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Identification of *trans*- and *cis*-Control Elements Involved in Regulation of the Carbon Monoxide Dehydrogenase Genes in *Mycobacterium* sp. Strain JC1 DSM 3803▽

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The *cutR* gene was identified 314 bp upstream of the divergently oriented *cutB1C1A1* operon encoding carbon monoxide (CO) dehydrogenase in *Mycobacterium* sp. strain JC1. Its deduced product was composed of 320 amino acid residues with a calculated molecular mass of 34.1 kDa and exhibits a basal sequence similarity to the regulatory proteins belonging to the LysR family. Using a *cutR* deletion mutant, it was demonstrated that CutR is required for the efficient utilization of CO by *Mycobacterium* sp. strain JC1 growing with CO as the sole source of carbon and energy. CutR served as a transcriptional activator for expression of the duplicated *cutBCA* operons (*cutB1C1A1* and *cutB2C2A2*) and was involved in the induction of the *cutBCA* operons by CO. The *cutBCA* operons were also subjected to catabolite repression. An inverted repeat sequence (TGTGA-N_6-TCACA) with a perfect match with the binding motif of cyclic AMP receptor protein was identified immediately upstream of and overlapping with the translational start codons of *cutB1* and *cutB2*. This palindrome sequence was shown to be involved in catabolite repression of the *cutBCA* operons. The transcription start point of *cutR* was determined to be the nucleotide G located 36 bp upstream of the start codon of *cutR*. Expression of *cutR* was higher in *Mycobacterium* sp. strain JC1 grown with glucose than that grown with CO.

Carboxydobacteria are a group of bacteria that are able to grow on carbon monoxide (CO) as the sole source of carbon and energy under aerobic conditions (18, 24). Carboxydotrophic bacteria were identified in both Gram-negative and -positive bacteria (4, 15, 16, 18, 21, 24). We have previously shown that several mycobacteria, including *Mycobacterium* sp. strain JC1 and *Mycobacterium tuberculosis* H37Ra, were able to grow on CO as the sole source of carbon and energy (5, 27).

The key enzyme for the utilization of CO by carboxydobacteria is CO dehydrogenase (CO-DH), which catalyzes oxidation of CO to CO_2, using H_2O as the oxidant (18, 24). The electrons produced by the oxidation of CO are transferred to the respiratory electron transport chain to generate proton motive force, and CO_2 is assimilated for the carbon source (24). The CO-DH has the quaternary structure of $L_2M_2S_2$ with molybdopterin cytosine dinucleotide, flavin adenine dinucleotide (FAD), and two different [Fe-S] centers as cofactors (9, 15).

Mycobacterium sp. strain JC1, a nonpathogenic, fast-growing mycobacterium isolated in Korea, is a carboxydotrophic mycobacterium capable of growing aerobically on CO as the sole carbon and energy source (5, 37). This bacterium can also grow methylotrophically on methanol and heterotrophically using various organic compounds (32). The CO-DH of *Mycobacterium* sp. strain JC1 is similar in molecular weight, subunit structure, cofactor composition, and other properties to those of Gram-negative carboxydobacteria, but it differs in stability when exposed to oxygen (19). The CO-DH of this bacterium also has been shown to be immunologically unrelated with the enzymes of Gram-negative carboxydobacteria such as *Oligotropha carboxidovorans* and *Pseudomonas carboxydohydrogena* but related to other mycobacterial CO-DHs (19). It was demonstrated that the synthesis of heme oxygenase-1 is induced in mouse macrophages infected with *M. tuberculosis*, as well as at the site of infection in a mouse model (23). The resulting increase in the local concentration of CO (a by-product of heme oxygenase reaction) might affect negatively the growth of *M. tuberculosis* since CO inhibits heme-containing terminal oxidases of the respiratory electron transport chain. In this respect, the mycobacterial CO-DHs possibly serve as a CO defense system. Furthermore, the CO-DHs of *Mycobacterium* sp. strain JC1 and other mycobacteria possess the nitric oxide dehydrogenase (NO-DH) activity that may be involved in the protection of mycobacterial pathogens from nitrosative stress during infection, which is devoid in the Gram-negative carboxydobacterial CO-DHs (28). This unique property of mycobacterial CO-DHs supports the idea that the mycobacterial CO-DHs may have divergently evolved from the Gram-negative enzymes.

The genes encoding CO-DHs have been cloned from *Pseudomonas thermocarboxydovorans*, *O. carboxidovorans*, *Hydrog-*

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Source or reference
Strains		
Mycobacterium sp. strain JC1	Wild type (DSM 3803)	5, 37
Mycobacterium sp. strain ΔCutR	*cutR* deletion mutant derived from *Mycobacterium* sp. strain JC1	This study
E. coli DH5α	*supE44 lac16* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 Δthi relA1*	14
Plasmids		
pKO	Hyg^r; *sacB*, suicide vector	7
pNBV1	Hyg^r; 5.8-kb plasmid derived from p16R1	12
pCV77	Km^r; promoterless *lacZ*	MediImmune
pNC	Hyg^r; promoterless *lacZ*	38
pTS19	pBluescript II SK(+) containing the 995-bp *cutR* and *cutB1* of *Mycobacterium* sp. strain JC1	38
pTS49	pBluescript II SK(+) containing the 873-bp *cutB2* of *Mycobacterium* sp. strain JC1	38
pSJ1	pBluescript II KS(+)::1,304-bp EcoRI-BamHI fragment containing *cutR*	This study
pSJ2	pBluescript II KS(+)::1,039-bp EcoRI-BamHI fragment containing the Δ*cutR* mutation	This study
pSJ3	pKO::1,051-bp HindIII-BamHI fragment containing the Δ*cutR* mutation	This study
pCUTR	pNBV1::1,316-bp HindIII-BamHI fragment containing *cutR*	This study
pCUTB1LACZ2	pNC::1,192-bp ClaI-XbaI fragment containing the *cutB1* and *cutR* intergenic region	38
pCUTB2LACZ2	pNC::872-bp ClaI-XbaI fragment containing the *cutB2* and *orf1* intergenic region	38
pCUTB2up461	pNC::461-bp upstream region of *cutB2*	This study
pCUTB2up82	pNC::82-bp upstream region of *cutB2*	This study
pCUTB2up58	pNC::58-bp upstream region of *cutB2*	This study
pCUTB2CBSPM	The same construct as pCUTB2up461 except point mutations in the putative CRP-binding site upstream of *cutB2*	This study
pBScutB2	pBluescript II KS(+)::899-bp BamHI-ClaI fragment from pCUTB2LACZ2	This study

[a] Hyg^r, hygromycin resistance; Km^r, kanamycin resistance.

enophaga pseudoflava, and *Mycobacterium* sp. strain JC1 and sequenced (4, 15, 34, 38). The open reading frames for the three subunits of CO-DH (either *cutA*, *cutB*, and *cutC* or *coxL*, *coxM*, and *coxS* for large, medium, and small subunits, respectively) form an operon in the transcriptional order of *cutBCA* (*coxMSL*). Two copies of the *cutBCA* operons encoding the structural genes of CO-DH occur in *Mycobacterium* sp. strain JC1 (38). The open reading frames encoding amino acid sequences similar to those of the CO-DH structural genes were also identified in the sequenced genomes of other mycobacterial species, which is in good agreement with the fact that the members of the genus *Mycobacterium* have an intrinsic ability of carboxydotrophic growth (20, 27, 38).

Although the CO-DHs were synthesized in *Mycobacterium* sp. strain JC1 and *H. pseudoflava* during growth on organic substrates even in the absence of CO, synthesis of the enzymes was further induced by CO (16, 31, 38). The regulatory molecule(s) and mechanism, by which the CO-DH genes are induced by CO or repressed in the presence of organic substrates, have not been reported yet. We first report *cis*- and *trans*-acting elements involved in the regulation of the *cutBCA* operons in *Mycobacterium* sp. strain JC1 and then discuss their implications in the regulation of the operons.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation conditions. The bacterial strains and plasmids used here are listed in Table 1. *Mycobacterium* sp. strain JC1 DSM 3803 was grown at 37°C in standard mineral base (SMB) medium with a gas mixture of 30% (vol/vol) CO–70% (vol/vol) air (SMB-CO) or SMB supplemented with 0.2% (wt/vol) glucose (SMB-glucose) as described previously (18, 27). For the construction of a mutant, Middlebrook 7H9 medium (Becton Dickinson, Sparks, MD) supplemented with 0.2% (wt/vol) glucose (7H9-glucose) was used to cultivate *Mycobacterium* sp. strain JC1. *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani medium (LB). Ampicillin (50 μg/ml for *E. coli*) and hygromycin (70 μg/ml for *Mycobacterium* sp. strain JC1 and 200 μg/ml for *E. coli*) were added to culture medium when required.

DNA manipulation and electroporation techniques. Standard protocols or manufacturer's instructions were followed for recombinant DNA manipulations (33). The introduction of plasmids into *Mycobacterium* sp. strain JC1 was carried out as described elsewhere (36).

Construction of a *cutR* mutant. For the construction of a deletion mutation of *cutR*, a 1,304-bp DNA fragment containing the *cutR* gene was amplified from pTS19 by PCR using *PFU* Turbo DNA polymerase and the primers cutREcoRI+ (5′-CCCGGAATTCCTCGTCTGATCCGCGGT-3′) and cutRBamHI− (5′-GC TAGGATCCGTTGCCGCCGCGATGG-3′). The PCR product was digested with EcoRI and BamHI and cloned into pBluescript II KS(+) to give the plasmid pSJ1. pSJ1 was digested with PstI and self-ligated to delete the 256-bp internal PstI fragment within *cut*R, yielding pSJ2. Finally, a 1,051-bp HindIII and BamHI fragment from pSJ2 was cloned into the suicide vector pKO1, which was restricted with the same enzymes. The resulting plasmid pSJ3 was introduced into *Mycobacterium* sp. strain JC1 by electroporation. Heterogenotes of the strain JC1, generated by a single recombination event, were selected for their hygromycin resistance, and homogenotes were obtained from the heterogenotes after a second recombination for sucrose resistance on 7H9-glucose plates containing 10% (wt/vol) sucrose. The allelic exchange in the homogenotes that produced isogenic *cutR* deletion (ΔCutR) mutants was verified by PCR.

Construction of plasmids. (i) pCUTR. A 1,051-bp BamHI/HindIII fragment containing the *cutR* gene was isolated from pSJ1 and cloned into the shuttle vector pNBV1 digested with BamHI and HindIII, resulting in pCUTR, which was used to complement the ΔCutR mutant (ΔCutR mt).

(ii) pCUTB2up461, pCUTB2up82, pCUTB2up58, and pCUTB2CBSPM. pCUTB2up461, pCUTB2up82, and pCUTB2up58 are the *lacZ* transcriptional fusion plasmids that contain the 5′ portion of *cutB2*, as well as 461-, 82-, and 58-bp DNA sequences upstream of the *cutB2* start codon, respectively. In order to construct these plasmids, the promoter regions of the corresponding lengths were amplified by PCR using the forward primers with an XbaI restriction site

and the reverse primers with a ClaI restriction site. pCUTB2LACZ2 was used as the template for PCR. The PCR products were restricted with XbaI and ClaI and cloned into pNC, resulting in the plasmids pCUTB2up461, pCUTB2up82, and pCUTB2up58. To construct pCUTB2CBSPM, site-directed mutagenesis was performed using the plasmid pBScutB2 as the template and the QuikChange site-directed mutagenesis kit (Stratagene). pBScutB2 was constructed by cloning of a 899-bp BamHI/ClaI fragment from pCUTB2LACZ2 into pBluescript II KS(+). Synthetic oligonucleotides, 33 bases long and containing TA in place of AC in the middle of their sequences, were used to mutagenize the putative CRP-binding site located upstream of *cutB2*. Mutation was verified by DNA sequencing. A 0.85-kb XbaI/ClaI fragment from the mutated pBScutB2 was cloned into pNC, resulting in the plasmid pCUTB2CBSPM, which has the same construct as pCUTB2up461 except for the point mutations.

RNA isolation and primer extension. RNA isolation and primer extension were performed as described previously (38). The primer PER30 (5′-TACGTG CATTGATATTCCGG-3′), which is complementary to the nucleotide position 88 to 107 downstream of the *cutR* start codon, was used for both primer extension and sequencing.

Enzyme assays and protein determination. Cultures of *Mycobacterium* sp. strain JC1 were grown to an appropriate growth phase. Cells were harvested, resuspended in an appropriate buffer, and disrupted by passage through a French pressure cell twice. Cell-free crude extracts were obtained by centrifugation for 15 min at 20,000 × *g*.

CO-DH. CO-DH activity was assayed spectrophotometrically by measuring the CO-dependent reduction of 2-(4-indophenyl)-3-(4-nitrophenyl)-2*H*-tetrazolium chloride (INT; $\varepsilon_{496} = 17.981\ mM^{-1}\ cm^{-1}$) by the method of Kraut et al. (22).

β-Galactosidase. The β-galactosidase activity was assayed spectrophotometrically by determining the initial conversion rate of the substrate analog ONPG (*o*-nitrophenyl-β-D-galactopyranoside) to *o*-nitrophenol at 420 nm and 30°C for 1 min with a spectrophotometer (26).

Protein concentration was determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL), with bovine serum albumin as a standard.

Activity staining of CO-DH. Staining by activity of CO-DH was performed as described previously (17).

Immunoblotting analysis. SDS-PAGE and Western blotting with antiserum to the CO-DH of *Mycobacterium* sp. strain JC1 (19) were performed as described elsewhere (25).

RESULTS

Identification of the *cutR* gene. Two copies of the structural genes (*cutB1C1A1* and *cutB2C2A2*) encoding CO-DH have been identified in *Mycobacterium* sp. strain JC1 (38). The *cutB*, *cutC*, and *cutA* genes code for the medium, small, and large subunits of CO-DH, respectively, and form a transcriptional unit. The open reading frame designated *cutR* was identified to be located 314 bp upstream of *cutB1* with the transcriptional orientation opposite to the *cutB1C1A1* operon. Its deduced protein product consists of 320 amino acid residues with a calculated molecular mass of 34.1 kDa and an isoelectric point of 9.72. A BLAST search using the deduced amino acid sequence of CutR showed the highest overall resemblances (73 to 74% identity and 82 to 84% similarity) to the CutR homologs from *Mycobacterium bovis*, *Mycobacterium marinum*, *M. tuberculosis*, *Mycobacterium smegmatis*, and *Mycobacterium ulcerans*. CutR is a member of the family of LysR-type transcriptional regulators (LTTRs). A plausible helix-turn-helix (HTH) motif, which represents the DNA-binding domain, occurs in the NH_2-terminal part (amino acids 1 to 60) of CutR. The COOH-terminal part (amino acids 90 to 290) of CutR exhibited some homology to the substrate-binding domain of the LTTR (35).

Phenotypic features of a *cutR* mutant. To investigate the function of the *cutR* gene in *Mycobacterium* sp. strain JC1, a mutant carrying a deletion within the *cutR* (ΔCutR) was constructed. The ΔCutR mutant (ΔCutRmt) grew heterotrophically with glucose or mixotrophically with glucose and CO at the approximately same rate as the wild-type (WT) strain JC1. However, under lithoautotrophic conditions where CO was supplied as the sole source of carbon and energy, the doubling time of the ΔCutRmt (58.7 h) was considerably longer than that of the WT strain (25.5 h). This result suggests that CutR is required for efficient utilization of CO by *Mycobacterium* sp. strain JC1.

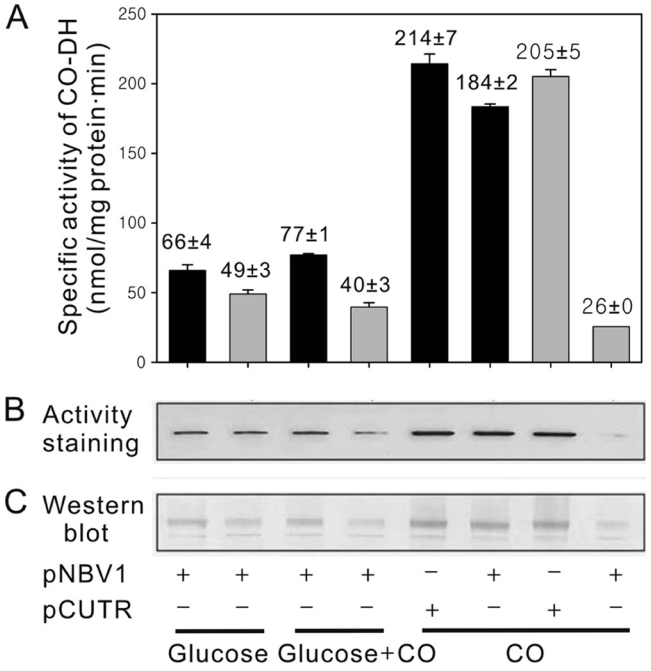

FIG. 1. Synthesis of CO-DH in the WT and ΔCutRmt strains of *Mycobacterium* sp. strain JC1 grown on various growth substrates. The strains were grown to an OD_{600} of 0.7 in SMB medium supplemented with 0.2% (wt/vol) glucose (glucose), 0.2% (wt/vol) glucose plus 30% (vol/vol) CO (glucose + CO), or 30% (vol/vol) CO (CO). The ΔCutRmt with pNBV1 grew with CO as the sole source of carbon so slowly that the mutant was grown to an OD_{600} of 0.3. (A) CO-DH activities. The specific activity indicates nanomoles of INT reduced per milligram protein per minute. The black and gray bars indicate the enzyme activities detected in the WT and ΔCutRmt strains, respectively. The error bars indicate the standard deviation of the values obtained from two independent determinations. (B) Activity staining of CO-DH. Crude extracts (10 μg) were subjected to nondenaturing PAGE on 7.5% (wt/vol) acrylamide gel, and the gel was subsequently subjected to activity staining. (C) Western blot analysis with a polyclonal CO-DH antibody. Crude extracts (10 μg) were subjected to denaturing SDS-PAGE on 12.5% (wt/vol) acrylamide gel. The bands represent the large subunit of CO-DH. "+" and "–" indicate the presence or absence, respectively, of the corresponding plasmids in *Mycobacterium* sp. strain JC1 strains.

The possible role of CutR in the production of CO-DH was investigated by determining the activity and amount of CO-DH synthesized in the WT and ΔCutRmt strains grown on various conditions, since CO-DH is the key enzyme for CO utilization. As shown in Fig. 1, the WT strain harboring the empty vector pNBV1 (WT-pNBV1), which was grown lithoautotrophically with CO, showed a 2.8-fold increase in CO-DH activity, compared to WT-pNBV1 grown on glucose. When WT-pNBV1 was grown mixotrophically with glucose and CO, CO-DH activity detected in WT-pNBV1 was marginally increased relative

to that in WT-pNBV1 grown on glucose. The levels of the CO-DH protein determined by activity staining and immunoblotting were well correlated with CO-DH activity detected in the strain grown under the corresponding growth conditions. This result indicates that the synthesis of CO-DH is induced in the presence of CO and repressed by glucose. The ΔCutRmt carrying pNBV1 (ΔCutRmt-pNBV1) grown heterotrophically or mixotrophically exhibited lower CO-DH activities than WT-pNBV1 grown under the same growth conditions. It is especially noteworthy that the synthesis of CO-DH was not induced in the ΔCutRmt-pNBV1 grown lithoautotrophically. Introduction of the plasmid-borne *cutR* gene into the ΔCutRmt (ΔCutRmt-pCUTR) led to restoration of CO-DH activity to the level detected in WT-pCUTR, indicating that incapability of the ΔCutRmt in the induction of CO-DH synthesis by CO is caused by the inactivation of the *cutR* gene. Taken together, these results indicate that the CutR protein is involved in the induction of CO-DH synthesis in the presence of CO.

Transcriptional regulation of the *cutBCA* operons. In order to elucidate the control of each of the *cutBCA* operons at the transcriptional level, the *cutB1*::*lacZ* and *cutB2*::*lacZ* transcriptional fusions in pCUTB1LACZ2 and pCUTB2LACZ2, respectively, were used to study the expression of the operons in WT and ΔCutRmt strains grown under various growth conditions (Fig. 2). The WT and ΔCutRmt strains harboring the empty vector pNC served as the negative controls, and virtually no β-galactosidase activity was detected in these strains grown heterotrophically, mixotrophically, and lithoautotrophically (data not shown). The considerable levels of the promoter activities of *cutB1* and *cutB2* were detected in the WT strain grown heterotrophically with glucose even in the absence of CO. In agreement with increased activity and protein levels of CO-DH in the lithoautotrophically grown cells, the promoter activities of *cutB1* and *cutB2* in the WT strain grown lithoautotrophically with CO were increased by 1.8- and 3.5-fold, respectively, compared to those in the strain grown on glucose. When grown under mixotrophic conditions with glucose and CO, the WT strain showed the intermediate promoter activities of *cutB1* and *cutB2* between those detected in WT grown heterotrophically and lithoautotrophically, indicating that the expression of *cutB1* and *cutB2* is induced in the presence of CO and repressed by glucose. In the ΔCutRmt the promoter activities of both *cutB1* and *cutB2* were significantly lower than those observed for WT grown under the corresponding growth conditions. Interestingly, the ΔCutRmt strain grown mixotrophically showed the induction of neither *cutB1* nor *cutB2*, compared to the ΔCutRmt grown heterotrophically. However, induction of both *cutB1* and *cutB2* was observed in the ΔCutRmt grown lithoautotrophically with CO, when their promoter activities were compared to those in the mutant grown heterotrophically. All of the results presented in Fig. 2 suggest the following: (i) CutR serves as an activator for expression of both the *cutBCA* operons; (ii) CutR is involved in the induction of the *cutBCA* operons by CO; and (iii) in addition to CutR, a regulatory pathway independent of CutR is involved in the induction of the *cutBCA* operons in the strain grown lithoautotrophically with CO.

As shown in Fig. 3A, CO-DH activity was increased as the growth phase proceeded, when the WT and ΔCutRmt strains were grown heterotrophically with glucose. However, the activity detected in the ΔCutRmt was lower than that in the WT strain. The highest activities of CO-DH in both strains were observed in the late stationary phase. In good agreement with this observation, the promoter activities of *cutB1* and *cutB2* in the WT and ΔCutRmt strains grown on glucose were increased in a growth-dependent manner, although the expression of both genes was significantly impaired in the ΔCutRmt strain (Fig. 3B and C). This result implies that the induction of the *cutBCA* operons in the stationary phase in the absence of CO is mediated by a regulatory system other than CutR and that CutR is required for optimal expression of the *cutBCA* operons.

The induction of the *cutBCA* operons in cells growing in the stationary phase in the absence of CO can result from low level of oxygen or nutritionally poor environments in cultures. To ascertain whether the induction of the *cutBCA* operons was

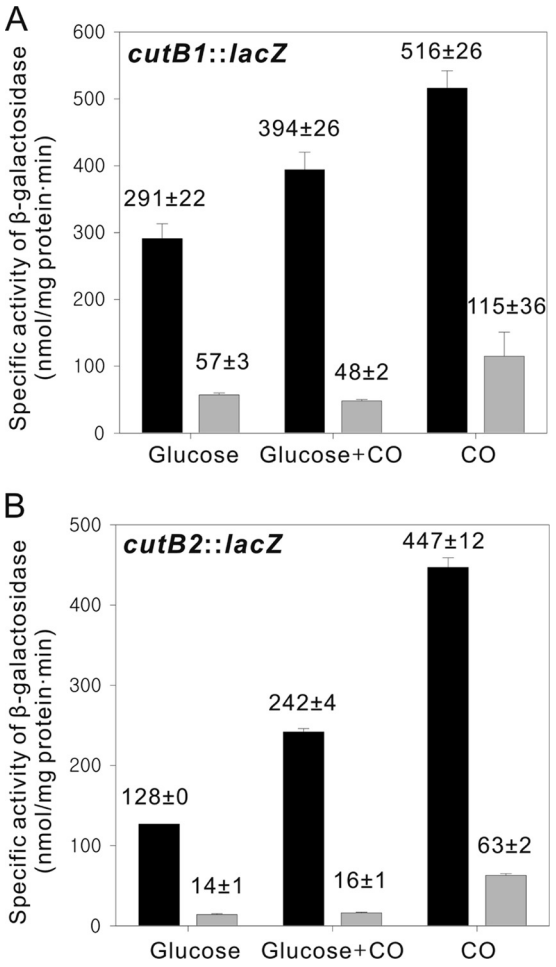

FIG. 2. Expression of the *cutBCA* operons in the WT and the ΔCutRmt strains of *Mycobacterium* sp. strain JC1 grown on various growth substrates. For the determination of the promoter activities for the *cutB1* (A) and *cutB2* (B) genes, the strains harboring pCUTB1LACZ2 and pCUTB2LACZ2, respectively, were grown as described in Fig. 1, and the β-galactosidase activities were determined. The black and gray bars indicate the enzyme activities detected in the WT and ΔCutRmt strains, respectively. All values provided are the means of two independent determinations. Error bars represent the standard deviations from the mean.

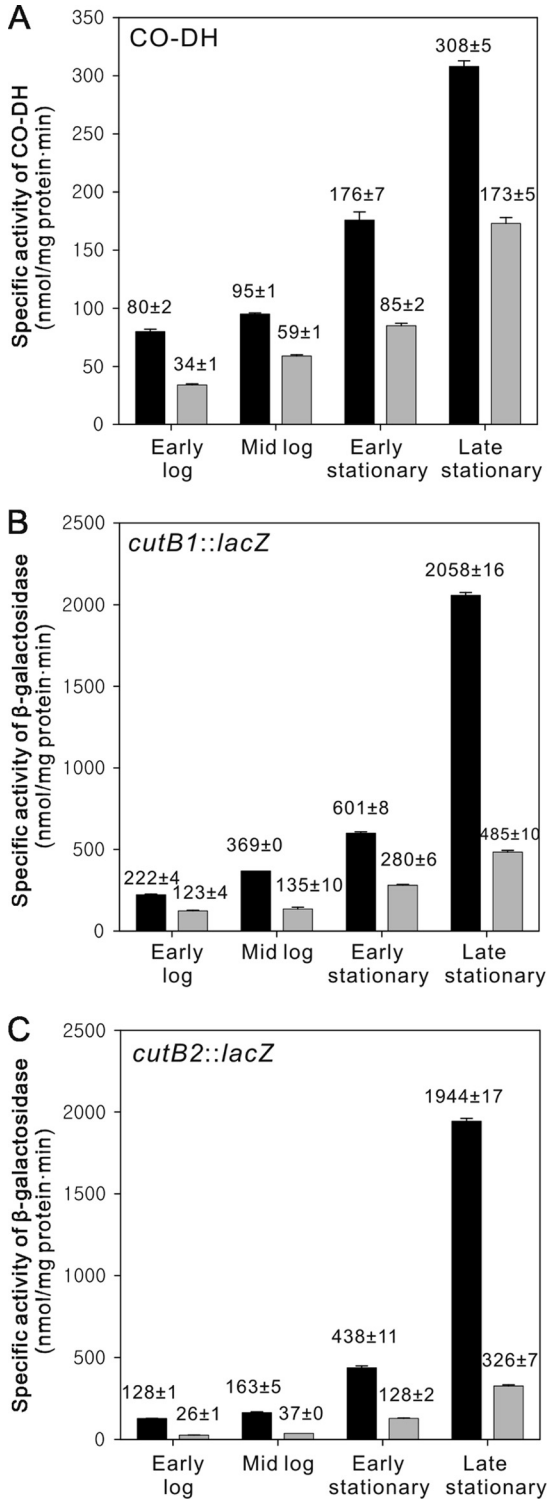

FIG. 3. Effect of the growth phase on the expression of the *cutBCA* operons in the WT and the ΔCutRmt strains of *Mycobacterium* sp. strain JC1. The WT (black bar) and ΔCutRmt (gray bar) strains were grown to an OD_{600} of 0.3 (early log), 0.6 (mid log), and 2.3 (early stationary) in SMB medium supplemented with 0.2% (wt/vol) glucose. For the late stationary culture, cells which had reached the early stationary phase, were further grown for additional 50 h. (A) CO-DH activities; (B) promoter activities of *cutB1*; (C) promoter activities of *cutB2*. All values provided are the means of two independent determinations. Error bars represent the standard deviationd from the mean.

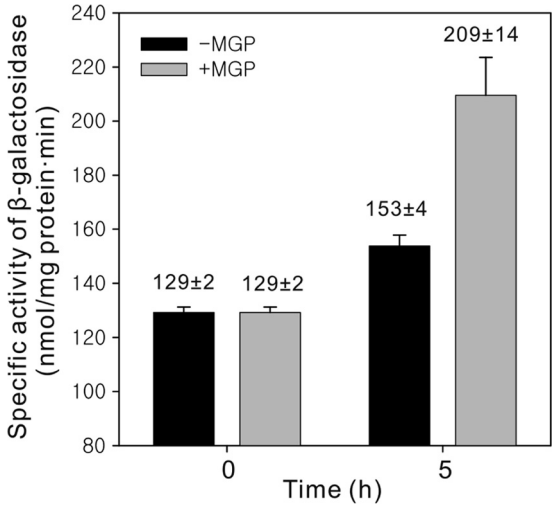

FIG. 4. Expression of the *cutB1* gene in *Mycobacterium* sp. strain JC1 under glucose-limiting conditions. The WT strain carrying pCUTB1LACZ2 was grown in SMB medium supplemented with 0.1% (wt/vol) glucose to an OD_{600} of 0.5. The culture was divided into two groups. The control group (black bar) was grown for additional 5 h under the same conditions. In the case of the experimental group (gray bar), methyl-α-D-glucopyranoside (MGP), a nonmetabolizable glucose homolog, was added to the culture to a final concentration of 10% (wt/vol), and the culture was further grown for additional 5 h. The β-galactosidase activities in cells grown to an OD_{600} of 0.5 (0 h) and for 5 h thereafter (5 h) were measured to determine expression of *cutB1*. Error bars indicate the standard deviations of the results obtained from two independent determinations.

brought about in response to low oxygen tensions, we determined the CO-DH activity in the WT strain subjected to low aeration (for 15 h). The CO-DH activity was not increased in the strain exposed to hypoxic conditions compared to that detected in the WT strain grown aerobically (data not shown). However, when the WT strain grown on 0.2% (wt/vol) glucose was further grown on 0.002% (wt/vol) glucose for 5 h, the CO-DH activity was increased by 1.6-fold relative to that detected in the same strain grown on 0.2% (wt/vol) glucose for additional 5 h as a control (data not shown), indicating that the induction of CO-DH synthesis in the stationary phase in cells growing with glucose is related to glucose depletion in the growth medium. When the WT strain grown heterotrophically on 0.1% (wt/vol) glucose was treated with a 100-fold excess amount (10%) of methyl-α-D-glucopyranoside (MGP), which is a nonmetabolizable structural homolog of glucose, the promoter activity of *cutB1* was 1.4-fold higher than that in the control group (Fig. 4), reinforcing the possibility that glucose starvation can lead to the induction of expression of the *cutBCA* operons.

Determination of the transcriptional start point of *cutR*. The transcriptional start point of the *cutR* gene was determined by primer extension analysis, which was performed with total RNA isolated from glucose- and CO-grown cells of *Mycobacterium* sp. strain JC1. As shown in Fig. 5, an extension product corresponding to the nucleotide G located 36 bp upstream of the start codon of *cutR* was detected. The extension analysis also indicated that expression of *cutR* was higher in cells grown under heterotrophic conditions with glucose than that grown

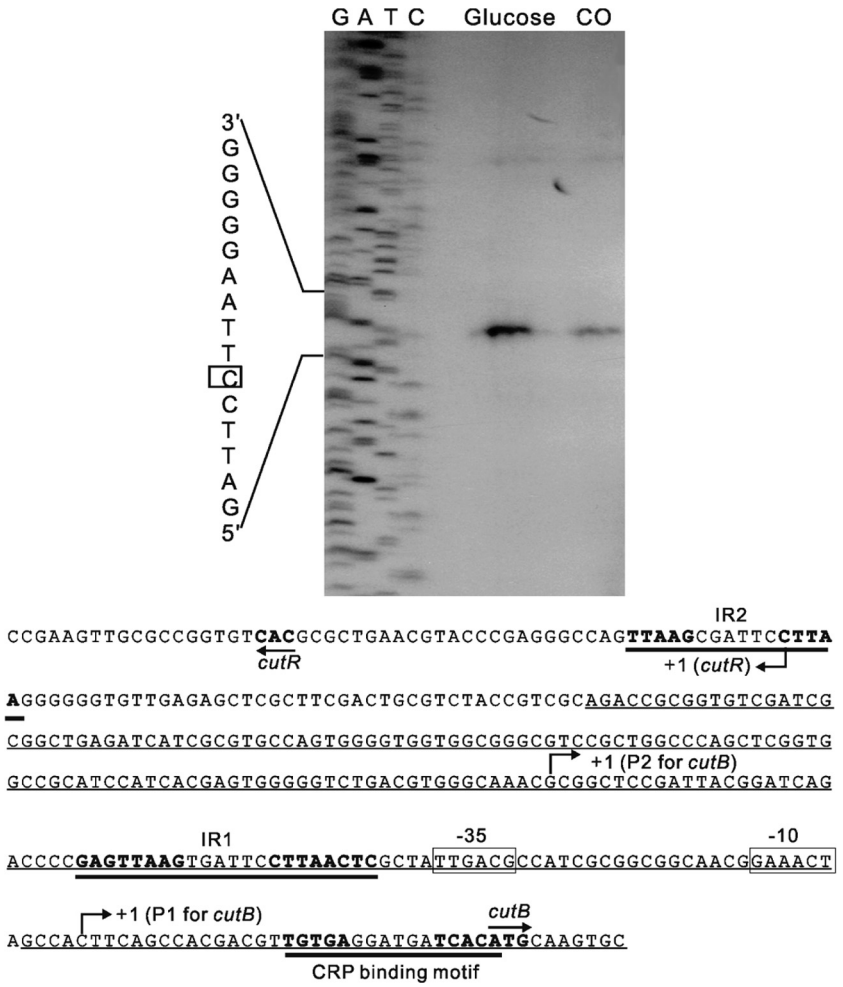

FIG. 5. Mapping of the 5′ ends of the *cutR* gene transcripts by primer extension and the nucleotide sequence of the intergenic region between *cutR* and *cutB1*. The total RNA isolated from *Mycobacterium* sp. strain JC1 grown on CO or glucose was subjected to primer extension analysis. The boxed base (+1) indicates the location of the identified transcriptional start point. 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 starts (ATG and GTG in boldface) of *cutB* and *cutR* are indicated by the arrows with the gene names. Three inverted repeat sequences (IR1, IR2, and CRP site) are marked by thick underlining. The 233-bp upstream region of *cutB1* is identical to that of *cutB2*, and the corresponding nucleotide sequence is underlined.

under CO-lithoautotrophic conditions, which was confirmed by promoter assay using a *cutR*::*lacZ* transcriptional fusion. The expression level of *cutR* in cells grown under glucose conditions was 3.5 times as high as that grown under CO conditions (data not shown).

Identification of *cis*-control elements upstream of the *cutBCA* operons. It has been demonstrated that the *cutB* genes have two transcriptional start points on the basis of two extension products identified by primer extension analysis (38). One (P1) is strongly induced under lithoautotrophic growth conditions with CO, and the other (P2) is constitutively expressed regardless of the presence or absence of CO (Fig. 5). Sequence regions resembling the mycobacterial −35 and −10 regions were identified upstream of P1. An inverted repeat sequence (IR1, GAGTTAAG-N_6-CTTAACTC) is located immediately upstream of the putative −35 region. A similar inverted repeat sequence (IR2, TTAAG-N_6-CTTAA) was also identified 274 bp upstream of the translational start of *cutB1* but not *cutB2*. Since expression of both *cutB1* and *cutB2* are regulated in the identical manner, the presence of IR2 appears not to be necessary for the regulation of *cutB1* expression by CO and glucose. There is another inverted repeat sequence (TGTGA-N_6-TCACA) with a perfect match with the binding motif of cyclic AMP (cAMP) receptor protein (CRP), which is located immediately upstream of the translational starts of *cutB1* and *cutB2*.

Using several *cutB2*::*lacZ* transcriptional fusions, we next investigated the role of the identified *cis*-acting regulatory elements (promoter elements, IR1, and CRP-binding site) in the expression of *cutB2* and its induction in late stationary phase (Fig. 6). The *cutB2* gene was chosen for the experiment since only one IR1 sequence occurs upstream of *cutB2*, in contrast to *cutB1* whose upstream sequence contains both IR1 and IR2. As a positive control, the WT strain harboring pCUT2up461 (WT-pCUT2up461) was used, which contains 461 bp of the upstream sequence of *cutB2*. Expression of *cutB2* in WT-pCUT2up461 was shown to be strongly induced, when it was grown to the late stationary phase. The transcriptional fusion

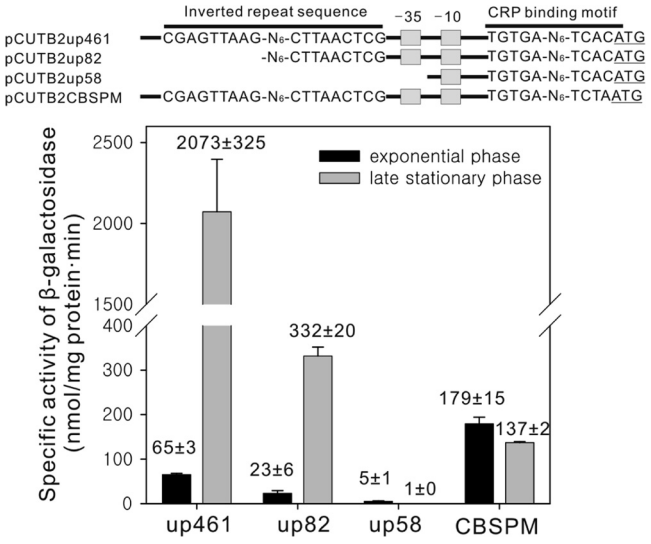

FIG. 6. Promoter activities from the serially deleted and point-mutated promoters of *cutB2* in *Mycobacterium* sp. strain JC1 grown on glucose. The strains harboring the corresponding transcriptional fusion plasmids were grown on SMB medium with 0.2% (wt/vol) glucose either to mid-exponential phase (black bar) or to late stationary phase (gray bar) as described in Fig. 3. pCUTB2up461, pCUTB2up82, and pCUTB2up58 contain the 461-, 82-, and 58-bp upstream sequences of *cutB2*, respectively. pCUTB2CBSPM has the same construct as pCUTB2up461 except that the CRP-binding sequence is point mutated. The β-galactosidase activities in cells were measured to determine expression of *cutB2*. Error bars indicate the standard deviations of the results obtained from two independent determinations.

plasmid pCUTB2up82 contains an 82-bp upstream sequence of *cutB2* with the 5′-half portion of IR1 removed. Expression of *cutB2* was induced in the WT strain with pCUTB2up82 (WT-pCUTB2up82) grown to the late stationary phase in comparison to that in WT-pCUTB2up82 grown to the exponential phase, although the expression levels of *cutB2* in WT-pCUTB2up82 were significantly lower than those in WT-pCUTB2up461. Deletion of the putative −35 region (pCUTB2up58) completely abolished the expression of *cutB2* in the WT strain grown to exponential or late stationary phase, indicating that the region between 58 and 82 bp upstream of *cutB2* contains the promoter element(s). Interestingly, expression of *cutB2* was not induced in late stationary phase when the CRP-binding site was point mutated (pCUTB2CBSPM). Furthermore, the WT strain with pCUTB2CBSPM (WT/pCUTB2CBSPM) grown to exponential phase showed increased levels of promoter activity compared to the WT-pCUT2up461 grown to the same growth phase. These results suggest that a regulatory transcription factor binding to the putative CRP binding site rather than to IR1 is responsible for the induction of the *cutB* genes in the late stationary phase and that the CRP-like transcription factor serves as an activator in the late stationary phase.

DISCUSSION

CutR is a transcriptional activator for the induction of the *cutBCA* operons. The *cutR* gene is located divergently to the *cutB1C1A1* operon. The modular structure (HTH motif in the NH_2-terminal domain and LysR substrate binding domain in the central and COOH-terminal domains) and the overall sequence similarity of CutR to those of known LTTRs suggest that CutR belongs to the LTTR family (35). Both *cutBCA* operons were not induced in the CutRmt grown mixotrophically with glucose and CO compared to the mutant grown heterotrophically with glucose, indicating that CutR is responsible for the induction of the *cutBCA* operons in the presence of CO. Furthermore, inactivation of CutR led to decreased expression of the *cutBCA* operons irrespective of the presence or absence of CO. Both observations allow us to suggest that CutR is an activator for the expression of the *cutBCA* operons even in the absence of CO and that it mediates the induction of the *cutBCA* operons by CO. Two very similar, 16-bp long sequence segments (IR1 and IR2) occur between *cutB1* and *cutR*. They share the consensus sequence 5′-TTAAG(C/T)GATTC CTTAA-3′ and contain the pentamers (underlined) of inverted repeats on both ends. IR1 is located at positions −58 to −43 relative to the transcriptional start points of *cutB1* and *cutB2*. IR2 is located far upstream of *cutB1* and is not present upstream of *cutB2*. In the case of σ^{70}-dependent promoters, transcriptional activators bind predominantly to positions between −80 and −30 relative to the transcriptional start points (29). Since both *cutB1* and *cutB2* are regulated in the same way in terms of their induction in the presence of CO and in the stationary phase, IR2 appears not to be required for the regulation of the *cutBCA* operons. In addition to the *cutR* genes, the conserved IR1 sequences (GAGTTAAG-N_6-CTT AACTC) are present upstream of all mycobacterial *cutBCA* operons (Fig. 7). This finding strongly indicates that the IR1 and IR2 sequences might be the binding sites for the CutR activator, which is corroborated by the presence of a LysR motif (T-N_{11-12}-A) within the IR1 and IR2 sequences (11). A binding assay of CutR to IR1 and IR2 is necessary to prove that the IR sequences serve as the CutR binding sites.

The *cutBCA* operons are subjected to catabolite repression. The presence of glucose in the growth medium suppressed the expression of the *cutBCA* operons in the presence of CO. The fact that the excess amount of MGP relative to glucose derepressed the expression of *cutB1* strongly suggests that the *cutBCA* operons are subjected to catabolite repression (8). We determined the promoter activity of *cutB2* in the WT strain of *Mycobacterium* sp. strain JC1 grown to early log phase (optical density at 600 nm [OD_{600}] of 0.3) in SMB medium containing either 0.2% glucose or 0.2% glycerol. The expression level of *cutB2* in cells grown in glycerol-SMB medium was 2.18-fold higher than that detected in cells grown in glucose-SMB medium, confirming catabolite repression of the *cutBCA* operons by glucose (data not shown). The strong induction of the *cutBCA* operons in *Mycobacterium* sp. strain JC1 grown lithoautotrophically with CO appears to result from the combinatory effect of both CO induction by CutR and the abolishment of catabolite repression in the absence of glucose. Intriguingly, an inverted repeat sequence (TGTGA-N_6-TCACA) with a perfect match to the binding motif of CRP (13) was found to be centered at the −7 position with respect to the start codons of both *cutB1* and *cutB2*. The 3′-end portion of the putative CRP-binding site overlaps with the start codons of *cutB1* and *cutB2*. The CRP-binding motifs also occur immediately upstream of the *cutB* gene homologs of fast-growing mycobacteria such as

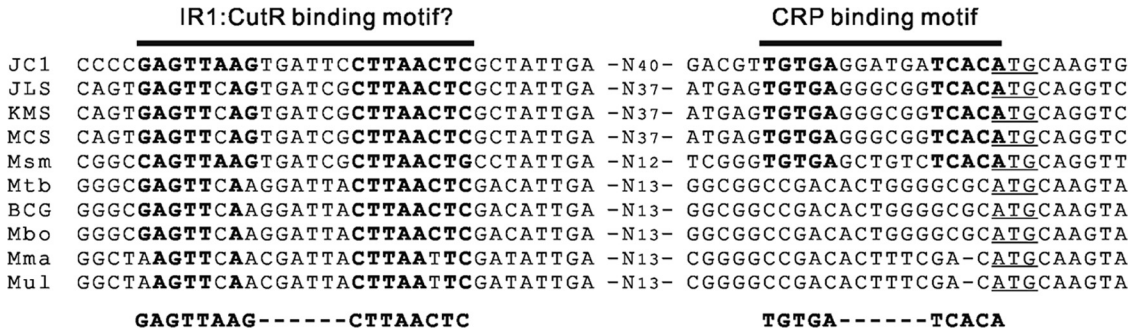

FIG. 7. Inverted repeat sequences identified upstream of the *cutB* genes of various mycobacteria. The conserved inverted repeats are indicated in boldface. The consensus sequences of the conserved inverted sequences are given below the aligned sequences. The start codons of the *cutB* genes are underlined. Abbreviations: JC1, *Mycobacterium* sp. strain JC1; JLS, *Mycobacterium* sp. strain JLS; KMS, *Mycobacterium* sp. strain KMS; MCS, *Mycobacterium* sp. strain MCS; Msm, *M. smegmatis*; Mtb, *M. tuberculosis* H37Rv; BCG, *M. bovis* BCG; Mbo, *M. bovis*; Mma, *M. marinum*; Mul, *M. ulcerans*.

M. smegmatis and *Mycobacterium* sp. strains JLS, KMS, and MCS, whereas they are not found in the upstream control regions of the *cutB* homologs of *M. tuberculosis*, *M. bovis*, *M. marinum*, and *M. ulcerans* (Fig. 7). The mutation of TGTGA-N_6-TCACA to TGTGA-N_6-TCTAA completely abolished the induction of *cutB2* in late stationary phase, indicating that this consensus CRP-binding site might be related to catabolite repression.

The genes for the CRP homologs, *rv3676* and *mb3700*, were identified in the genomes of *M. tuberculosis* and *M. bovis*, respectively (6, 10). The Rv3676 protein was shown to bind to the consensus sequence of *E. coli* CRP-binding site (TGTGA-N_6-TCACA) (2). cAMP enhanced the binding affinity of Rv3676 to its target DNA sequences and led to allosteric alterations in its conformation (2, 3). The Rv3676 binding motifs were identified in 73 promoter regions regulating 114 genes in the *M. tuberculosis* genome (2). It is noteworthy that 34 genes involved in nutrient starvation response have the Rv3676 binding motifs in their upstream control regions, implying that Rv3676 might be important in regulation of the starvation response. It has been also demonstrated that Rv3676 plays an important role in the proliferation of *M. tuberculosis* in macrophages and in mice (30). We recently identified and cloned the gene encoding the Rv3676 homolog in *Mycobacterium* sp. strain JC1 (J. H. Kim and Y. M. Kim, unpublished data). The nucleotide sequence of Rv3676 homolog of *Mycobacterium* sp. strain JC1 showed 96% identity to that of *M. tuberculosis* Rv3676. The binding of CRP homolog to the promoter region of the CO-DH genes was already predicted by computational analysis on the *M. smegmatis* genome (1). Although we present here the first indication that the Rv3676 homolog is involved in the regulation of the *cutBCA* operons in *Mycobacterium* sp. strain JC1, experimental validation with an Rv3676 homolog knockout mutant will be required to provide direct evidence.

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