

Analysis of *cutA* mutants

Mycobacterium sp. JC1 mutant strains YK001 and YK002, with deletions of *cutA1* alone and of both *cutA1* and *cutA2*, respectively, were constructed and analysed for their ability to grow on CO as a source of carbon and energy. Strain

Fig. 4. Analysis of *cutA* mutants and a complemented strain. (a, b) Defect in growth of *cutA* mutants and recovery of growth in a complemented strain. Cells of *Mycobacterium* sp. JC1 wild-type (●), a *cutA1*⁻ mutant (○, YK001), a *cutA1*⁻ *cutA2*⁻ double mutant (■, YK002) and the *cutA1*⁻ *cutA2*⁻ double mutant complemented with *cutA1* (□, YK003) were grown in SMB-CO medium (a) or 7H9-CO medium (b). Growth was monitored by measuring OD₄₃₆. (c, d) CO-DH activity and expression of CO-DH subunits in *cutA* mutants. Twenty micrograms of cell-free extract from *Mycobacterium* sp. JC1 wild-type (lane 1), YK001 (lane 2) and YK002 (lane 3), grown to mid-exponential phase in SMB-glucose medium, were subjected to staining based on CO-DH activity after non-denaturing PAGE on a 7.5% acrylamide gel [(c), Coomassie brilliant blue-stained gel on the left], and Western blotting with antisera raised against purified CO-DH of *Mycobacterium* sp. JC1 following SDS-PAGE on a 12.5% acrylamide gel (d). (e) Recovery of CO-DH activity in a complemented strain. Twenty micrograms of cell-free extract from *Mycobacterium* sp. JC1 wild-type (lane 1) and YK003 (lane 2) grown to mid-exponential phase in SMB-glucose medium were subjected to staining based on CO-DH activity after non-denaturing PAGE on a 7.5% acrylamide gel (Coomassie brilliant blue-stained gel on the left).

YK001 was not greatly affected, but strain YK002 was completely inhibited in its ability to grow on CO (Fig. 4a), suggesting that *cutA2*, at least, is necessary for growth of *Mycobacterium* sp. JC1 on CO. Meanwhile, the defect in growth on CO of YK002 was restored by complementation with *cutA1* (Fig. 4b), indicating that either *cutA1* or *cutA2* alone is sufficient to support the growth of *Mycobacterium* sp. JC1 on CO. These results suggest that both the duplicated CO-DH genes are functional in *Mycobacterium* sp. JC1. Activity assays for CO-DH and immunoblot analysis using rabbit antisera raised against the purified CO-DH of *Mycobacterium* sp. JC1 showed that the mutant YK002 did not produce the large subunit of CO-DH and exhibited no CO-DH activity (Fig. 4c, d), while the mutant complemented with *cutA1* produced CO-DH and exhibited CO-DH enzyme activity (Fig. 4e).

Transcriptional regulation of the *cutBCA* operons

The three CO-DH structural genes in *O. carboxidovorans* and *H. pseudoflava* have previously been shown to constitute an operon (Kang & Kim, 1999; Schübel *et al.*, 1995). Northern blot analysis using total RNA prepared from cells of *Mycobacterium* sp. JC1 grown in 7H9-glucose and DIG-labelled DNA probes complementary to the transcriptional products of *cutB*, *cutC* and *cutA* revealed that the *cutBCA* genes in *Mycobacterium* sp. JC1 were transcribed as a monocistronic mRNA (data not shown).

It was previously observed that CO-DH is synthesized in *Mycobacterium* sp. JC1 during the stationary growth phase on glucose in the absence of CO, and the amount of CO-DH synthesized is increased when CO is supplied as the sole source of carbon and energy (Kim & Kim, 1989). This evidence suggests that the CO-DH in *Mycobacterium* sp. JC1 is produced even in the absence of CO, and that the synthesis of the enzyme is induced by CO. In good agreement with this previous result, a significant level of CO-DH activity was detected from *Mycobacterium* sp. JC1 grown to mid-exponential phase on SMB-glucose even in the absence of CO [66.4 nmol INT reduced (mg protein)⁻¹ min⁻¹]. The bacterium grown to mid-exponential phase on SMB-CO exhibited a 2.8-fold higher CO-DH activity [184 nmol INT reduced (mg protein)⁻¹ min⁻¹] than the strain grown on glucose. To elucidate the expression pattern of each of the *cutBCA* genes at the transcriptional level, the pCUTB1LACZ2 and pCUTB2LACZ2 plasmids, containing the *cutB1::lacZ* and *cutB2::lacZ* transcriptional fusions, respectively, were constructed, introduced into *Mycobacterium* sp. JC1, and then tested to determine the expression levels of *cutB1* and *cutB2* in cells grown with glucose or CO. While the bacterium harbouring the empty vector pNC exhibited virtually no β -galactosidase activity (Fig. 5), considerable promoter activities of both *cutB1* and *cutB2* were detected from the bacterium grown heterotrophically on glucose. The promoter activities of *cutB1* and *cutB2* in the bacterium grown on CO were increased by 1.4- and 2.7-fold, respectively, compared with those in the

Fig. 5. Expression of the *cutB1C1A1* and *cutB2C2A2* operons in *Mycobacterium* sp. JC1. For the determination of the promoter activities of *cutB1C1A1* and *cutB2C2A2*, *Mycobacterium* sp. JC1 harbouring pCUTB1LACZ2 (*cutB1::lacZ*) and pCUTB2LACZ2 (*cutB2::lacZ*), respectively, was grown to OD₆₀₀ 0.7 on SMB-glucose (open bars) or SMB-CO (solid bars), and β -galactosidase activities were measured. *Mycobacterium* sp. JC1 transformed with pNC was used as a negative control (Promoterless). All values provided are the averages of two independent measurements. Error bars, SD.

bacterium grown on glucose, which is consistent with the result that the CO-DH activity was increased in cells grown with CO. The present results indicate that the *cutB1C1A1* and *cutB2C2A2* operons in *Mycobacterium* sp. JC1 are expressed even in the absence of CO, and that their expression is also inducible by CO, with *cutB2C2A2* being more strongly induced by CO than *cutB1C1A1*.

Determination of the transcriptional start points of the *cutBCA* operons

The potential transcriptional start sites of the *cutB1C1A1* and *cutB2C2A2* operons were determined by primer extension analysis using total RNAs prepared from glucose- and CO-grown cells of *Mycobacterium* sp. JC1. Since the 233 bp DNA sequences upstream of the start codons of *cutB1* and *cutB2* are identical, primer extension analysis for both *cutB1* and *cutB2* was performed at the same time. Two extension products, a strong and a weak product, transcribed from the nucleotides corresponding to 116 bp (G) and 30 bp (C) upstream of the translational start sites of the *cutB* genes, respectively, were identified (Fig. 6a). The strong product (P2) was expressed regardless of the presence or absence of CO, whereas the weak product (P1) was strongly induced in CO-grown cells compared with glucose-grown cells. The primer extension results suggest the following: (i) two promoters are present in the region upstream of *cutB1* and *cutB2*; and (ii) transcription of P1 is inducible by CO, and transcription of P2 accounts for the

Fig. 6. Promoter regions of the *cutBCA* operons. (a) Primer extension analysis of *cutB* mRNA. Total RNA isolated from *Mycobacterium* sp. JC1 grown on SMB-glucose (Glu) or SMB-CO (CO) was subjected to primer extension analysis employing an oligonucleotide primer complementary to the 5' portion of the *cutB* gene. Two transcription initiation sites, P1 and P2, were identified. The bases corresponding to the transcriptional start sites are in upper-case type. (b) Nucleotide sequence of the 5'-flanking regions of *cutB1* and *cutB2*. The positions of the identified transcriptional start sites are marked by +1. The putative -35 and -10 regions of CO-induced P1 are boxed. The translational start site (ATG) of *cutB* is indicated by the arrow with the gene name. Three inverted repeat sequences (IR1, IR2 and IR3) are marked by thick underlining. The 233 bp upstream region of *cutB1* is identical to that of *cutB2*, and the nucleotide sequence in *cutB1* that is duplicated upstream of *cutB2* is underlined.

expression of the *cutBCA* operons in the absence of CO. Sequences resembling the mycobacterial -35 and -10 regions (Agarwal & Tyagi, 2006) were identified immediately upstream of P1 (Fig. 6b), but potential promoter sequences resembling the mycobacterial -35 and -10 regions were not identified immediately upstream of P2. Interestingly, an inverted repeat sequence (CGAGTTAAG-N₆-CTTAAGTCG; IR1) was found immediately upstream of the putative -35 region (Fig. 6b). A similar inverted repeat sequence (TTAAG-N₆-CTTAA; IR2) was also identified 274 bp upstream of the translational start site of *cutB1*, but not that of *cutB2*. The IR1 and IR2 sequences contain a LysR motif (T-N₁₁₋₁₂-A; Schell, 1993), and therefore are assumed to be the binding sites for a LysR-family transcriptional regulator. Another inverted repeat sequence (TGTGA-N₆-TCACA; IR3) is located immediately upstream of the translational start of *cutB1* and *cutB2* (Fig. 6b). The sequence is completely identical to the known binding site for Crp/Fnr-family proteins (Akhter *et al.*, 2008; Bai *et al.*, 2005).

In this study, we cloned and characterized the duplicated genes for CO-DH in *Mycobacterium* sp. JC1. This is believed to be the first report to show the duplication of CO-DH genes with identical sequences and in close

proximity in carboxydobacteria and to present the genetic evidence for the function of the CO-DH genes in mycobacteria. Phylogenetic analysis of CO-DHs revealed that mycobacterial CO-DHs form a subclade distinct from CO-DHs in other carboxydobacteria, possibly implying their unique NO-DH activity. In addition to the *cutBCA* genes encoding CO-DH, several other genes which could be associated with CO oxidation, including a possible positive regulator (*cutR*) of *cutBCA* gene expression, were found to be clustered in the genomic region. Transcriptional analysis of the *cutBCA* genes and computational analysis of the promoter region revealed several sequence elements that may serve as the binding sites for regulatory proteins of gene expression. Further studies on the putative gene products in terms of their interaction with the sequence elements should provide invaluable information on the regulatory mechanism of gene expression involved in CO oxidation, about which very little is yet known.

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