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Biochemical characterization of a chromosomal toxin–antitoxin system in *Mycobacterium tuberculosis*

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Abstract In the present paper, we report the biochemical characterization of a chromosomal toxin–antitoxin (TA) system in *Mycobacterium tuberculosis*, consisting of the *Rv1991c* gene and its upstream open reading frame (ORF) termed *Rv1991a*. Rv1991c was characterized as a toxin with ribonuclease activity and Rv1991a as the antitoxin against Rv1991c. Rv1991a interacted with Rv1991c to form a complex. A promoter located immediately upstream of *Rv1991a* was identified. Both Rv1991a and the Rv1991a–Rv1991c complex were able to bind to the promoter region of the *Rv1991a–Rv1991c* operon, indicating that the expression of the *Rv1991a–Rv1991c* operon can be autoregulated.

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1. Introduction

A typical toxin–antitoxin (TA) system consists of two genes, one for a stable toxin and the other for an unstable antitoxin. The toxin and antitoxin interact with each other to form a complex. Both the antitoxin–toxin complex and the antitoxin alone can bind to promoter region to autoregulate the expression of TA system at transcription level. TA systems, which were originally identified on plasmids, have been detected on chromosomes of numerous bacteria, and the chromosomal TA systems are proposed to be involved in the bacterial cell growth control or bacterial programmed cell death under the stress conditions [1–3].

Up to one-third of humans worldwide infected by *Mycobacterium tuberculosis* are in a latent state and at risk for tuberculosis when immune-compromised [4,5]. The latency of tuberculosis infections is attributed to the ability of *M. tuberculosis* to persist at a non-replicating state in a host lesion under the stress condition with reduced availability of nutrients and oxygen [6,7]. By sequence homology, 38 toxin–antitoxin systems have been identified in *M. tuberculosis* H37Rv and 36 systems in *M. tuberculosis* CDC1551 [1]. We have reported that seven *M. tuberculosis* genes encode the proteins homologous to MazF, the toxin in *Escherichia coli* mazEF TA system. Four of the MazF homologues, Rv2801, Rv1991c, Rv0659c and Rv1102c, were identified as toxins, among which Rv2801c and Rv1102c were characterized as ribonucleases cleaving RNA in a sequence-specific manner [8]. An open reading frame (ORF) locates immediately upstream of *Rv199c* and overlaps with *Rv1991c* by 7 base pairs. Recently *Rv1991c* and its upstream ORF have been proposed to form a putative toxin–antitoxin pair [9], but the biochemical properties and molecular mechanism of such a TA system remain unknown. In this work, we characterized Rv1991c as a toxin with ribonuclease activity. The ORF located upstream of *Rv199c*, termed as *Rv1991a*, encoded the antitoxin Rv1991a, which inhibited the ribonuclease activity of Rv1991c by forming Rv1991a–Rv1991c complex. Both Rv1991a–Rv1991c complexes and Rv1991a alone could bind to the promoter region of *Rv1991a–Rv1991c* operon. These data indicate that the *Rv1991a–Rv1991c* operon is a typical TA system on *M. tuberculosis* chromosome.

2. Materials and methods

2.1. Strains and media

Mycobacterium smegmatis mc^{2}155 was grown in Middlebrook 7H9 medium with 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H10 agar medium with 0.5% glycerol, supplemented with OADC (Difco). All *E. coli* strains were grown in M9 or LB medium. Hygromycin was added as required at a concentration of 150 mg/L for *E. coli* and 50 mg/L for mycobacteria. Kanamycin was added as required at a concentration of 50 mg/L for *E. coli* and 25 mg/L for mycobacteria.

2.2. Construction of vectors

M. tuberculosis Rv1991c gene was cloned into the NdeI-XhoI sites of pET28a, creating pET28a-$(His)_6$Rv1991c for expression of $(His)_6$Rv1991c in *E. coli.* The *Rv1991a* ORF was cloned into the NdeI-XhoI sites of pET28a, creating pET28a-$(His)_6$Rv1991a for expression of $(His)_6$Rv1991a in *E. coli.* Expression vector pACYCDuet (Novage) has two multi-cloning sites (MCS). The *Rv1991c* gene was cloned into MCS II of pACYCDuet, creating pACYC-1991c. The *Rv1991a* ORF was cloned into MCS I of pACYC-1991c, creating pACYC-1991a–1991c. The *Rv1991a* ORF was cloned into the BamHI–HindIII sites of pMV261, creating pMV261-Rv1991a. The *Rv1991c* gene was cloned into the BamHI-ClaI sites of pACE, creating pACE-Rv1991c for inducible expression of Rv1991c in *M. smegmatis*. The *M. tuberculosis Rv0707* gene was cloned into the BamHI-HindIII sites of pET17b, creating pET17b-Rv0707. The 99 base pair sequence located upstream of *Rv1991a* was cloned into the XbaI-SphI sites ahead of the promoterless *lacZ* gene in pSD5B [10], creating pSD5B-99bp.

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Abbreviations: TA, toxin–antitoxin; ORF, open reading frame; IPTG, isopropyl β-D-thiogalactopyranoside; MCS, multi-cloning site; EMSA, electrophoretic mobility shift assay

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2.3. Native PAGE and Tricine SDS–PAGE

$(His)_6$Rv1991a and $(His)_6$Rv1991c were mixed in binding buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 5% glycerol) at 4 °C for 30 min. The compositions of the stacking gel and the separation gel are same as described previously [11]. Electrophoresis was performed at constant voltage (150 V) at 4 °C. Tricine SDS–PAGE was carried out for the resolution of low molecular mass proteins as described previously [11].

2.4. In vitro RNA cleavage by Rv1991c

The DNA fragment containing a T7 promoter followed by the *M. tuberculosis Rv0707* gene was obtained by PCR amplification with the T7 primer 5′-AGATCTCGATCCCGCAAATTAAT-3′ and the primer Rv0707-2 5′-TCAGCTCTCCGTGCTCTG-3′ with pET17b-Rv0707 plasmid as template. Another DNA fragment containing a T7 promoter followed by the *E. coli era* gene was obtained by PCR amplification with the T7 primer 5′-AGATCTCGATCCCGCAAATTAAT-3′ and the primer era-2 5′-TTAAAGATCGTCAACGTAACCG-3′ with pET28a-Era plasmid [12] as template. The *Rv0707* mRNA and *era* mRNA were prepared from these two DNA fragments respectively with the RiboMAX™ T7 large scale RNA production system (Promega). RNA substrates were incubated with $(His)_6$Rv1991c at 37 °C for 15 min. Each reaction mixture (10 μl) contained 1.5 μg of RNA substrate, 100 pmol $(His)_6$Rv1991c, 1 μl of RNase inhibitor, 20 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 1 mM DTT. The reaction mixture was then subjected to 5% PAGE.

2.5. Determination of promoter activity

Promoter activity was determined by β-galactosidase activity assays. Assays for β-galactosidase activity were performed as described by Miller [13].

2.6. Electrophoretic mobility shift assays (EMSA)

The DNA fragment consisting of the 99 base pairs located upstream of *Rv1991a* was 5′ end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase. The binding reactions were carried out at 4 °C for 30 min with purified proteins and [^{32}P]-labeled 99-bp DNA in the binding buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol and 10 μg/ml poly(dI-dC)). Electrophoresis was performed with 8% native polyacrylamide gel in TAE buffer.

3. Results and discussion

3.1. Characterization of toxin Rv1991c and antitoxin Rv1991a

The effects of Rv1991c and Rv1991a on bacterial cell were tested in *E. coli* and *M. smegmatis*. The pACYCDuet-1 vector has two multi-cloning sites (MCS), each of which is under an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible promoter. In the presence of IPTG, *E. coli* cells harboring pACYC- 1991c with *Rv1991c* at MCS II could not grow on

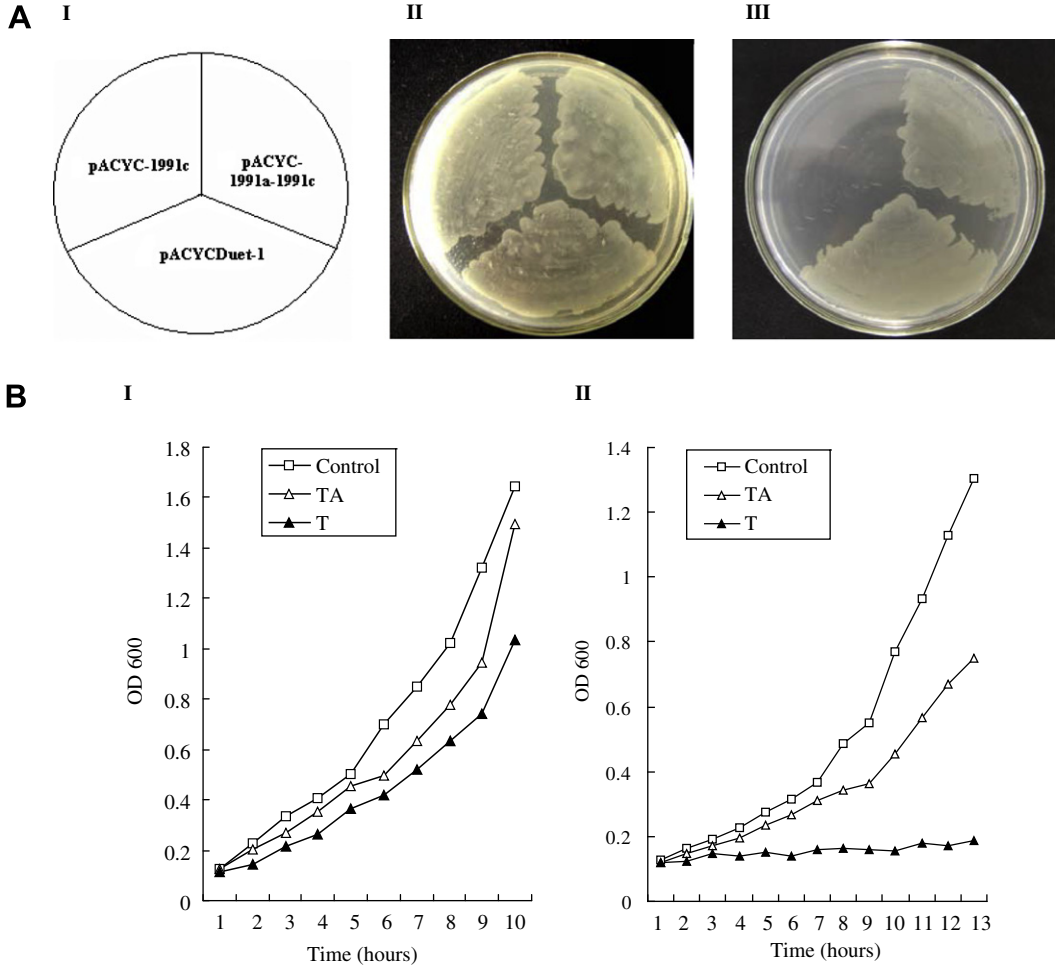

Fig. 1. Toxic effect of Rv1991c and antitoxic effect of Rv1991a. (A) Plasmids were transformed into *E. coli* BL21(DE3) as indicated in each sector (I). *E. coli* BL21(DE3) strains harboring different plasmids were grown on M9 plates without IPTG (II) or with 0.1 mM IPTG (III). (B) The *M. smegmatis* mc^2155 cells harboring the plasmids as indicated were grown in 7H9 middlebrook medium (Difco) without acetamide (I) or with 0.2% acetamide (II). Control (□), *M. smegmatis* harboring pACE and pMV261; TA (△), *M. smegmatis* harboring pACE-Rv1991c and pMV261-Rv1991a; T (▲), *M. smegmatis* harboring pACE-Rv1991c and pMV261.

the agar plate, while the *E. coli* cells harboring pACYC-1991a–1991c with *Rv1991a* at MCS I and *Rv1991c* at MCS II were able to grow (Fig. 1A). To assess the toxic effect of Rv1991c on mycobacteria cell, the *Rv1991c* gene was cloned under the inducible acetamidase promoter in pACE, an *E. coli–M. smegmatis* shuttle vector. When *M. smegmatis* cells harboring pACE-Rv1991c were cultured in the presence of acetamide, the cell growth was significantly arrested. The growth inhibition was released when pMV261-Rv1991a with *Rv1991a* under the hsp60 promoter was introduced into *M. smegmatis* cells harboring pACE-Rv1991c (Fig. 1B), indicating that the simultaneous expression of Rv1991a prevents the toxicity of Rv1991c. These results suggest that Rv1991c is toxic to bacterial cell, while Rv1991a functions as an antitoxin against Rv1991c.

3.2. Rv1991c and Rv1991a form a complex

$(His)_6$Rv1991a and $(His)_6$Rv1991c were expressed in *E. coli* BL21(DE3) and purified by affinity chromatography. $(His)_6$Rv1991a and $(His)_6$Rv1991c are referred as Rv1991a and Rv1991c in the following in vitro experiments. When the purified Rv1991a and Rv1991c were subjected to native PAGE respectively, Rv1991a was found as a band at the bottom of the gel (Fig. 2A, lane 2), while Rv1991c could not be detected (Fig. 2A, lane 1). It may be due to the highly positive charge of Rv1991c, which keeps Rv1991c from running into the gel under the experimental condition. When the mixture of Rv1991a and Rv1991c was subjected to native PAGE, a new band appeared at the position near the top of the gel (Fig. 2A, lanes 3 and 4). The gel corresponding to the new band was cut out, and then put on the top of Tricine SDS–PAGE gel to run a second dimensional electrophoresis to analyze the protein components. Two bands corresponding to Rv1991c and Rv1991a were observed (Fig. 2B, lanes 3 and 4). These data demonstrate that Rv1991a can interact with Rv1991c to form a stable complex. As shown in Fig. 2A, Rv1991a was totally recruited to form the complex when the molar ratio of Rv1991a to Rv1991c was 1:2. Although the exact composition of Rv1991a–Rv1991c complex is still unknown, it is possible that, as same as the molar ratio of MazE to MazF in MazE-MazF complex, the molar ratio of Rv1991a to Rv1991c is 1:2 in Rv1991a–Rv1991c complex.

3.3. Rv1991c has the ribonuclease activity

Rv1991c has the sequence similarity with Rv2801c, Rv1102c and *E. coli* MazF. In respect that Rv2801c, Rv1102c and MazF have been characterized as ribonucleases, the RNA cleavage activity of Rv1991c was tested. When *E. coli era* mRNA was used as substrate, the RNA was digested after incubated with Rv1991c (Fig. 3A, lane 3). Rv1991a inhibited the RNA cleavage activity of Rv1991c in a dose-dependent manner (Fig. 3A, lanes 4 and 5). The ribonuclease activity of Rv1991c was almost totally inhibited when the molar ratio of Rv1991a to Rv1991c was increased to 1:2 (Fig. 3A, lane 5), supporting the hypothesis that the molar ratio of Rv1991a to Rv1991c in Rv1991a–Rv1991c complex is about 1:2. *M. tuberculosis* is a pathogen with high GC content in its genome. It is interesting to test the ribonuclease activity of Rv1991c against *M. tuberculosis* mRNAs. As same as the *era* mRNA, the mRNA of *M. tuberculosis Rv0707* gene (GC 67.4%) was digested by Rv1991c, and protected by Rv1991a in a dose-dependent manner (Fig. 3B). Same results were observed when the mRNA of *M. tuberculosis Rv0527* gene (GC 69.2%) and the total RNA from *M. tuberculosis* H37Rv were used as substrates (data not shown). These data indicate that Rv1991c is a toxic ribonuclease arresting bacterial cell growth through the mechanism of RNA cleavage, and that Rv1991a inhibits the ribonuclease activity of Rv1991c by forming Rv1991a–Rv1991c complex.

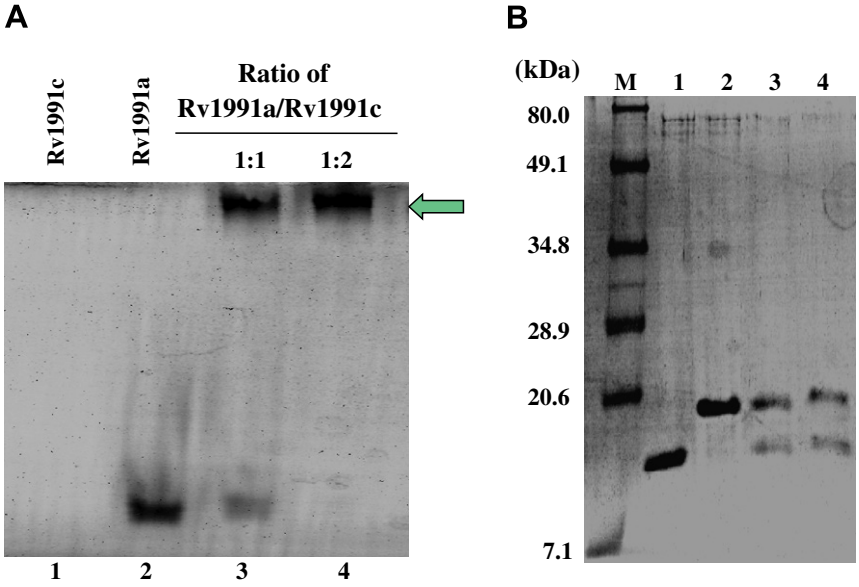

Fig. 2. Rv1991a–Rv1991c complex formation. (A) Native PAGE. Lane 1, $(His)_6$Rv1991c only; lane 2, $(His)_6$ Rv1991a only; lanes 3 and 4, the mixture of $(His)_6$Rv1991a and $(His)_6$Rv1991c with different molar ratios as indicated. The position of the complex is indicated by arrow. (B) Tricine SDS–PAGE. The gel corresponding to the band of the complex was cut out, and then subjected to Tricine SDS–PAGE to analyze the protein components. Lane M, protein molecular mass markers; lane 1, $(His)_6$Rv1991a; lane 2, $(His)_6$Rv1991c; lane 3, the complex from lane 3 of panel A; lane 4, the complex from lane 4 of panel A.

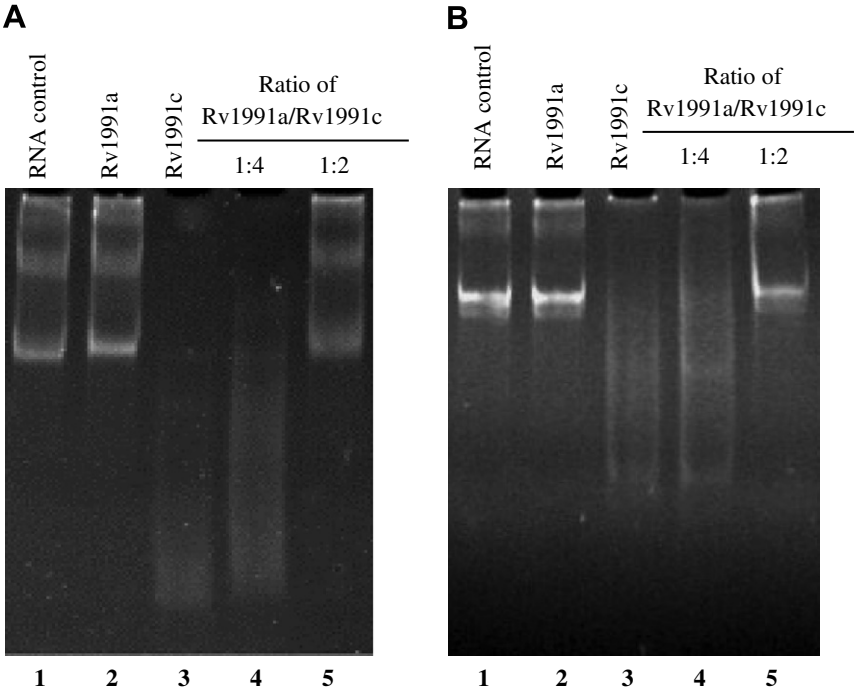

Fig. 3. Ribonuclease activity of Rv1991c. (A) *E. coli era* mRNA was used as substrate; (B) *M. tuberculosis Rv0707* mRNA was used as substrate. Each reaction was performed at 37 °C for 15 min with 1.5 μg RNA. The reaction products were analyzed by 5% PAGE. Lane 1, control, RNA alone; lane 2, RNA incubated with 50 pmol $(His)_6$Rv1991a; lane 3, RNA incubated with 100 pmol $(His)_6$Rv1991c; lanes 4 and 5, RNA incubated with 100 pmol $(His)_6$Rv1991c together with 25 pmol and 50 pmol $(His)_6$Rv1991a, respectively.

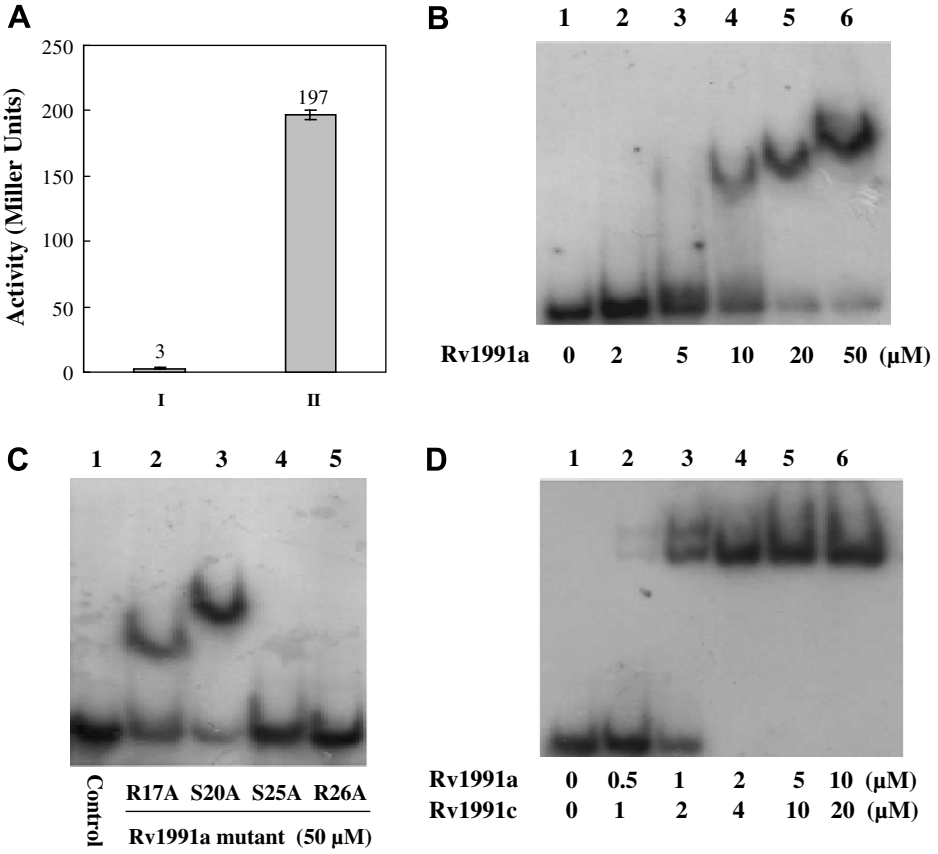

Fig. 4. Binding abilities of Rv1991a and Rv1991a–Rv1991c complex to the promoter DNA. (A) Analysis of the promoter activity. *M. smegmatis* mc^2155 cells containing pSD5B (I) or pSD5B-99bp (II) were grown in 7H9 Middlebrook medium with 2.0% glucose till mid-log phase before harvest. The β-galactosidase activity assays were done in triplicates, and the data represent the averages. (B–D) Binding abilities of Rv1991a (B), Rv1991a mutants (C) and Rv1991a–Rv1991c complex (D) to the promoter DNA were determined by EMSA as described in Section 2.

3.4. Both Rv1991a–Rv1991c complex and antitoxin Rv1991a alone can bind to the promoter region

The 99 base pair sequence located immediately upstream of *Rv1991a* was cloned ahead of the promoterless *lacZ* gene in pSD5B vector to construct the promoter-reporter plasmid pSD5B-99bp, which was transformed into *M. smegmatis* for promoter activity determination by β-galactosidase assays. As shown in Fig. 4A, the promoter activity was detected from the 99-bp DNA fragment. In the updated annotation of *M. tuberculosis* H37Rv genome, *Rv1991a* has not been identified as an ORF. The existence of a promoter upstream of *Rv1991a* suggests that *Rv1991a* is an active ORF to form the *Rv1991a–Rv1991c* operon.

The DNA fragment consisting of the 99 base pairs located upstream of *Rv1991a* was 5′ end-labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase, and then used to test the promoter binding abilities of Rv1991a, Rv1991c and Rv1991a–Rv1991c complex by EMSA. The antitoxin Rv1991a could bind to the DNA fragment and shift it up (Fig. 4B), while the toxin Rv1991c could not (data not shown). Several substitution mutations were made in the N-terminal region of Rv1991a. The S25A and R26A mutation disrupted the DNA binding ability of Rv1991a (Fig. 4C, lanes 4 and 5), suggesting that the N-terminal region of Rv1991a is involved in DNA binding. The Rv1991a–Rv1991c complex was also able to bind to the 99-bp DNA fragment. Compared with Rv1991a alone, the Rv1991a–Rv1991c complex has significant higher binding affinity to the promoter region of *Rv1991a–Rv1991c* operon (Fig. 4D). These data indicate that the expression of *Rv1991a–Rv1991c* system can be autoregulated at transcription level.

Most *M. tuberculosis* infections do not immediately develop into acute diseases. The host immune response restricts the development of active infection by quarantining *M. tuberculosis* in granuloma, while the *M. tuberculosis* bacterium can persist in granulomatous lesions in a non-replicative state, with the ability to resume growth and activate disease [14]. It has been demonstrated that the stress conditions such as nutrient deprivation and oxygen limitation play a role in inducing non-replicacing persistence [15]. Under normal growth conditions, the antitoxin and toxin are coexpressed from a TA operon and form a stable complex. However, the stresses may affect the balance between toxin and antitoxin and release the free toxin, allowing cells to enter a persistent state. We have characterized the *Rv1991a–Rv1991c* operon as a TA system on *M. tuberculosis* chromosome, encoding antitoxin Rv1991a and toxin Rv1991c with ribonuclease activity, but we do not presently know which stringent regulator can activate the toxicity of endogenous Rv1991c. Further works will be done to investigate the expression control of *Rv1991a–Rv1991c* TA system in vivo, and to clarify its physiological function.

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