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Functional characterization of the *cutI* gene for the transcription of carbon monoxide dehydrogenase genes in *Mycobacterium* sp. strain JC1 DSM 3803

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Carbon monoxide dehydrogenase (CO-DH) in *Mycobacterium* sp. strain JC1 is a key enzyme for the carboxydotrophic growth, when carbon monoxide (CO) is supplied as a sole source of carbon and energy. This enzyme is also known to act as nitric oxide dehydrogenase (NO-DH) for the detoxification of NO. Several accessory genes such as *cutD*, *cutE*, *cutF*, *cutG*, *cutH*, and *cutI*, are clustered together with two copies of the CO-DH structural genes (*cutB1C1A1* and *cutB2C2A2*) in *Mycobacterium* sp. strain JC1 and are well conserved in carboxydotrophic mycobacteria. Transcription of the CO-DH structural and accessory genes was demonstrated to be increased significantly by acidified sodium nitrate as a source of NO. A *cutI* deletion (Δ*cutI*) mutant of *Mycobacterium* sp. strain JC1 was generated to identity the function of CutI. Lithoautotrophic growth of the Δ*cutI* mutant was severely affected in mineral medium supplemented with CO, while the mutant grew normally with glucose. Western blotting, CO-DH activity staining, and CO-DH-specific enzyme assay revealed a significant decrease in the cellular level of CO-DH in the Δ*cutI* mutant. Northern blot analysis and promoter assay showed that expression of the *cutB1* and *cutB2* genes was significantly reduced at the transcriptional level in the Δ*cutI* mutant, compared to that of the wild-type strain. The Δ*cutI* mutant was much more susceptible to NO than was the wild type.

***Keywords*:** CO dehydrogenase, CutI, carboxydobacteria, *Mycobacterium* sp. strain JC1, nitric oxide

Introduction

Carboxydobacteria are a group of bacteria capable of growing aerobically on carbon monoxide (CO) as a sole source of carbon and energy using CO dehydrogenase (CO-DH) as a key enzyme (Kim and Hegeman, 1983; Meyer and Schlegel, 1983). CO-DH catalyzes the oxidation of CO to CO_2 using H_2O as an oxidant (Kim and Hegeman, 1983; Meyer and Schlegel, 1983). The electrons from the oxidation of CO are transferred to the CO-insensitive respiratory electron transport chain for energy generation and CO_2 is assimilated via Calvin cycle for a carbon source (Kim and Hegeman, 1983; Meyer and Schlegel, 1983). CO-DHs were classified into three groups, mycobacterial CO-DHs (group I), Gram-negative bacterial CO-DHs (group II), and Gram-positive bacterial CO-DHs other than mycobacteria (group III) by means of phylogenetic analysis on the catalytic subunits of CO-DHs (Kim and Park, 2012). It has been known that only CO-DHs in group I have nitric oxide dehydrogenase (NO-DH) activity (Park *et al.*, 2007). All classes of CO-DHs are composed of three nonidentical subunits with a quaternary structure of $\alpha_2\beta_2\gamma_2$, and the three structural genes for CO-DH have been cloned and sequenced in several carboxydobacteria (King and Weber, 2007; Kim and Park, 2012).

Mycobacterium sp. strain JC1 (MJC1) is a fast-growing mycobacterium isolated from soil (Cho *et al.*, 1985; Song *et al.*, 2002) and has been well studied regarding physiological, biochemical, and molecular biological aspects for its carboxydotrophic growth (Park *et al.*, 2003, 2009; Lee *et al.*, 2009; Oh *et al.*, 2010; Song *et al.*, 2010; Kim and Park, 2012). The CO-DH structural genes in MJC1 are duplicated and form the separate transcriptional units (*cutB1C1A1* and *cutB2C2A2*). Several genes such as *cutR*, *cutD*, *cutE*, *cutF*, *cutG*, *cutH*, and *cutI*, which are located upstream or downstream of the duplicated CO-DH structural genes in MJC1, are conserved in carboxydotrophic mycobacteria (Song *et al.*, 2010; Kim and Park, 2012). However, among these seven conserved genes around the CO-DH structural genes, it was only the *cutR* gene that was studied for its function in MJC1 (Oh *et al.*, 2010; Song *et al.*, 2010). On the basis of sequence comparison and mutagenesis studies of the homologous genes in *Oligotropha carboxidovorans* OM5, some of the accessory genes (*cutD*, *cutE*, and *cutG*) were suggested to be implicated in biogenesis of the [$CuSMoO_2$] cofactor (Pelzmann *et al.*, 2009, 2014; Song *et al.*, 2010).

The heme-containing CooA and RcoM proteins in *Rhodospirillum rubrum* and *Burkholderia xenovorans*, respectively, have been demonstrated to serve as CO-sensing transcrip-

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tional regulators to induce expression of CO-DH genes in the presence of CO (Shelver *et al.*, 1997; Roberts *et al.*, 2004; Kerby *et al.*, 2008). In our research group, a LysR-type transcriptional regulator gene, *cutR*, was cloned and the function of CutR was identified as a transcriptional activator for the duplicated CO-DH structural genes in MJC1 (Oh *et al.*, 2010; Song *et al.*, 2010). In addition to CutR, the cAMP-receptor protein (CRP) protein was suggested to be involved in the positive regulation and catabolite repression of the CO-DH genes in MJC1 (Oh *et al.*, 2010).

The *cutI* gene was conserved in carboxydotrophic mycobacteria (Kim and Park, 2012). The *cutI* gene in MJC1 is 1,209 bp long and the deduced *cutI* gene product contains the von Willebrand domain that is known to be used for protein-protein interactions. This domain is also present in CoxE of *O. carboxidovorans* (Santiago *et al.*, 1999; Song *et al.*, 2010). However, the function of CutI still remains unknown. In this study, we examined the effects of NO on expression of the CO-DH gene cluster including the *cutI* gene, and constructed a *cutI* deletion mutant of MJC1 to examine the function of CutI in CO oxidation and NO detoxification in MJC1.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

MJC1 strains were grown at 37°C in either Middlebrook 7H9 medium (Difco) supplemented with 0.2% (w/v) glucose (7H9-glucose) or standard mineral base (SMB) medium supplemented with 0.2% glucose (SMB-glucose) or SMB medium supplemented with a gas mixture of 30% (v/v) CO–70% (v/v) air (SMB-CO) (Kim and Hegeman, 1981). To expose MJC1 strains to NO conditions, MJC1 strains were aerobically grown in 100 ml of SMB-glucose medium to an optical density at 600 nm (OD_{600}) of 0.6–0.7 (mid-exponential phase) on a gyratory shaker (200 rpm) and cells were harvested. The harvested cells were then resuspended in 100 ml of acidified SMB-glucose medium (pH 5.5) containing sodium nitrate at appropriate concentrations and further grown for 20 min. *Escherichia coli* strains were cultivated at 37°C in Luria-Bertani medium (LB). Ampicillin (50 mg/ml for *E. coli*) or hygromycin (50 mg/ml for MJC1 and 200 mg/ml for *E. coli*) were added to the growth medium when required. For phage infection, LB containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ was used.

DNA manipulation and transformation

Genomic DNA of MJC1 was isolated as described previously (Wilson, 1989). Plasmids were isolated from *E. coli* by the alkaline lysis method (Sambrook and Russell, 2001). Mycobacteria were transformed by electroporation of a 100 μl portion of competent cells in a 0.2 cm cuvette using an ECM 630 apparatus (Harvard Apparatus) set to 2.5 kV, 25 mF, and 1,000 W (Parish and Stoker, 1998).

Mutagenesis and complementation

A DNA fragment carrying the complete sequence of the *cutI* gene and their flanking regions was cloned by the ligation of a 4,386 bp *Sac*I fragment from lEMBL3-34 (Song *et al.*, 2010) into the pBluescript II SK(+) vector digested with *Sac*I to generate pSW57. After digestion of pSW57 with *Kpn*I, 4,020 and 2,738 bp DNA fragments were purified and ligated to yield pSW58, in which the 589 bp internal sequence of *cutI* is deleted. The resulting plasmid pSW58 was digested with *Pst*I, and a 2,167 bp DNA fragment carrying the impaired *cutI* gene and its flanking sequences, was finally ligated to the pKO vector (Sherman *et al.*, 2001) digested with *Pst*I to yield pKO-cutI. The resulting plasmid pKO-cutI was introduced into MJC1 by electroporation. Heterogenotes of MJC1, 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% (w/v) sucrose. The allelic exchange in the homogenotes that produced isogenic *cutI* deletion mutants (Δ*cutI*) was verified by PCR.

To construct the pSW59 plasmid carrying *cutI* for complementation of the Δ*cutI* mutant of MJC1, a PCR product of 1,539 bp containing *cutI* of MJC1 was amplified from chromosomal DNA using the cutI-CT-F (5′-AAGCTTAGTCCAGTCCGAACCCGAAC-3′; the *Hin*dIII site is underlined) and cutI-CT-R (5′-GGATCCCGAATAGGAAGCCAGCTTTC-3′; the *Bam*HI site is underlined) primers and ligated to pNBV1 (Howard *et al.*, 1995) restricted with *Hin*dIII and *Bam*HI, resulting in pSW59.

Survival assays with sodium nitroprusside

The wild-type and Δ*cutI* mutant strains of MJC1 were grown aerobically in 7H9 medium at 37°C to an OD_{600} of 0.5. A 180 μl portion of the culture was first mixed with 20 μl of 50 mM sodium nitroprusside (SNP) and then incubated at 25°C under the illumination of light. At regular time intervals, cells were spread onto 7H9 plates and incubated at 37°C to count the colony forming unit (CFU). The number of viable cells at each time point was presented as percentage of the CFU at time zero.

Enzyme assays

CO-DH activity was determined spectrophotometrically by measuring the CO-dependent reduction of 2-(4-indophenyl)-3-(4-nitrophenyl)-2H-tetrazolium chloride (INT: ε_{496} = 17.981 m/M/cm) as described elsewhere (Kraut *et al.*, 1989).

β-Galactosidase activity was assayed spectrophotometrically using *o*-nitrophenyl-D-galactopyranoside as described previously (Miller, 1972).

RNA isolation and Northern blot analysis

MJC1 strains were 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.). Northern blot analysis was performed as described previously (Sambrook and Russell, 2001). A 499 bp DNA fragment containing a portion of *cutB* obtained by PCR with the primers CutB-F (5′-CCGGACCATTCGAGTACGAG-3′) and CutB-R (5′-ACTAGCAACTCGTTGGGCTC-3′) was used as a probe

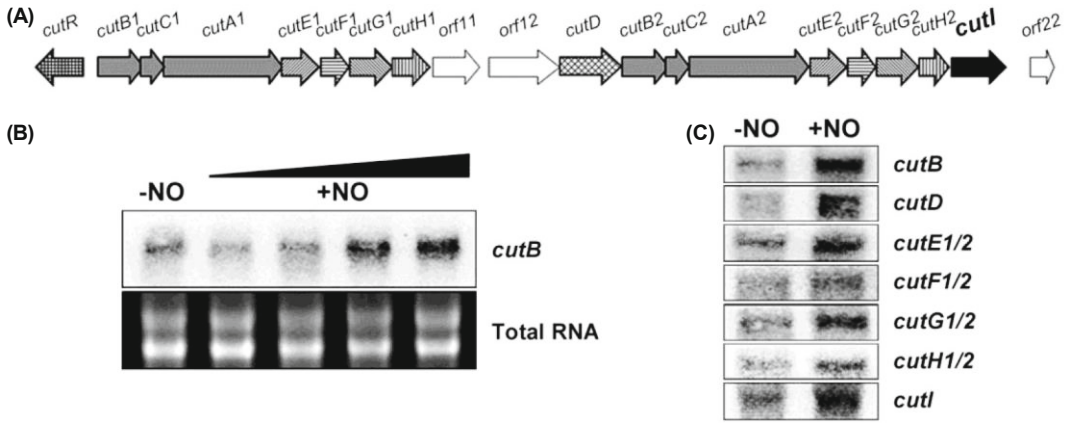

Fig. 1. NO-induced transcription of the CO-DH structural genes and their flanking accessory genes. (A) Genetic organization of the CO-DH gene locus in MJC1. The black and gray arrows represent the *cutI* gene and the CO-DH structural genes (*cutB1C1A1* and *cutB2C2A2*), respectively. The same shading patterns of the arrows indicate the duplicated genes with the same or similar nucleotide sequences. (B) Northern blot analysis of the *cutB* genes (*cutB1* and *cutB2*). The MJC1 strain grown in SMB-glucose to the mid-exponential phase was exposed to 0 (-NO) or 1.5, 3, 6, and 10 mM of sodium nitrate (+NO) at pH 5.5 for 20 min. (C) Northern blot analysis of the conserved genes around the CO-DH structural genes. The MJC1 strain grown in SMB-glucose to the mid-exponential phase was exposed to 0 (-NO) or 6 mM of sodium nitrate (+NO) at pH 5.5 for 20 min.

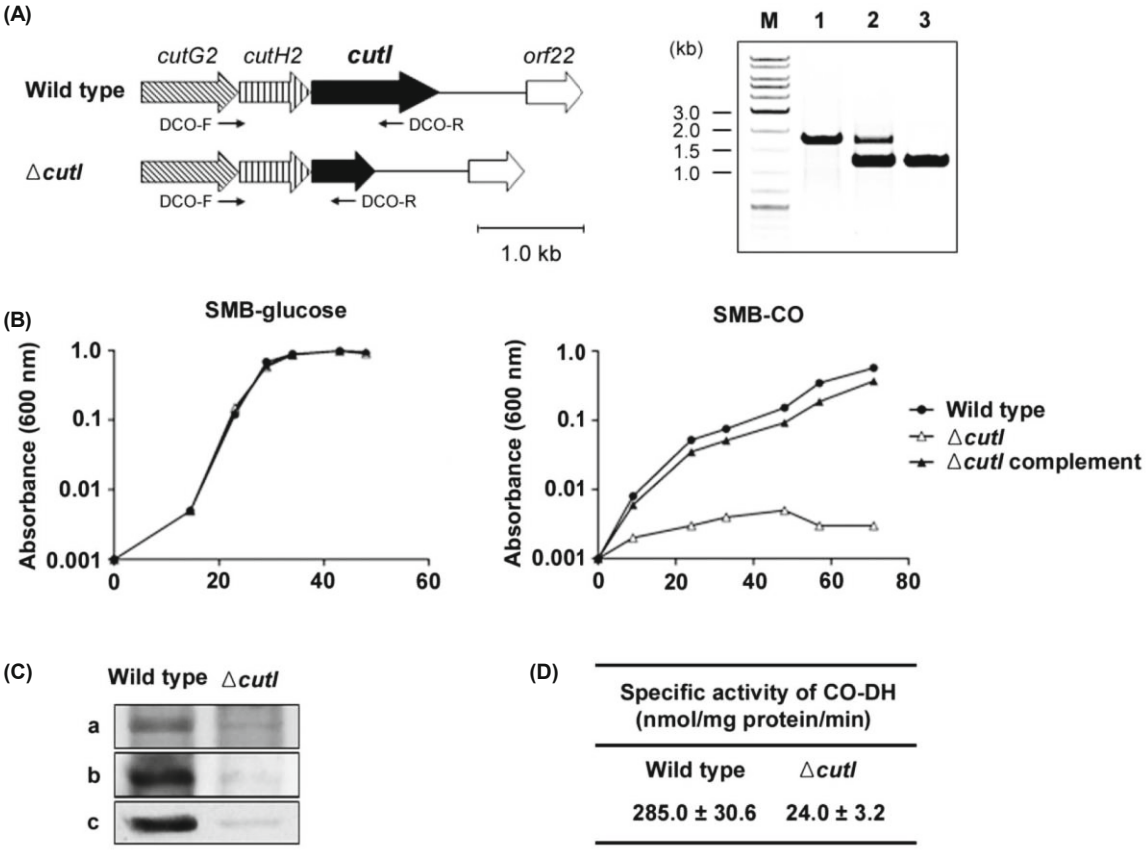

Fig. 2. Construction and characterization of the Δ*cutI* mutant of MJC1. (A) Construction of the Δ*cutI* mutant by homologous recombination as described in 'Materials and Methods'. The *cutI* gene is shown as the solid black arrows. The binding sites of the primers used for confirmation of double crossover mutants were marked as the arrows below the genes. The deletion of *cutI* in the Δ*cutI* mutant was confirmed by PCR with the primers, DCO-F (5′-CCGAGA CCATCGACTGGGTG-3′) and DCO-R (5′-GCGCATTACCGTGCGACGTG-3′). The expected size of the PCR products is 1,773 and 1,184 bp for the wild-type and Δ*cutI* mutant strains, respectively. Lanes: 1, wild type; 2, single crossover; 3, Δ*cutI* mutant; M, size maker for DNA. (B) Growth curves of the wild-type, Δ*cutI* mutant, and complemented Δ*cutI* mutant in SMB-glucose and SMB-CO. (C) Determination of CO-DH levels in the wild-type and Δ*cutI* mutant strains. Twenty micrograms of cell-free extracts from the wild-type and Δ*cutI* mutant strains grown to the mid-exponential phase in SMB-glucose, were subjected to non-denaturing PAGE on a 7.5% acrylamide gel. a, CBB staining; b, Western blot; c, CO-DH activity staining. (D) CO-DH activities were determined in the wild-type and Δ*cutI* mutant strains grown to the mid-exponential phase in SMB-glucose.

in Northern blot analysis.

CO-DH activity staining and Western blotting

Cell-free extracts were prepared from cells of MJC1 strains harvested at mid-exponential growth phase and subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE) at 4°C. Staining of gels by activity of CO-DH was performed in staining buffer (50 mM Tris-HCl [pH 7.5] containing 0.05% [w/v] phenazine methosulfate and 0.25% [w/v] nitroblue tetrazolium) flushed with CO as described previously (Kim and Hegeman, 1981). Rabbit antiserum raised against purified CO-DH of MJC1 was used for detection of CO-DH by Western blotting.

Results and Discussion

NO-induced transcription of the CO-DH structural genes and their flanking accessory genes

The synthesis of CO-DH in MJC1 was reported to be induced by SNP that is an NO generator (Park *et al.*, 2007). To confirm whether expression of the CO-DH structural genes is induced under NO exposure conditions, Northern blot analysis using a *cutB*-specific probe was performed with total RNA from cells exposed to various concentrations (0, 1.5, 3, 6, and 10 mM) of acidified sodium nitrate. The transcription of the *cutB* genes (*cutB1* and *cutB2*) was gradually induced with increasing concentrations of sodium nitrate and showed the maximum expression levels at 6 and 10 mM of sodium nitrate (Fig. 1B). Northern blot analysis also revealed that expression of the accessory genes (*cutD*, *cutE*, *cutG*, *cutH*, and *cutI*) was increased under NO exposure conditions (Fig. 1C), implying that all the conserved genes around the CO-DH structural genes are inducible by NO and that these accessory genes form transcriptional units with the *cutB1C1A1* and *cutB2C2A2* structural genes.

Construction and characterization of the Δ*cutI* mutant of MJC1

The last gene in the *cutB2C2A2* operon, *cutI*, was inactivated in MJC1 by homologous recombination as described in materials and methods and the deletion mutation of *cutI* was confirmed by PCR (Fig. 2A). The *ΔcutI* mutant did not have any growth defect in SMB-glucose. However, the growth of the mutant was severely affected in SMB-CO and this phenotype was complemented by the introduction of the intact *cutI* gene (Fig. 2B). To ascertain the effect of *cutI* inactivation on the synthesis of active CO-DH, Western blotting and CO-DH activity staining analyses were performed by using the wild-type and *ΔcutI* mutant strains of MJC1 grown in SMB-glucose. Western blotting and CO-DH activity staining showed that the synthesis and activity of CO-DH were significantly decreased in the *ΔcutI* mutant (Fig. 2C). This reduced protein level of CO-DH was also observed in Coomassie brilliant blue staining (Fig. 2C). CO-DH enzyme assay showed that CO-DH activity was reduced in the *ΔcutI* mutant by 91.6% relative to the wild-type strain (Fig. 2D). Taken together, the results from Western blotting, CO-DH activity staining and enzyme assay indicate that the growth defect of the *ΔcutI* mutant in SMB-CO is caused by the low cellular level of CO-DH.

Both CoxD and CoxE proteins in *O. carboxidovorans* OM5 were predicted to be involved in the posttranslational biogenesis of the [$CuSMoO_2$] cluster of CO-DH (Pelzmann *et al.*, 2009, 2014). Although CO-DH activity almost disappeared in both *coxD*- and *coxE*-defective mutants of *O. carboxidovorans* OM5, the protein level of CO-DH in the *coxD*- and *coxE*-defective mutants were same to that in the wild type (Pelzmann *et al.*, 2009, 2014), indicating that the transcription and translation of CO-DH genes were not affected by deletion of *coxD* or *coxE* and that the degradation rate of the inactive CO-DH lacking the [$CuSMoO_2$] cluster is not faster than that of the active CO-DH. In contrast to the *coxD*- and *coxE*-defective mutants of *O. carboxidovorans* OM5, the protein level of CO-DH was significantly reduced in the *ΔcutI* mutant, compared to that of the wild-type strain (Fig. 2C). From these results, we assumed that CutI is involved in expression of the CO-DH structural genes and not in posttranslational cofactor processing. To examine this assumption, Northern blot analysis was performed to determine transcription levels of the *cutB* genes in the *ΔcutI* mutant as well as in the wild-type strain. Northern blot analysis revealed that expression of the *cutB* genes were almost abolished in the *ΔcutI* mutant as contrasted with the wild-type strain (Fig. 3A). Furthermore, transcription of the *cutB* genes

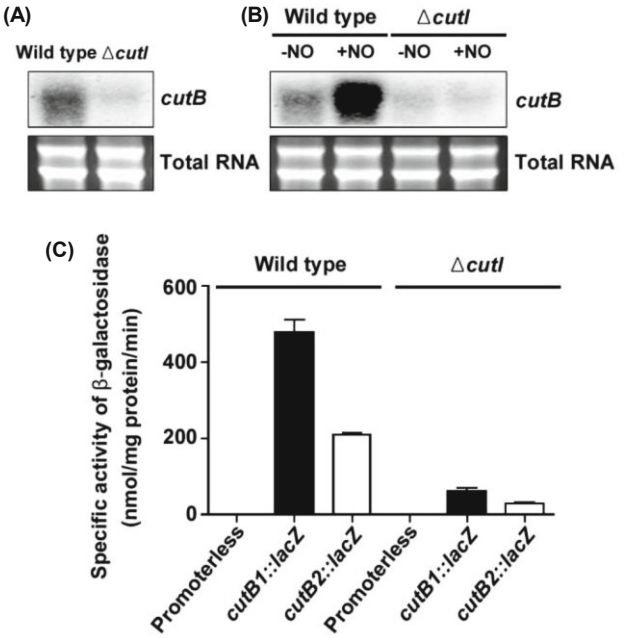

Fig. 3. Expression of the *cutB* genes in the wild-type and Δ*cutI* mutant strains of MJC1. (A and B) Northern blot analysis to determine the levels of transcripts containing *cutB1* and *cutB2*. Total RNAs were prepared from the wild-type and Δ*cutI* mutant strains grown to an OD_{600} of 0.6 to 0.7 in SMB-glucose (A) and then exposed to 0 (-NO) or 6 mM of sodium nitrate (+NO) at pH 5.5 for 20 min (B). (C) Promoter activities of *cutB1* and *cutB2*. Promoter activities were measured from the wild-type and Δ*cutI* mutant strains containing pNC (promoterless), pCUTB1LACZ2 (*cutB1*::*lacZ*, solid bars; Oh *et al.*, 2010), and pCUTB2LACZ2 (*cutB2*::*lacZ*, open bars; Oh *et al.*, 2010). The strains were grown to the stationary phase (OD_{600} = 1.0–1.2) in SMB-glucose. The error bars indicate the deviations from the averages of two independent experiments.

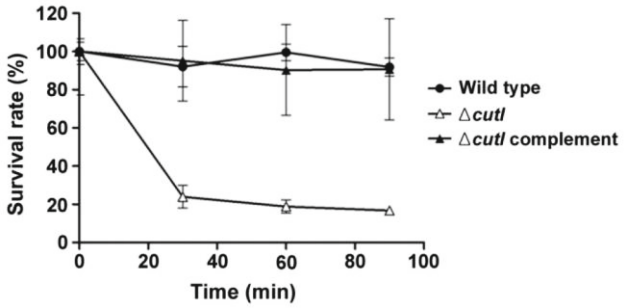

Fig. 4. Susceptibility of the wild-type and Δ*cutI* mutant strains of MJC1 to NO. The survival rates of the wild type (closed circles), Δ*cutI* mutant (open triangles), and complemented Δ*cutI* mutant (closed triangles) after NO exposure (5 mM SNP) were measured by counting the CFU. The number of viable cells at each time point was presented as percentage of the CFU at time zero. The error bars indicate the standard deviations from the averages of three independent experiments.

was not induced by NO in the *ΔcutI* mutant while expression of the genes was strongly induced in the wild-type strain (Fig. 3B). Therefore, CutI appears to participate in a regulatory network responsible for the transcriptional regulation of the CO-DH genes. Promoter activities were also measured for *cutB1* and *cutB2*, separately. Promoter assay showed that promoter activities of *cutB1* and *cutB2* in the *ΔcutI* mutant were decreased by 87.0 and 85.1%, respectively, relative to those in the wild-type strain (Fig. 3C). This result implies that CutI is implicated in the positive regulation of the duplicated CO-DH structural genes at the transcriptional level.

Survival of the Δ*cutI* mutant under NO exposure conditions

CO-DH in MJC1 possesses NO-DH activity, which implies the possibility that it might help the bacterium survive under NO exposure conditions by removing toxic NO molecules (Park *et al.*, 2007). We comparatively examined the survival rates of the wild-type and Δ*cutI* mutant strains of MJC1 under NO conditions. The wild-type strain, Δ*cutI* mutant, and complemented Δ*cutI* mutant grown in 7H9-glucose were exposed to 5 mM SNP for 0, 30, 60, and 90 min. The survival rate of the Δ*cutI* mutant was decreased by 76.0%, 81.2%, and 83.3% in 30, 60, and 90 min after the SNP treatment, respectively, as compared with the wild-type strain (Fig. 4). The survival rate of the Δ*cutI* mutant under NO exposure conditions was recovered by the complementation with the intact *cutI* gene to the level observed in the wild-type strain (Fig. 4).

In addition to CO-DH, some mycobacteria have NO dioxygenase and peroxinitritase as NO-detoxification systems (Hu *et al.*, 1999; Bryk *et al.*, 2000; Ouellett *et al.*, 2002). Since the survival rate of the Δ*cutI* mutant under NO exposure conditions was significantly reduced in comparison with the wild-type strain exposed to the same NO conditions (Fig. 4), CutI appears to play a critical role in the detoxification of NO by inducing expression of CO-DH under NO exposure conditions. Importance of CutI in survival under NO exposure conditions and NO-DH activities of mycobacterial CO-DH would open a possibility that CutI and CO-DH can be used as novel targets for the development of new anti-tuberculosis drugs.

Speculation of the possible role of CutI in the transcriptional activation of the *cut* operons

We previously demonstrated that CutR and CRP are involved in the positive regulation of both *cutB1C1A1* and *cutB2C2A2* operons (Oh *et al.*, 2010). It is not certain whether CutI directly regulates the CO-DH genes because CutI does not have similarity to any known DNA-binding regulatory proteins as judged by *in-silico* analysis. We previously reported that the inactivation of *cutR* led to a decrease in expression of *cutB1* and *cutB2* by 80.4 and 89.0%, respectively, at the similar level as observed in the *ΔcutI* mutant (Oh *et al.*, 2010), making it conceivable that CutI might be functionally related to CutR.

Our electrophoretic mobility shift assay revealed that both purified N-terminally His_6-tagged and C-terminally His_6-tagged CutR proteins did not bind to the *cutB2* control region containing the putative CutR binding motif (TTAAG-N_6-CTTAA) while purified CRP bound to the control region (data not shown). From the fact that the *cutI* product contains the von Willebrand domain that serves as a protein-protein interaction domain together with our results, we assume that protein-protein interactions between CutI and CutR might be necessary for the binding of CutR to its binding site. To examine this assumption, we attempted to overexpress *cutI* heterologously in *E. coli*. However, we failed to express and purify C-terminally His_6-tagged CutI. Experimental validation with purified CutI and CutR will be required to prove our assumption.

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