A TetR-like regulator broadly affects the expressions of diverse genes in Mycobacterium smegmatis

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ABSTRACT

Transcriptional regulation plays a critical role in the life cycle of Mycobacterium smegmatis and its related species, M. tuberculosis, the causative microbe for tuberculosis. However, the key transcriptional factors involved in broad regulation of diverse genes remain to be characterized in mycobacteria. In the present study, a TetR-like family transcriptional factor, Ms6564, was characterized in M. smegmatis as a master regulator. A conserved 19 bp-palindromic motif was identified for Ms6564 binding using DNaseI footprinting and EMSA. A total of 339 potential target genes for Ms6564 were further characterized by searching the M. smegmatis genome based on the sequence motif. Notably, Ms6564 bound with the promoters of 37 cell cycle and DNA damage/repair genes and regulated positively their expressions. The Ms6564-overexpressed recombinant strain yielded 5-fold lower mutation rates and mutation frequencies, whereas deletion of Ms6564 resulted in ~5-fold higher mutation rates for the mutant strain compared with the wild-type strain. These findings suggested that Ms6564 may function as a global regulator and might be a sensor necessary for activation of DNA damage/repair genes.

INTRODUCTION

Transcriptional regulation plays an important role in the life cycle of Mycobacterium tuberculosis (1,2), the causative microbe for tuberculosis (TB), which results in the death of ~2 million people globally each year (3). A unique DNA damage/repair mechanism has been proposed in M. tuberculosis (4). However, the regulations and consequence of these genes remain largely unclear. Mycobacterium smegmatis is a fast-growing non-pathogenic mycobacterium widely used as a model organism to study the biology of other virulent and extremely slow growing species like M. tuberculosis (5). In particular, the genome of M. smegmatis encodes more than 500 regulatory factors (GenBank accession number CP000480), which are strikingly more than the ~180 encoded by M. tuberculosis (1).

Generally, bacteria respond to DNA damage through an increase in the expression of a number of genes, resulting in a greater rate of survival. This response is regulated by the homologs of the Escherichia coli repressor protein LexA in many species (6). At least two mechanisms for DNA damage induction exist in M. tuberculosis (7); a LexA-regulated system dependent on RecA and a RecA/LexA-independent mechanism for DNA damage induction, which has yet to be characterized clearly (7). A few other genes have been reported to be upregulated in E. coli following DNA damage independent of LexA (8) or RecA (9). Interestingly, a global analysis of gene expression following DNA damage in both the wild-type strain and recA deletion mutant of M. tuberculosis demonstrated that the majority of inducible DNA repair genes in M. tuberculosis were induced independently of RecA (10). However, the target genes controlled by the majority of the transcription factors and the functional roles of these regulations in vivo remain largely unknown.

TetR is a large family of transcriptional regulators. Its prototype is TetR from the Tn10 transposon of E. coli, which functions to regulate the expression of a tetracycline efflux pump in Gram-negative bacteria (11). These proteins often serve as repressors and are widely distributed among bacteria, regulating a number of diverse processes (12). For example, Staphylococcus aureus QacR regulates the expression of a multidrug transporter (13). Mycobacterium tuberculosis EthR regulates the expression of a monoxygenase gene that catalyzes the activation of ethionamide, an antibiotic used in TB treatment (14,15). KstR, a highly conserved transcriptional repressor, in

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M. smegmatis and M. tuberculosis which also belongs to
the TetR family, directly controls the expression of 83
genes in M. smegmatis and 74 genes in M. tuberculosis
(16). SczA is one of the few examples of regulators from
the TetR family that function as a transcriptional activa-
tor (17).

In the present study, a new TetR family transcriptional
regulator, Ms6564, was examined in M. smegmatis.
Evidence was provided to show that Ms6564 is a can-
date for the broad regulation of gene expression including
cell cycle and RecA-dependent and RecA-independent
DNA damage/repair genes. In particular, Ms6564 was
demonstrated to function as a master activator and a
negative regulator of gene mutation rates.

MATERIALS AND METHODS

Strains, enzymes, plasmids and reagents

E. coli BL21 cells and pET28a were purchased from
Novagen and were used to express mycobacterial proteins.
pBT, pTRG vectors and E. coli XR host strains were
purchased from Stratagene. Restriction enzymes, T4
ligase, modification enzymes, Pyrobest DNA polymerase,
dNTPs and all antibiotics were obtained from TaKaRa
Biotech. The reagents for one-hybrid assay were pur-
chased from Stratagene. Polymerase Chain Reaction
(PCR) primers were synthesized by Invitrogen
(Supplementary Table S1) and Ni-NTA (Ni²⁺-
nitrilotriacetate) agarose was obtained from Qiagen.

Cloning of M. smegmatis transcription factors and
regulatory sequences of the target genes and bacterial
one-hybrid assays

About 505 transcription factors were predicted from the
genome of M. smegmatis mc² 155 National Center of
Biotechnology Information. All of these probable genes
were amplified using their respective primers and were
cloned into the pTRG vector (Stratagene). A subgenomic
library for M. smegmatis mc² 155 transcription factors was
produced by mixing these recombinant plasmids. The pro-
moters of the M. smegmatis mc² 155 genes were also
amplified using their primers (Supplementary Table S1)
and were cloned into pBXcmT vector (2). E. coli
XL1-Blue MRF’ Kan strain (Stratagene) was used for
the routine propagation of all pBXcmT and pTRG recom-
binant plasmids. BacterioMatch I One-Hybrid System
(Stratagene) was utilized to detect DNA–protein inter-
actions between pBXcmT and pTRG plasmids as
described previously (2). The recombinant plasmid
pBXcmT was used to screen the library for M. smegmatis
mc² 155 transcription factors. Positive growth
co-transformants were selected on a selective screening
medium plate containing 20 mM 3-AT, 16 µg/ml strepto-
mycin, 15 µg/ml tetracycline, 34 µg/ml chloramphenicol
and 50 µg/ml kanamycin. The plates were incubated at
30°C for 3–4 days. A co-transformant containing
pBX-R2031/pTRG-R3133 plasmids (2) was served as
positive control and a co-transformant containing empty
vector pBX and pTRG was also served as negative
control.

Expression and purification of recombinant proteins

Mycobacterium smegmatis mc² 155 genes were amplified
by PCR primers from genomic DNA (Supplementary
Table S1). The corresponding genes were cloned into
pET28a to produce recombinant vectors. Transformed
with the recombinant plasmid, E. coli BL21 cells were
grown in a 200 ml LB medium up to an OD₆₀₀ of 0.6.
Protein expression was induced by the addition of
0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).
The harvested cells were resuspended and sonicated in
binding buffer (100 mM Tris–HCl pH 8.0, 500 mM NaCl
and 10 mM imidazole) for his-tagged proteins. The lysate
was centrifuged at 10 000g for 30 min, and the cleared
supernatant was loaded on the affinity column. The
column-bound protein was washed with a wash buffer
(100 mM Tris–HCl pH 8.0, 500 mM NaCl and 40 mM
imidazole) for his-tagged proteins. The protein was then
ecluted using an elution buffer (100 mM Tris–HCl pH 8.0,
500 mM NaCl and 250 mM imidazole) for his-tagged
proteins. The elution was dialyzed overnight and stored
at −80°C. Protein concentration was detected by
Coomassie Brilliant Blue assay.

DNA substrate preparation and electrophoretic mobility
shift assay

The DNA fragments for the DNA-binding activity assays
were amplified by PCR from M. smegmatis mc² 155
 genomic DNA or directly synthesized by Invitrogen
(Supplementary Table S2). The amplified products were
purified with BioFlux PCR DNA Purification kit
(BioFlux) labeled with T4 polynucleotide kinase (Takara)
and [γ-³²P] Adenosine Triphosphate (ATP) following the
manufacturer’s instructions. The mixture was treated at
65°C for 7 min to inactivate the protein kinase in the re-
actions. The labeled DNA substrates were then stored at
−20°C until use. The synthesized oligonucleotide was
radioactively labeled with T4 polynucleotide kinase
(Takara) and [γ-³²P] ATP. The labeled oligonucleotide
was purified as described previously (18). The 1.2-fold un-
abeled reverse oligonucleotide was added and incubated
at 95°C for 10 min to allow complete annealing. The DNA
substrates were stored at −20°C until use. Labeled DNA
fragments were incubated at 25°C for 30 min or 1 h with
various amounts of proteins in a total volume of 20 µl
electrophoretic mobility shift assay (EMSA) buffer
consisting of 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂,
1 mM DTT and 50 mM NaCl. The mixtures were then
directly subjected to 5% native Polyacrylamide Gel
Electrophoresis containing 0.5 × Tris–borate–EDTA
buffer. Electrophoresis was performed at 150 V at 25°C.
Images were acquired by Typhoon Scanner (GE
Healthcare).

DNase I footprinting assays

The 189 bp promoter regions Ms6564p4-1 (coding strand)
and Ms6564p4-2 (non-coding strand) (Supplementary
Table S2) were amplified by PCR using their primers
labeled with Fluorescein Isothiocyanate (Supplementary
Table S1). The amplified products were purified with
BioFlux PCR DNA Purification kit (BioFlux) and then subjected to the same binding reaction as in EMSA. DNasel footprinting was performed as described previously (19). The ladders were produced using the Sanger dideoxy method and Ms6564p4f1 and Ms6564p4r2 primers (Supplementary Table S1).

Construction of the Ms6564 deletion mutant of M. smegmatis mc²155 and Southern blot analysis

Knockout of the Ms6564 gene from M. smegmatis mc²155 (20) was performed as described previously (21). A pMind (22) derived suicide plasmid carrying a hygromycin resistance gene was constructed and a sacB gene was inserted to confer sensitivity to sucrose as a negative selection marker. The recombinant plasmid pMindMs6564 was electrophorated into M. smegmatis mc²155 and selected on 7H10 medium containing 100 ?g/ml hygromycin and 4% sucrose. Genomic DNA from allelic-exchange mutants in which the Ms6564 gene had been deleted was identified by restriction digestion and confirmed by PCR analysis using the primers on each side of Ms6564 and the hygromycin gene.

The deleted Ms6564 gene was identified by Southern blot analysis. Approximately 10 ?g genomic DNA was digested overnight with an excess of NarI, and the fragments were separated by electrophoresis through 0.8% agarose gels. Southern blotting was carried out in 10x Saline Sodium Citrate (SSC) using Hybond-N+ nylon membranes (Amersham). The probe consisted of a 392 bp fragment of the upstream region of the Ms6564 gene amplified using a pair of its primers (Supplementary Table S1). The Prime a Gene labeling system (Amersham) and 5 ?Ci digolan were used to label the probe. Prehybridization and hybridization were carried out at 65°C using 5xSSC, 5xDenhardt’s solution and 0.5% Sodium Dodecyl Sulfate (SDS). Serial 15 min washes were performed at 65°C as follows: two washes with 2xSSC and 0.1% SDS and two washes with 1xSSC and 1% SDS. The filter was developed and photographed.

Quantitative real-time PCR

Isolation of mRNA and cDNA from Msm/pMV261 and Msm/pMV261-Ms6564 (Msm/WT and Msm/Ms6564::hyg) strains was performed as described previously (23). For real-time PCR analysis, gene-specific primers (Supplementary Table S3) were used, and first-strand cDNAs were synthesized using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Each PCR reaction (20 ?l) contained 10 ?l of 2xSYBR Green Master Mix Reagent (Applied Biosystems), 1.0 ?l of cDNA samples and 200 nM gene-specific primers. The reactions were performed in Bio-Rad IQ5 RT-PCR machine. The thermocycling conditions were 95°C for 5 min and 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. Amplification specificity was assessed using melting curve analysis. Different gene expressions were normalized to the levels of 16S rRNA gene transcripts (24). The degrees of expression change were calculated using the 2^-ΔΔCt method (25).

Quantitative chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (26). Mycobacterium smegmatis mc²155 cells were grown in a 100 ml 7H9 medium up to an OD600 of 1.0, fixed with 1% formaldehyde for 20 min and stopped with 0.125 M glycine for 5 min. Crosslinked cells were harvested and resuspended in 1 ml Tris-Buffered Saline supplemental with Tween-20 and Triton-X 100 (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20, 0.1% Triton X-100). The sample was sonicated on ice and the average DNA fragment size was determined to be ~0.5 kb. A 100 ?l sample of the extract was saved as the input fraction, whereas the remaining 900 ?l was incubated with 10 ?l of antibodies against Ms6564 or preimmune serum under rotation for 3 h at 4°C. The complexes were immunoprecipitated with 20 ?l 50% protein A agarose for 1 h under rotation at 4°C. The immunocomplex was recovered by centrifugation and resuspended in 100 ?l TE (20 mM Tris–HCl pH 7.8, 10 mM EDTA, 0.5% SDS). Crosslinking was reversed for 6 h at 65°C. The DNA samples of the input and ChIP were purified, resuspended in 50 ?l TE and analyzed by PCR with Platinum Taq (Invitrogen). Each experiment was performed in duplicate and repeated twice. The amplification protocol included one denaturation step of 5 min at 95°C, then 32 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C.

Analysis of β-galactosidase activity

β-galactosidase activity experiments were performed in M. smegmatis by creating operon-lacZ fusions based on the expression vector of pMV261 (27). Promoter sequences were first cloned into pMV261 backbone by XbaI/EcoRI and then the reporter gene lacZ was cloned by HindIII/NheI. The reporter plasmids were transformed into ΔMs6564 strain to obtain the corresponding recombinant reporter strains ΔY0, ΔY1, ΔY2, ΔY3, ΔY4, ΔY5 and ΔY6. They were transformed into wild-type M. smegmatis to obtain the corresponding reporter strains Y0, Y1, Y2, Y3, Y4, Y5 and Y6 (Supplementary Table S4). All strains were grown in 7H9-Tw-glycerol-Kan liquid medium at 37°C and 155 cells were grown in a 100 ml 7H9 medium up to an OD600 of 1.0, fixed with 1% formaldehyde for 20 min and stopped with 0.125 M glycine for 5 min. Crosslinked cells were harvested and resuspended in 1 ml Tris-Buffered Saline supplemental with Tween-20 and Triton-X 100 (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% Tween 20, 0.1% Triton X-100). The sample was sonicated on ice and the average DNA fragment size was determined to be ~0.5 kb. A 100 ?l sample of the extract was saved as the input fraction, whereas the remaining 900 ?l was incubated with 10 ?l of antibodies against Ms6564 or preimmune serum under rotation for 3 h at 4°C. The complexes were immunoprecipitated with 20 ?l 50% protein A agarose for 1 h under rotation at 4°C. The immunocomplex was recovered by centrifugation and resuspended in 100 ?l TE (20 mM Tris–HCl pH 7.8, 10 mM EDTA, 0.5% SDS). Crosslinking was reversed for 6 h at 65°C. The DNA samples of the input and ChIP were purified, resuspended in 50 ?l TE and analyzed by PCR with Platinum Taq (Invitrogen). Each experiment was performed in duplicate and repeated twice. The amplification protocol included one denaturation step of 5 min at 95°C, then 32 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C.

Estimation of mutation frequencies and rates

The mutation frequency of the streptomycin-resistant gene in both the wild-type strain and mutant strains was examined as reported previously (29). Briefly, single colonies from M. smegmatis strains were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% (v/v) albumin-dextrose-catalase (Merk), 0.2% glycerol and 0.1% Tween-80 to an optical density at 600 nm of 1.2–1.5. Then 1 ml of the cultures were plated in triplicate onto a 7H10 solid medium containing Str
(50 µg/ml) to monitor spontaneous mutation frequencies. The remaining cultures were diluted to 10−6, and 200 µl of the dilution was plated in triplicate onto a 7H10 antibiotic-free solid medium for Colony-forming unit (CFU) determination. Mutation frequencies were calculated as reported previously (29).

The rates of the spontaneous mutation of M. smegmatis strains to streptomycin (Str) resistance were determined by Luria–Delbrück fluctuation analysis (30) using the method described by Machowski et al. (31) with slight modification. Single colonies from M. smegmatis wild-type strain, Ms6564 deletion strain and Ms6564 deletion strain complemented with a Ms6564 expression plasmid pMindD6564 were grown in Middlebrook 7H9 medium (Difco) supplemented with 10% (v/v) albumin-dextrose-catalase (Merck), 0.2% glycerol and 0.1% Tween-80 to an optical density at 600 nm of 1.2–1.5. To stabilize pMindD6564 in the complemented mutant strain, additional kanamycin (50 µg/ml) was placed in the medium. For the selection of mutant clones, the entire contents of a culture tube were plated on Str-supplemented medium following the removal of a 100 µl aliquot for CFU determination. The final number of cells in the culture (N0) and the observed number of mutant in the same culture (r) were determined by plating with and without streptomycin, respectively. Mutation rates were calculated by the method of the median (32) using the following formula: mutation rate = m/N0, where m was calculated from r via the Lea–Coulson equation: r/m − ln(m) − 1.24 = 0 using the web tool Fluctuation AnaLysis CalculatOR (33).

RESULTS

Ms6564 interacts with the promoters of RecA-dependent and RecA-independent DNA repair genes and its own promoter region

We used a bacterial one-hybrid system (2), which detected protein–DNA interactions based on transcriptional activation of reporter genes of HIS3 and aadA, to search for the potential common transcriptional factors involved in the broad regulation of the expressions of both RecA-dependent and RecA-independent DNA repair genes in M. smegmatis. The promoter region of Ms2312, a reported RecA-dependent DNA repair gene (10) or Ms4925, a RecA-independent gene was cloned into the upstream of HIS3–aadA in the reporter vector pBXcmT (2). The library of predicted transcriptional regulators from M. smegmatis was screened using these two promoters, Ms2312p and Ms4925p, as a bait sequence. In a bacterial one-hybrid assay, a putative TetR-like transcriptional factor, Ms6564, interacted with both promoters in Figure 1A. This result was evident in the co-transformants with pTRG-Ms6564/pBX-Ms2312p and pTRG-Ms6564/pBX-Ms4925p that grew very well in the screening medium. The positive control, composed of co-transformants with pTRG-Rv3133c/pBX-Rv2031p (2), also grew well in the medium (Figure 1A). The binding of Ms6564 with its own promoter was likewise examined because most members of the TetR family presented an auto-regulation mechanism. The co-transformants with pTRG-Ms6564/pBX-Ms6564p grew very well in the screening medium as shown in Figure 1A. By contrast, no growth was observed for their self-activated controls. Therefore, Ms6564 can bind to the promoters of RecA-dependent and RecA-independent DNA repair genes and it can also bind with its own promoter.

Ms6564 specifically binds to the target promoters both in vivo and in vitro

ChIP assay was subsequently conducted to examine the binding of Ms6564 to Ms2312p, Ms4925p and Ms6564p in vivo. As shown in Figure 1B, Ms6564 can be crosslinked to Ms2312p, Ms4925p and Ms6564p. These promoter DNAs can be particularly recovered by immunoprecipitation through the specific Ms6564 antiserum (Figure 1B, lane 2). By contrast, the preimmune serum failed to precipitate significant amounts of DNA (Figure 1B, lane 3). Ms1432p, the promoter of an unrelated gene used as negative control, cannot be recovered by the Ms6564 antiserum. Further EMSA assays confirmed the binding of the purified Ms6564 protein to these target promoter DNAs in vitro. As shown in Figure 1C, when 3 nM Ms6564 promoter DNA substrates were co-incubated with increasing amounts of Ms6564 (0, 0.05, 0.1, 0.2 and 0.4 µM), clear shifted bands were observed (Figure 1C, lanes 2–5). By contrast, the heat-denatured Ms6564 protein lost most of its binding activities (Figure 1C, lane 6). Ms6564 cannot bind with an unrelated Ms821p promoter DNA (Figure 1C, lanes 7 and 8). Therefore, Ms6564 can bind with its promoter DNA. It can also bind with Ms2312p (Figure 1C, lanes 9–11) and Ms4925p (Figure 1C, lanes 12–14) forming a clear protein–DNA complex on the gel. A competition assay confirmed the specificity of Ms6564 binding with its promoter DNA. Unlabeled cold Ms6564 or unspecific Ms821 promoter DNA substrates were used to compete with the labeled Ms6564 promoter DNA. As shown in Figure 1D, cold Ms6564 promoter DNA, but not Ms821 promoter DNA, could competitively inhibit the binding of Ms6564 to the labeled Ms6564 promoter DNA substrate.

The above findings strongly suggested that Ms6564 can bind with the promoters of both RecA-dependent and RecA-independent DNA repair genes, as well as its own promoter region.

Ms6564 binds with DNA fragments containing a palindrome sequence motif

A series of truncated DNA substrates within the promoter region of Ms6564, designated as p1–p7 (Supplementary Figure S1A), was produced to characterize the DNA-binding motif for Ms6564 protein. After two cycles of EMSA assays, the binding region was mapped further into Ms6564 p6 as evidenced by an obvious DNA-binding activity on the 39 bp-length substrate p6, but not on p5 or p7 (Supplementary Figure S1B).

The binding motif for the recognition of Ms6564 was characterized by further DNaseI footprinting assays. As shown in Figure 2A, when increasing amounts of Ms6564 protein (0–2 µM) were co-incubated with DNaseI, the
region around AACGAGACGGTACGTCTCGT was obviously protected on the coding strand. This result indicates that the DNA fragment contained a potential binding motif for Ms6564. Similarly, the region around CCACAAGACGAGACGT ACCGTCTCGTT was protected when the non-coding strand DNA was used as substrate (Figure 2A, right panel). The protected DNA region was extended from position $+66$ to $+37$ in the coding strand and from position $-65$ to $-39$ in the non-coding strand (Figures 2B and C). A palindromic motif formed by two inverted repeats (IR, 5’-ACGAGACG-3’) separated from each other by three nucleotides (Figures 2B and C) was found from an analysis of this protected sequence. Further EMSA assays were conducted to confirm the significance of the motif for the specific recognition by Ms6564. As shown in Figure 2C (right panel, lanes 5–8), Ms6564 lost the capability to bind with the Ms6564-p8 in which two inverted repeats were replaced by the random sequences CTTGACTA and TTCAGTGC. Therefore, the putative binding sites for Ms6564 contained a specific palindromic sequence motif.

Ms6564 binds with the promoters of many DNA damage/repair and cell cycle genes

The intergenic regions of the M. smegmatis genome were searched based on the sequence motif. A total of 339...
potential target genes were characterized (Supplementary Table S5 and Supplementary Figure S2). We further analyzed the classification and percentage of these target genes in the context of Cluster of Orthologous Groups of proteins (COG) categories. As shown in Figure 3A and B, among several defined functional categories, notably, it included 37 promoters which regulated expression of cell cycle and DNA damage/repair genes. Using the WebLogo tool (34), a logo assay was conducted to search for a more general conserved motif for Ms6564 binding. An inverted repeat sequence was characterized within the motif as shown in Figure 3C. Further in vivo ChIP assay established that 29 of all 30 target promoters of DNA damage/repair genes and seven cell cycle genes can be specifically recovered by immunoprecipitation through specific Ms6564 antiserum (Figure 3D). Only one promoter, Ms2723p, was not validated successfully. A negative control, Ms1432p, cannot be recovered by the Ms6564 antiserum. Therefore, these results proved that Ms6564 can regulate a large number of target genes.
Ms6564 positively regulates the expression of DNA damage/repair and cell cycle genes

An Ms6564-deleted mutant *M. smegmatis* strain was produced by gene replacement strategy (Figures 4A and B) to examine further the regulation of Ms6564 on the target genes. A knockout plasmid containing the Up and Down regions of the Ms6564 gene and the selective hygromycin resistance gene (Hgr) was constructed and transformed into *M. smegmatis*. A ΔMs6564 strain in which the Ms6564 gene was deleted was successfully produced using this method (Figure 4C). Southern blot assay was then conducted to confirm the deletion of Ms6564 in the ΔMs6564 strain. As shown in Figure 4D, a signal band of ~1.7 kb was detected (Figure 4D, right panel) using a 317 bp probe from the NarI-digested genomic DNA of the mutant *M. smegmatis* strain. By contrast, a signal band of only ~1.2 kb was seen in the wild-type strain (Figure 4D, right panel). This finding is consistent with the band sizes expected upon replacement of the Ms6564 gene with the Hygro^{r} gene, indicating that the Ms6564 gene was successfully deleted in the mutant strain.

A comparison of the expressions of some cell cycle and DNA damage/repair genes in both wild-type and Ms6564-deleted mutant mycobacterial strains was
conducted using quantitative real-time (qRT)-PCR assays. As shown in Figure 5A, compared with the expression in the wild-type strain, those of most of the tested genes were significantly downregulated ($P$-value $< 0.05$) in the ΔMs6564 *M. smegmatis* strains, with the exceptions of dinB and dinP. By contrast, the expression of the negative control gene, Ms6900, had not significantly change. This finding suggested that although two genes were upregulated unexpectedly, Ms6564 can function as a positive regulator for most target genes in *M. smegmatis* (Figure 5A). Further overexpression assay was conducted to examine the regulatory function of Ms6564. As shown in Figure 5B, the expressions of all tested genes were significantly upregulated ($P$-value $< 0.05$) when Ms6564 was overexpressed (~5-fold) through a pMV261-derived recombinant plasmid in *M. smegmatis* strains compared with the wild-type strain. This finding is consistent with the above assay in the ΔMs6564 *M. smegmatis* strains. Relative gene expression levels in response to DNA damage were also measured by qRT-PCR before and after induction by 5 mM H$_2$O$_2$ for 3 h in the ΔMs6564 or wild-type *M. smegmatis* strains (Supplementary Figure S3). Most of these cell cycle and DNA damage/repair target genes were found to be DNA damage inducible.

A series of promoter-β-galactosidase reporter plasmids was constructed using β-galactosidase as reporter gene in *M. smegmatis* to confirm further the positive regulation of Ms6564 on the target gene expressions. As shown in Figure 5C, the strong promoter hsp60 strikingly promoted the expression of lacZ in both wild-type and ΔMs6564 *M. smegmatis* strains compared with the non-promoter...
Figure 5. Expression assays of DNA damage and repair genes in wild-type and Ms6564-deleted mutant strains. qRT-PCR assay for the relative expression levels of DNA damage and repair genes in ΔMs6564 M. smegmatis strains (A) and in Ms6564-overexpressed strains (B). The mycobacterial cDNA was amplified as described in Materials and Methods’ section. The relative expression levels of the genes were normalized using 16S rRNA gene as an invariant transcript, and an unrelated promoter gene Ms6900 was used as negative control. Data were analyzed using the 2^{-ΔΔCT} method as described previously (25). As a positive control, total DNA of each strain was used as template for PCR amplification. The cDNA of the mutant strains and the recombinant strain containing an empty pMV261 vector was used as template in the negative controls. The P-values of the relative expression data were calculated by unpaired two-tailed Student’s t-test using GraphPad Prism 5. (C) The effect of Ms6564 on the gene expression of representative DNA repair genes was assayed by constructing a series of lacZ alone or promoter-lacZ co-expression plasmids. The included genes in the same operon were presented in the following brackets. The activity of β-galactosidase was further examined and presented as Miller units. The values presented were the averages of three independent experiments. For statistical analysis, two-way analysis of variance with Bonferroni multiple comparison tests were performed using a P-value of ≤0.05.
lacZ plasmid. The strain appeared deep blue and had high β-galactosidase (~700 Miller units), indicating that the report system worked well (Figure 5C, bottom of the panel). Six promoters including Ms6564p itself and an additional negative control, as well as an unrelated promoter of Rv0757p, were also used to promote expression of lacZ. As shown in Figure 5C, the expression of lacZ was downregulated in the Ms6564-deleted mutant M. smegmatis strains compared with the wild-type strain under all four promoters: Ms4232p, Ms4235p, Ms6806p and Ms6564p. However, there was no significant difference in the expression of lacZ between the wild-type and mutant strains when a negative control, Rv0757p, was used as promoter.

These results strongly suggested that Ms6564 can function as a positive regulator and it affected the expression of DNA damage and repair genes in M. smegmatis.

**Ms6564 negatively regulates gene mutation frequencies and rates**

Ms6564 was shown to bind directly to the promoter regions of many cell cycle and DNA damage and repair genes. This finding suggests that Ms6564 regulates genes necessary for DNA repair and, therefore, could indirectly affect spontaneous mutation rates and other repair processes. The gene mutation frequencies and rates in both Ms6564 overexpression and gene deletion mutant M. smegmatis strains were compared to examine this result further. Ms6564-overexpressed M. smegmatis tained a 5.1-fold lower streptomycin-resistant gene mutation frequencies (2.5 ± 0.3 × 10–9), whereas the Ms6564-deleted strain obtained 5-fold higher mutation frequency (6.06 ± 0.3 × 10–9) compared with the wild-type strains (12.8 ± 0.4 × 10–9) (Table 1). Similar results were obtained with an assay of mutation rates using a fluctuation experiment (Table 2). The P-values of the rates were calculated to be <0.05 (Table 2), indicating that these differences were statistically significant. Interestingly, when expressing the Ms6564 gene through a pMind in ΔMs6564 strain, the recombinant strain of M. smegmatis ΔMs6564/pMindD6564 re-obtained a closer frequency (14.1 ± 0.4 × 10–9) (Table 1) or mutation rates (2.6 × 10–9) (Table 2) to the wild-type strain, with no significant difference between these changes (P > 0.05). The empty pMV261 plasmid or overexpression of an unrelated gene, Ms3452, had no significant effect on mutation frequency. Therefore, these results suggest that Ms6564 regulates genes responsible for repair of spontaneous mutations in M. smegmatis mc2155.

**DISCUSSION**

The fast-growing M. smegmatis contains a large number of regulatory factors, and it has been widely used as a model organism to study the gene regulatory mechanism of the virulent M. tuberculosis (5). In the present study, M. smegmatis Ms6564 was confirmed as a candidate for the broad regulation of gene expression including cell cycle and DNA damage/repair genes. Some broad regulators have been reported from M. smegmatis and other bacterial species. For example, Sharon et al. (16) characterized a KstR repressor involved in regulating a total of 159 genes and in directly controlling the expression of 83 genes in M. smegmatis and 74 genes in M. tuberculosis. The motifs within the target operator for the TetR-like transcriptional factor were demonstrated to have an internal palindromic symmetry with an extra central base pair (12,16). A 19 bp-palindromic motif for specific recognition by Ms6564 was identified in the current study using DNaseI footprinting experiment combined with EMSA assays. Similar to many other transcription activators, Ms6564 bound to inverted repeats of an operator sequence upstream of and only partially overlapping

### Table 1. The mutation frequencies of wild-type and recombinant M. smegmatis strains

| Strain             | SM mutation frequency × 10⁻⁹ |
|--------------------|------------------------------|
| Ms6564:: hyg       | 60.6 ± 0.3*                  |
| Ms6564::hyg/pMV261 | 9.3 ± 0.2                    |
| Ms6564/pMV261      | 2.5 ± 0.3*                   |
| Ms6564/pMV261-Ms6564| 12.8 ± 0.4                  |
| Ms6564            | 12.2 ± 0.2                   |
| Ms6564 WT         | 10.9 ± 0.9                   |
| Ms6564::hyg        | 10.9 ± 0.9                   |

P-values of the results were calculated by unpaired two-tailed student’s test GraphPad Prism 5. *P-values of the results were <0.05.

### Table 2. The mutation rates of wild-type and recombinant M. smegmatis strains

| Fluctuation Expt no. | Strain             | No. of cultures | Nₐ-valueᵃ | Lea-Coulson m-valueᵇ | Mutation rateᶜ |
|----------------------|--------------------|-----------------|------------|----------------------|----------------|
| 1                    | Ms6564 WT         | 30              | 1.2 × 10⁹  | 4                    | 2.0 × 10⁻⁹    |
| 2                    | Ms6564 WT         | 30              | 1.0 × 10⁹  | 5                    | 2.5 × 10⁻⁹    |
| 3                    | Ms6564 WT         | 30              | 1.2 × 10⁹  | 8                    | 0.7 × 10⁻⁹a   |
| 4                    | Ms6564 WT         | 30              | 1.0 × 10⁹  | 6                    | 2.2 × 10⁻⁹    |
| 5                    | Ms6564 WT         | 30              | 1.1 × 10⁹  | 7.6                  | 6.9 × 10⁻⁹a   |
| 6                    | Ms6564 WT         | 30              | 0.8 × 10⁹  | 2.1                  | 2.6 × 10⁻⁹    |

ᵃFinal number of cells in the culture.
ᵇNumber of mutations per culture.
ᶜProbability of mutation per cell per generation.

P-values of the results were calculated by unpaired two-tailed student’s test GraphPad Prism 5. *P-values of the results were <0.05.
Ms6564 in Supplementary Figure S3). When overexpressing were found to be DNA damage inducible corrections (10,37). In the present study, most of these cell repair genes in mycobacteria are RecA-independent activations (10,37). In the current study, based on qRT-PCR and β-galactosidase activity analysis, Ms6564 may function as an activator different from most typical TetR-like regulators. However, the expressions of dinB and dinP were shown to be negatively regulated by Ms6564 in contrast to other genes (Figure 5A). We further compared the location of the binding site for Ms6564 in the promoters of dinB and dinP with that of other target genes. However, no obvious difference was observed (Supplementary Figure S4), thus, the mechanism for the repression or activation of dinB and dinP remains to be characterized. Interestingly, a recent study suggested that mycobacterial DinB homologs were substantially different from their E. coli counterparts and deletion of these genes did not affect bacterial growth and survival (36).

Two different mechanisms, the RecA/LexA-dependent and RecA-independent mechanisms, have been described for DNA damage repair in the bacterial Save Our Ship response. The RecA/LexA-dependent mechanism has been reported (7), but the majority of inducible DNA repair genes in mycobacteria are RecA-independent activations (10,37).

In summary, a TetR-like family transcriptional factor, Ms6564, in M. smegmatis, was found to be a master regulator affecting mycobacterial gene mutation rates. About 339 promoters of M. smegmatis genes or operons were characterized as its potential targets. Notably, Ms6564 was found to be involved in regulating the expressions of 37 cell cycle and DNA damage/repair genes. Mycobacterial gene mutation rates were also confirmed to correlate significantly with the expression level of Ms6564. These findings suggested that Ms6564 may function as a global regulator and may activate DNA repair genes.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online: Supplementary Tables S1–S5, Supplementary Figures S1–S5.

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