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Characterization of a Bifunctional β-Lactamase/Ribonuclease and Its Interaction with a Chaperone-Like Protein in the Pathogen *Mycobacterium tuberculosis* H37Rv

Lei Sun, Lei Zhang, Hua Zhang, and Zheng-Guo He*

National Key Laboratory of Agricultural Microbiology, Center for Proteomics Research, College of Life Science and Technology, Huazhong Agricultural University, Wuhan 430070, China; fax: +86-27-8728-0670; E-mail: hezhengguo@mail.hzau.edu.cn; he.zhengguo@hotmail.com

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Abstract—Most mycobacteria appear to be naturally resistant to β-lactam antibiotics such as penicillin. However, very few β-lactamases and their regulation have been clearly characterized in *Mycobacterium tuberculosis* H37Rv. In this study, a unique bifunctional protein, Rv2752c, from *M. tuberculosis* showed both β-lactamase and RNase activities. Two residues, D184 and H397, appear to be involved in Zn^{2+}-binding and are essential for the dual functions. Both activities are lost upon deletion of the C-terminal 100 a.a. long Rv2752c tail, which contains an additional loop when compared with the RNase J of *Bacillus subtilis*. A chaperone-like protein, Rv2373c, physically interacted with Rv2752c and inhibited both activities. This is the first report of characterization of a bifunctional β-lactamase and its regulation in mycobacteria. These data offered important clues for further investigation of the structure and function of microbial β-lactamases. Increased understanding of this protein will provide further insights into the mechanism of microbial drug resistance.

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Key words: *Mycobacterium tuberculosis*, metallo-β-lactamase, ribonuclease

One of the most important mechanisms in microbial drug resistance results from the presence of a β-lactamase that catalyzes the degradation of β-lactam antibiotics such as penicillin [1, 2]. However, the spread of drug-resistant bacterial strains has been accelerated due to the excessive use and abuse of β-lactam antibiotics. The recent appearance of multidrug-resistant strains has introduced great challenges in the treatment of infectious diseases. Understanding the regulation of this class of enzymes in pathogens would therefore be of great benefit in fighting diseases.

β-Lactamases (EC 3.5.2.6) can be divided into four classes – A, B, C, and D [3]. Class B consists of metalloenzymes with one or two zinc cofactor ions. Resolution of the X-ray crystal structures of several β-lactamases has revealed a common αβ/βα sandwich folding pattern [4-8]. Metal ions are important in regulating the activity of metallo-β-lactamase [5, 9-13]; for example, Hu et al. found that Zn(II) can flexibly regulate the transposition of different metal ions, allowing the organism to withstand many different β-lactam drugs [14]. Several proteins with structures that place them in the metallo-β-lactamase superfamily have not yet been confirmed to show β-lactamase activity; instead, they clearly show RNase activity. One example is RNase E, an essential endoribonuclease involved in the maturation of stable RNA and the degradation of messenger RNA in *Escherichia coli*. In *Bacillus subtilis*, both RNase J1 and J2 share functional homologies with RNase E, but no sequence similarity [15]. RNase J1 is an essential ribonuclease involved in the maturation of *B. subtilis* 16S rRNA [16], and its conserved sites have been identified [17]. RNase J has recently been shown to display an exonucleolytic activity, and the three-dimensional structure of *Thermus thermophilus* RNase J has been resolved [18, 19]. There is no report of a *Mycobacterium tuberculosis* RNase J.

Mycobacterium tuberculosis is a highly successful human pathogen and its multiple drug resistance is the most serious challenge facing current tuberculosis treatment programs. Several genes encoding β-lactamase

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; GST, glutathione-S-transferase; Ni-NTA, Ni^{2+}-nitrilotriacetate.

* To whom correspondence should be addressed.

activity have been found in the genome of *M. tuberculosis*, and primary research on their biochemical characteristics has been launched [20-22]. For example, Tremblay et al. analyzed co-crystallization of BlaC, which belongs to the class A β-lactamases, with clavulanate [22].

This paper presents the first report of a bifunctional enzyme, Rv2752c, from *M. tuberculosis* that shows both β-lactamase and RNase activities. Several critical residues and domains of Rv2752c are shown to have important effects on both activities. In addition, we also report identification and characterization of a chaperone-like protein, Rv2373c, that physically and functionally interacts with Rv2752c.

MATERIALS AND METHODS

Strains, enzymes, plasmids, and reagents. *Escherichia coli* BL21 (DE3) cells and pET28a were purchased from Novagen (USA) and were used to express *M. tuberculosis* proteins. The pBT and pTRG vectors and *E. coli* XR host strains were purchased from Stratagene (USA). DNA polymerase, dNTPs, restriction enzymes, and all antibiotics were obtained from TaKaRa Biotech (China). PCR primers were synthesized by Invitrogen (USA) (table). Ni-NTA (Ni^{2+}-nitrilotriacetate) and GST agarose were obtained from Qiagen (USA). The reagents for two-hybrid assay were obtained from Stratagene (USA).

Cloning and purification of *M. tuberculosis* proteins. Prokaryotic recombinant vectors expressing Rv2752c and its mutant proteins were constructed. *Escherichia coli* BL21 (DE3) cells were used as the host strain to express their proteins as previously described [23]. Protein concentrations were determined by spectrophotometric absorbance at 280 nm according to Gill and Hippel [24].

Bacterial two-hybrid analysis. BacterioMatch II Two-Hybrid System (Stratagene) was used to detect protein–protein interaction between Rv2752c and Rv2373c proteins. The bacterial two-hybrid analysis was carried out according to the procedure supplied with the commercial kit and our previously published procedures [25]. Archaeal genes were amplified by PCR using their specific primer pairs (table) from genomic DNA of *M. tuberculosis*. The pBT and pTRG vectors containing *M. tuberculosis* genes were generated. Positive-growth co-transformants were selected on Screening Medium plate containing 5 mM 3-amino-1,2,4-triazole (3-AT) (Stratagene), 8 μg/ml streptomycin, 15 μg/ml tetracycline, 34 μg/ml chloramphenicol, and 50 μg/ml kanamycin.

Co-expression/co-purification and western blotting assays of protein–protein interactions. Rv2372c and Rv2752c were cloned into the pHEX-derived and pGEX-derived vectors, respectively, as described in an earlier report [26]. A pair of recombinant vectors was co-transformed into competent *E. coli* BL21 (DE3). Protein co-expression and GST-affinity co-purification were performed according to our previously published procedures [26] with modifications. Fractions were pooled and stored at −80°C for further assays. The above co-purified sample was run on SDS-PAGE for Western blot analysis. The pro-

Nucleotide sequences for PCR primers

Gene	Sequences	Enzyme
Rv2752c	CCGCCCGAATTCGCGTGGATGTAGACCTTCCC	*Eco*RI
Rv2752c	CCGCCCTCTAGATCACACCTCGATGACCGTCG	*Xba*I
H397V	AACGTGATGCCGGTGGTCGGAACCTGGCGGAT	–
H397V	ATCCGCCAGGTTCCGACCACCGGCATCACGTT	–
D184A	ACGGCCGTCCCACCGCTCTACCGGGCATGTC	–
D184A	GACATGCCCGGTAGAGCGGTGGGACGGCCGT	–
NTD(1–269 a.a.)*	CCGCCCTCTAGATCAGTTGCGCACCATCGA	*Xba*I
CTD(270–558 a.a.)**	CCGCCCGAATTCGCATGCGCGTCGCAAGGC	*Eco*R
ΔNΔC(270–450 a.a.)*	CGCCGGCGATTCTAGAGTCGACGAACATCTTG	*Xba*I
Rv2373c	CAGGAATTCGTGGCACGCGATTAT	*Eco*RI
Rv2373c	CATTCTAGATTAGCGCCCGGTGAA	*Xba*I

Note: Forward and reverse primers are shown for each gene.
* and **, only forward or only reverse primer is shown for these genes, respectively.

tein bands were transferred to a nitrocellulose membrane. Anti-Rv2373c was used as primary antibody (1 : 1000) and IgG-HRP (goat anti-rabbit) (1 : 10,000) was used as secondary antibody. To quantify the protein, the signal was developed using 3,3′-diaminobenzidinde detection reagent, and it was photographed to serve as a record.

Starch/iodine coloration assay. Starch/iodine coloration assay of β-lactamase activity was carried out according to a previously published procedure [23]. The 0.6 ml reaction mixture contained 100 mM Tris-HCl (pH 7.5), 0.2 M NaCl, 100 μg/ml β-lactamase, 1 mg/ml penicillin, 1% (w/v) starch, I_2/KI, and 10 mM $ZnSO_4$. A solution containing 10,000 U of penicillin G per ml of phosphate buffer was freshly prepared and dispensed in 1.5-ml small tubes. A time-course assay was performed, and the reaction mixture was rotated for up to 50 min at room temperature. Rapid decolorization occurred if the penicillin was hydrolyzed by β-lactamase, indicating a positive β-lactamase activity.

Determination of β-lactamase activity by spectrophotometric assay. Spectrophotometric assay for β-lactamase was carried out using a modification of previously published procedures [21]. The working solution of nitrocefin (500 μg/ml; Oxoid, USA) was diluted 16-fold in buffer (0.1 M sodium phosphate, 1 mM EDTA, pH 7.0). The reaction mixture contained 600 μl nitrocefin solution and the indicated concentrations of wild-type Rv2752c or mutant proteins. The changes in absorbance at 486 nm were recorded at 25°C each 30 sec for 10 min.

RNase activity assay. *Escherichia coli* BL21 (DE3) total RNA was extracted according to the procedure supplied with the commercial kit (BioTek Corporation, China) and was used as a substrate for the RNase activity assay of Rv2752c. Wild-type Rv2752c or its mutant proteins were co-incubated with RNA substrate in the reaction buffer (500 mM Tris-HCl (pH 7.5), 100 mM $MgCl_2$, 10 mM dithiothreitol, 1 M NaCl) at 37°C for 30 min. RNase A was used as a positive control. Samples were analyzed on a 0.7% agarose gel and then stained with ethidium bromide.

Homology structure modeling. The structure of Rv2752c was modeled computationally using the automated comparative protein modeling web server SWISS-MODEL [27]. The *B. subtilis* RNase J protein [19] was used as a template (PDB ID: 3bk1).

RESULTS

Rv2752c contains critical amino acid residues for β-lactamase activity. In a previous report, we characterized Rv2752 as a Zn^{2+}-dependent metallo-β-lactamase [23]. When comparing the sequence of Rv2752c with the RNase J gene from *B. subtilis* [21], several conserved residues, including D184 and H397, were found that could potentially be involved in Zn^{2+}-binding [28] (Fig. 1a). To test this possibility, we made site-directed mutants, Rv2752c-D184A and Rv2752c-H397V, and examined their β-lactamase activities. As shown in Fig. 1b, using a starch/iodine colorimetric assay, neither mutant protein showed significant activity as no obvious color change occurred (lanes *3* and *4*). In contrast, a substantial decolorization occurred within 45 min with the wild-type Rv2752c (lane *2*). No color change occurred in the negative control (starch/iodine + penicillin) (lane *1*). These results show that both D184 and H397 residues are essential for the β-lactamase activity of Rv2752c. Spectrophotometric analysis confirmed this, as shown in Fig. 1c, where the rate of penicillin degradation of the wild-type Rv2752c was 0.32 $mg \cdot liter^{-1} \cdot sec^{-1}$, but was only 0.12 and 0.07 $mg \cdot liter^{-1} \cdot sec^{-1}$ for Rv2752c-D184A and Rv2752c-H397V, respectively.

The C-terminus of Rv2752c is essential for its β-lactamase activity. Based on the assumed structure (Fig. 1a), Rv2752c contains C-terminal tail of about 100 a.a. length, in addition to the two major domains of metallo-β-lactamase and RMMBL. Several truncated mutants that included the C-terminal domain (Rv2752c-CTD), N-terminal domain (Rv2752c-NTD), or only an RMMBL core (Rv2752c-ΔCΔN) (Fig. 1a) were constructed to examine the contribution of different domains to the β-lactamase activity of Rv2752c. As shown in Fig. 1b, when either purified Rv2752c-CTD or Rv2752c-NTD was added to the reaction mixture, rapid decolorization was observed (within about 15 min), indicating that both mutant proteins had enhanced β-lactamase activities (lanes *5* and *6*). In contrast, Rv2752c-ΔCΔN mutant, which lacks more of the C-terminal tail than does Rv2752c-CTD, showed almost no β-lactamase activity (lane *7*). Therefore, the C-terminal tail appeared to be essential for Rv2752c metallo-β-lactamase. Spectrophotometric analysis, as shown in Fig. 1c, confirmed that Rv2752c-CTD had a 4-fold higher activity for penicillin degradation (1.20 $mg \cdot liter^{-1} \cdot sec^{-1}$) than the wild-type protein (0.32 $mg \cdot liter^{-1} \cdot sec^{-1}$) while Rv2752c-NTD had an 8-fold higher activity (2.40 $mg \cdot liter^{-1} \cdot sec^{-1}$). In contrast, Rv2752c-ΔCΔN again showed extremely low penicillin degradation activity (0.0058 $mg \cdot liter^{-1} \cdot sec^{-1}$).

Rv2752c possesses an RNase activity. The sequence of the Rv2752c β-lactamase is similar to that of the RNase J gene from *B. subtilis* [18, 19], suggesting an additional function of RNA hydrolysis. We have characterized the RNase activity of Rv2752c and the effects of several critical residues or domains on it. As shown in Fig. 2a (lanes *4-7*) and Fig. 2b, when 100 ng RNA was mixed with different amounts of Rv2752c, a clear RNA hydrolysis occurred in a stepwise manner with increasing concentrations of Rv2752c (0.4-1.6 μM). The overall activity was similar to the RNase A positive control (Fig. 2a, lanes *3* and *18*, and Fig. 2b). Rv2752c-CTD, which contained a RMMBL core and the additional C-terminal tail, was also capable of significant RNA hydrolysis (Fig. 2a, lanes

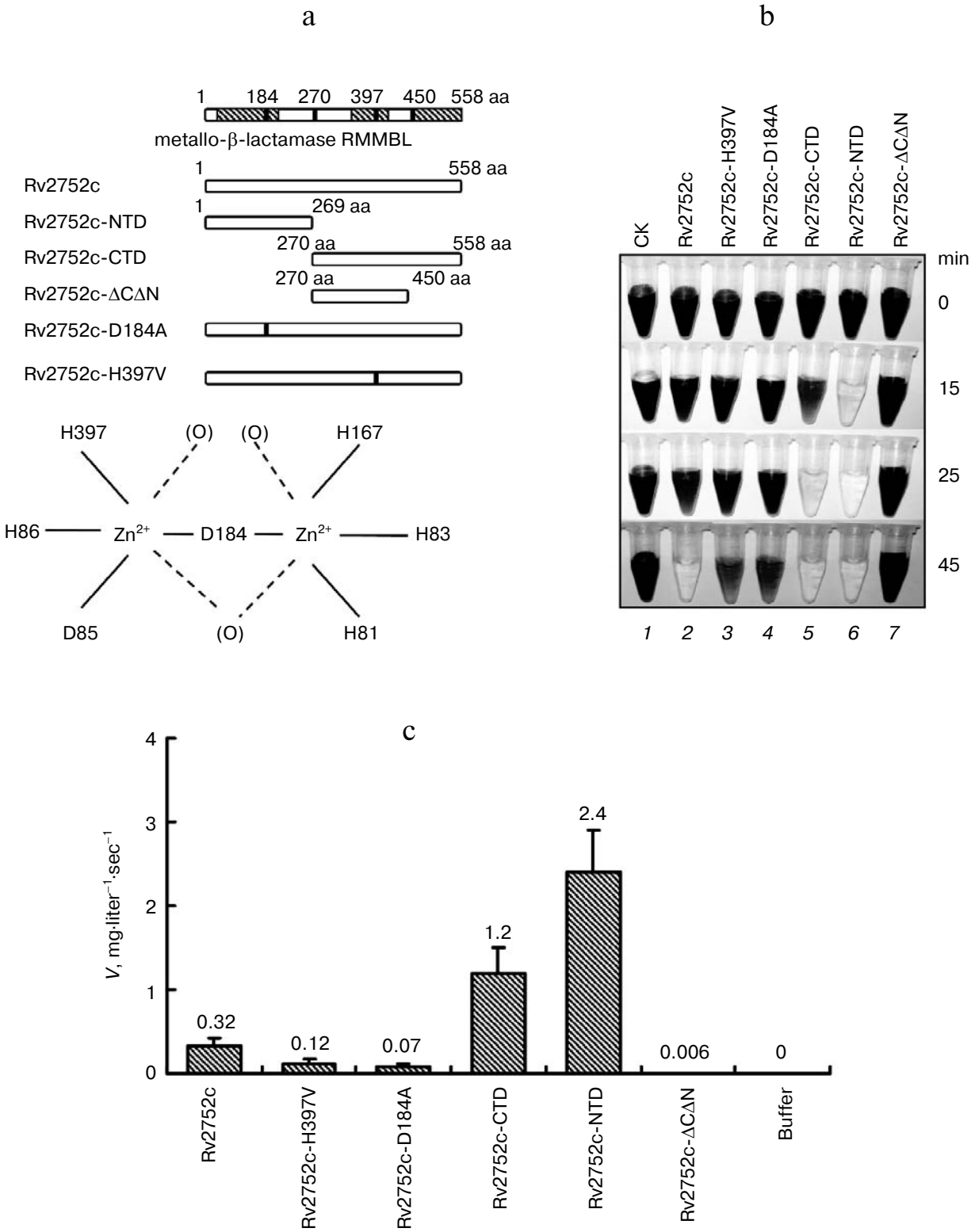


Fig. 1. β-Lactamase activity assays for Rv2752c and its mutants. a) Schematic representation of Rv2752c and several mutants. The names given to mutants are listed to the left of the panel. The amino acid regions that have been deleted are shown. b) Starch/iodine coloration assay for β-lactamase activity. Reaction times (min) are listed to the right of the panel. CK, starch + I_2/KI + penicillin. c) Spectrophotometric assay for β-lactamase activity.

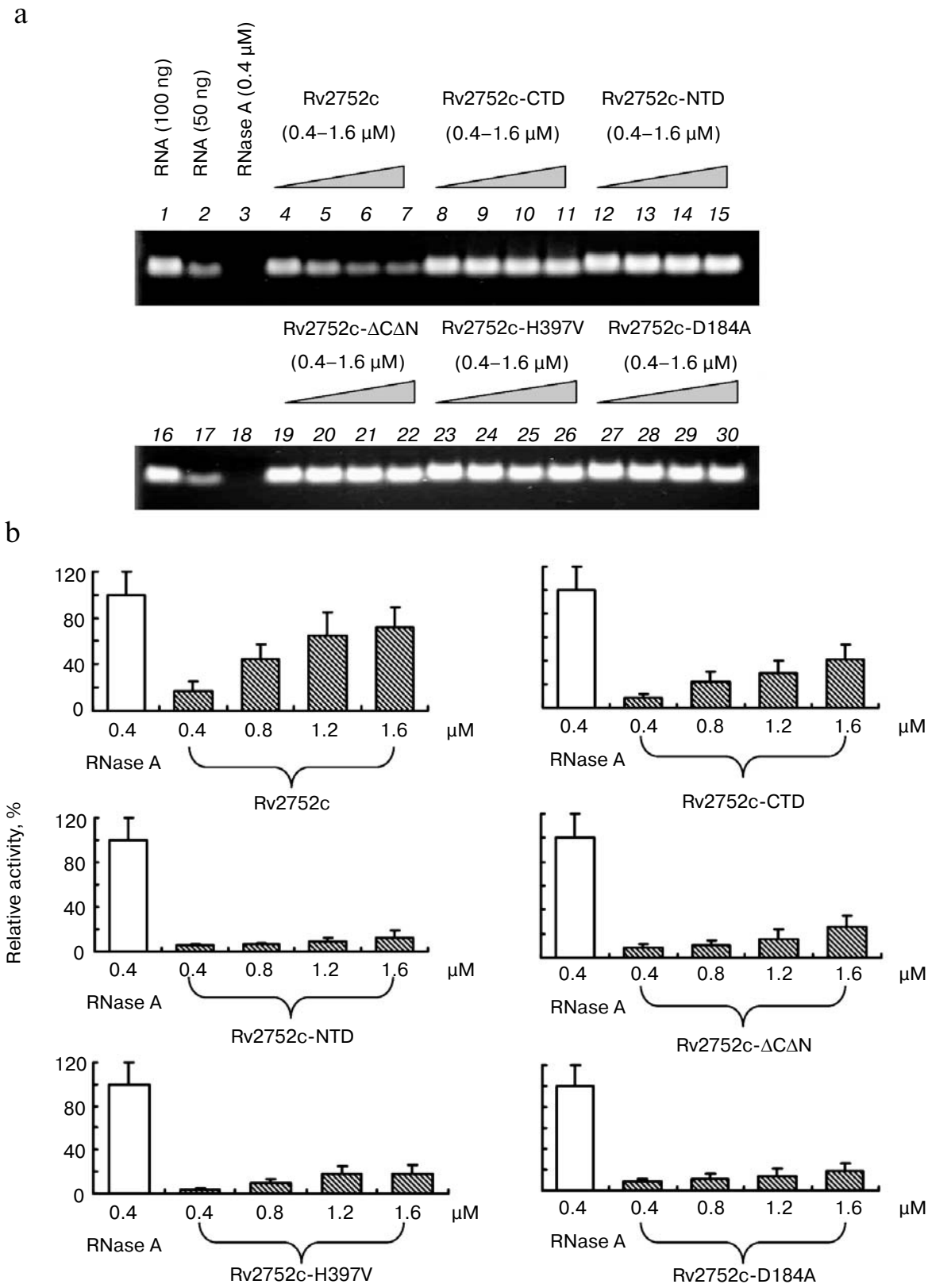


Fig. 2. RNase activity assays for Rv2752c and its mutants. The proteins and their concentration are indicated. RNase A was used a positive control. a) Agarose gel assays for the activities of Rv2752c and its mutants. b) A relatively quantitative result of the agarose gel assays.

8-11, and Fig. 2b). In contrast, only very weak RNA hydrolysis was observed for several other mutants, including Rv2752c-ΔCΔN, Rv2752c-NTD, Rv2752c-D184A, and Rv2752c-H397V (Fig. 2a, lanes *12-15* and *19-30*, and Fig. 2b).

Rv2752c clearly possesses RNase activity in addition to its β-lactamase activity. The major functional domain for RNA hydrolysis is situated in the C-terminal domain of the protein. Residues, D184 and H397, both needed for the β-lactamase, are also required for RNase activity.

Rv2373 interacts with Rv2752c and inhibits β-lactamase and RNase activities. An interaction between Rv2752c and a *M. tuberculosis* protein, Rv2373c, was evident in bacterial two-hybrid screening. As shown in Fig. 3a, the co-transformant strain with both genes grew well on the screening medium, but the negative control (CK^-) and all self-activation controls did not grow. The positive control (CK^+) also grew well on the medium. The interaction of Rv2752c with Rv2373c was confirmed with a further GST affinity co-purification/Western blotting assay. As shown in Fig. 3b, His-tagged Rv2373c protein could be readily co-purified with GST-tagged Rv2752c protein (Fig. 3b, lane *2*). In contrast, the His-tagged protein could not be obtained with GST alone (Fig. 3b, lane *3*).

These physical interactions suggested a functional link between these two proteins. The effects of Rv2373c on the activity of Rv2752c are shown in Fig. 3c. Rv2752c showed clear β-lactamase activity (lane *3*), whereas Rv2373c alone did not (lane *2*). However, the activity of Rv2752c was clearly inhibited when combined with 0.75 μM Rv2373c (lane *4*). When a higher concentration of Rv2373c (1.5 μM) was added, there was no β-lactamase activity detected until 50 min (lane *5*). Spectrophotometric analysis showed similar results, as very low activity for Rv2752c was observed when either 25 or 50 nM Rv2373c was added to the penicillin degradation reactions (Fig. 3d). Addition of Rv2373c also inhibited the RNase activity of Rv2752c. As shown in Fig. 3e, no RNA hydrolysis activity was detected for Rv2373c alone (lane *4*), but it was clearly present for Rv2752c (lane *5*) or RNase A (lane *3*). An increasing amount of Rv2373c protein (from 1 to 3 μM) was mixed with Rv2752c, a stepwise decrease in RNA hydrolysis was noted (lanes *6* and *7*).

DISCUSSION

Most of the mycobacteria appear to be naturally resistant to β-lactam antibiotics such as penicillin, presumably due to the presence of an active β-lactamase that can catalyze the hydrolysis of β-lactams [20, 29, 30]. However, little is known regarding which proteins can catalyze the hydrolysis of β-lactams and the related regulation mechanisms in the human pathogen *M. tuberculosis* H37Rv. In the present study, we identified Rv2752c to be a unique bifunctional protein with both β-lactamase and RNase activities. Two residues, D184 and H397, were essential for both activities. The C-terminal 100 a.a. fragment of Rv2752c also significantly contributed to both functions. A chaperone-like protein, Rv2373c, was also identified that physically interacts with Rv2752c and can functionally inhibit both activities. These findings offer important clues for understanding the *M. tuberculosis* β-lactamase activity and its regulation. In a previous study, Nampoothiri et al. [17] reported on several *M. tuberculosis* β-lactamases. Although we recently characterized Rv2752c as a Zn^{2+}-dependent metallo-β-lactamase [23], no significant sequence similarity was found between these two classes of β-lactamases based on a blast assay (Fig. 4a). Instead, Rv2752c was clearly similar to the *B. subtilis* RNase J, both of which contain a metallo-β-lactamase domain and a RMMBL domain, as well as several conserved motifs (Fig. 4b). In the present study, Rv2752c has been characterized as a bifunctional enzyme; it is both a β-lactamase and an RNase. Two residues, D184 and H397, presumably are involved in Zn^{2+}-binding. This is the first report that shows a β-lactamase to also possess RNase activity in the unique human pathogen *M. tuberculosis*. Our results suggest Zn^{2+}-binding ability is likely to be important for both functions.

Another critical finding from the current study is that the C-terminal 100 a.a. long tail of Rv2752c is essential for both activities. The 3D structure of Rv2752c has not yet been determined. However, a modeling structure of Rv2752c (Fig. S1; available on the site of the journal http://protein.bio.msu.ru/biokhimiya) can be obtained through an automated comparative protein modeling [23] using the *B. subtilis* RNase J protein as a template (PDB ID: 3bk1) (identities = 38%). Interestingly, Rv2752c had an additional loop structure within its C-terminal tail when compared to RNase J (Fig. S1b). Although the function of the C-terminal domain of the *B. subtilis* RNase J has not yet been characterized, our data suggest that the unique loop structure may contribute a regulatory role. Clearly, further detailed study is needed.

The mechanism underlying regulation of a dual function β-lactamase/RNase such as Rv2752c is not known. In the current study, we found clear evidence for a regulatory function of a chaperon-like protein, Rv2373c, which interacts with and inhibits both the β-lactamase and RNase activities of Rv2752c. A previous report identified two *M. tuberculosis* genes, Rv0352 and Rv2373c, that encode Hsp40 homologs [31]. Rv0352, designated as DnaJ1, strongly stimulated the transcription of hsp70/dnaK and hsp60/groE. In contrast, no effect on transcription was observed when Rv2373c, designated as DnaJ2, was overexpressed [28]. In the present study, we now show that Rv2373c can directly interact with target proteins and regulate their activities, including the dual function β-lactamase/RNase of Rv2752c of *M. tuberculosis*.

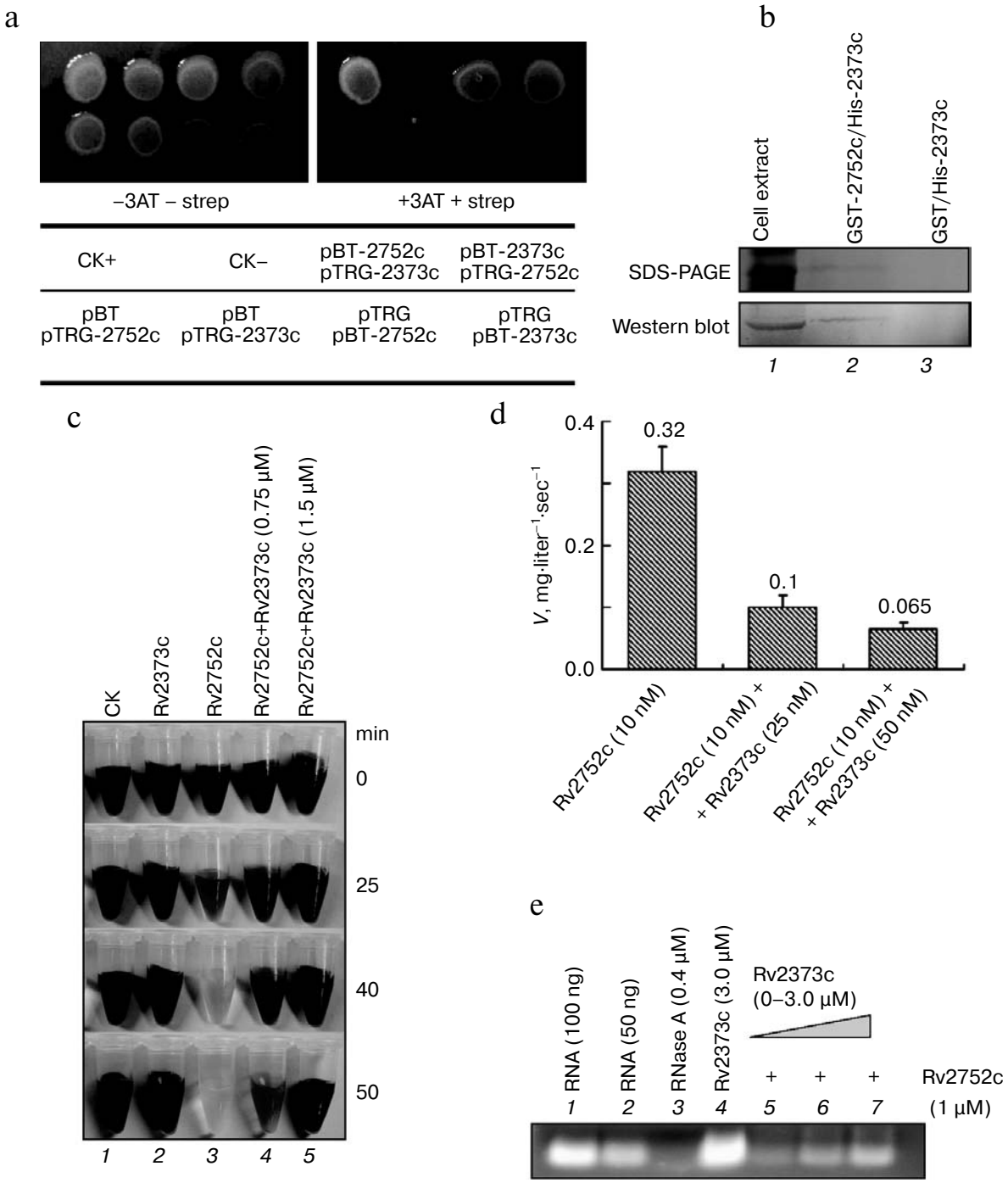
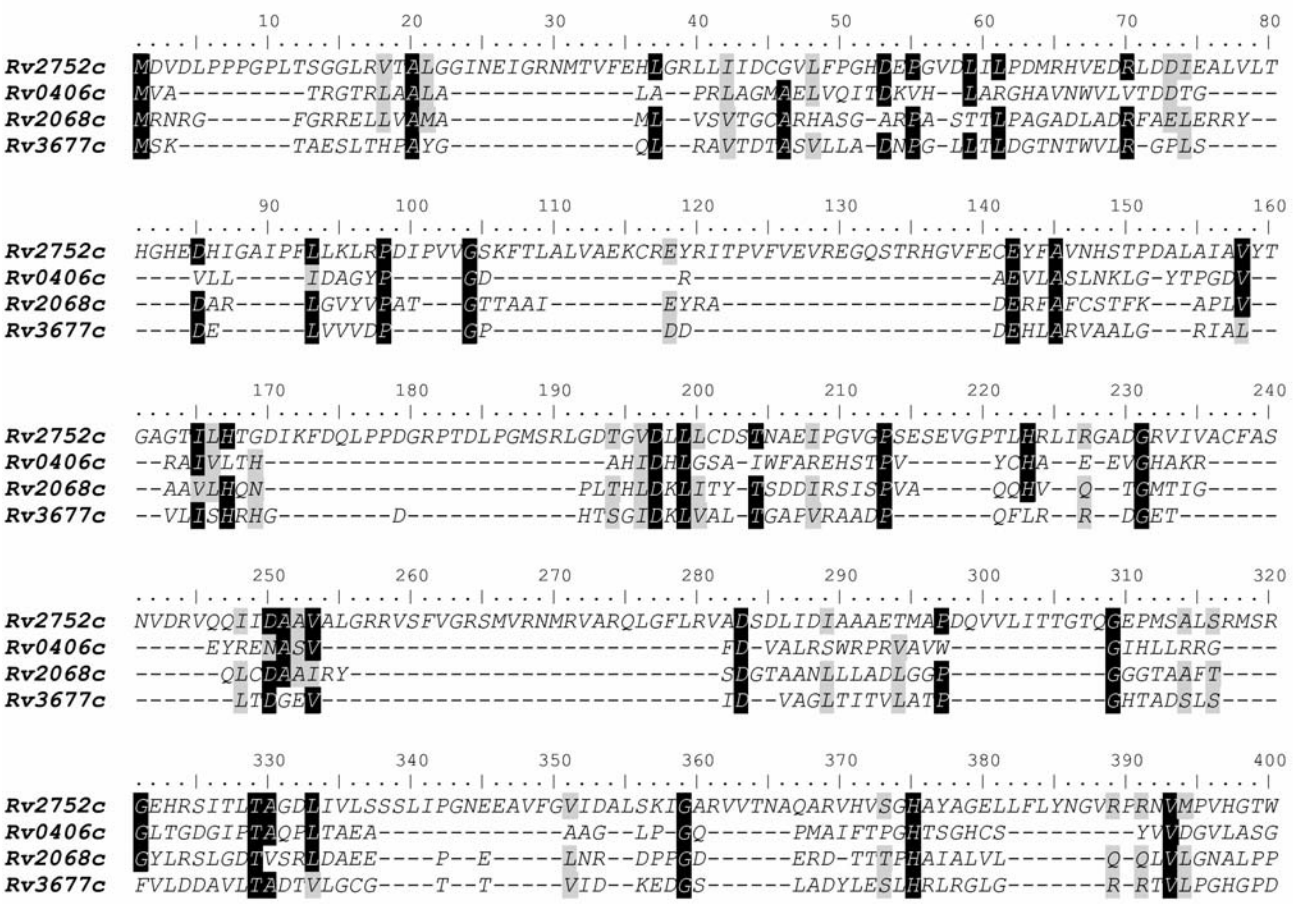


Fig. 3. Assay for the interaction between Rv2752c and Rv2373c. a) Bacterial two-hybrid assay. CK^+, co-transformant containing pBT-LGF2 and pTRG-Gal11P as a positive control; CK^-, co-transformant containing pBT and pTRG as a negative control. b) GST affinity co-purification/Western blotting assay. All samples were subjected to SDS-PAGE and the protein bands were transferred to a nitrocellulose membrane for Western blot analysis. c) Starch/iodine coloration assay for β-lactamase activity. Reaction times (min) are listed to the right of the panel. CK, starch + I_2/KI + penicillin. d) Spectrophotometric assay for β-lactamase activity. The activity is indicated on top of the columns. e) Assay for the effect of Rv2373c on the RNase activity of Rv2752c. The proteins and their concentration are indicated. RNase A was used a positive control.

a



b

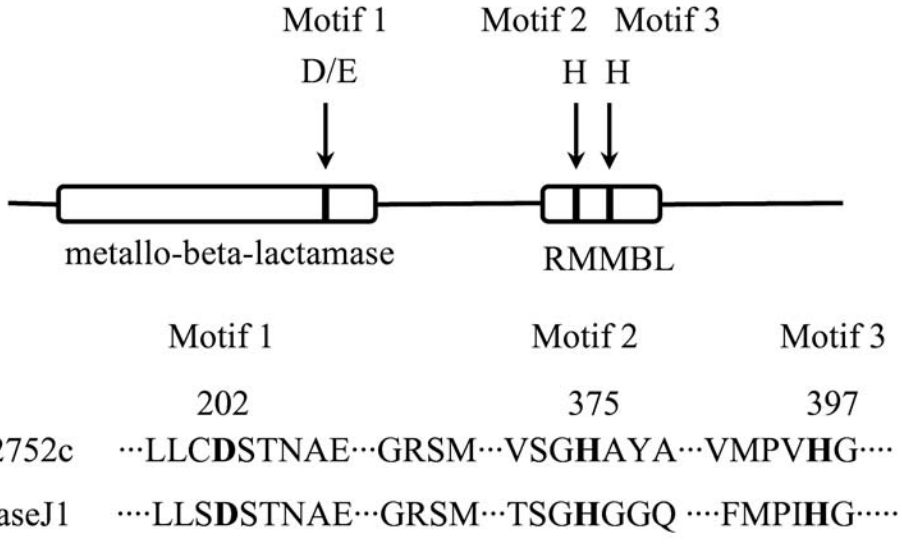


Fig. 4. Blast and domain assays for Rv2752c. a) Blast assay for several reported β-lactamases from *M. tuberculosis*. b) Rv2752c contains conserved sequence motifs of metallo-β-lactamase and RMMBL domains (RNase J of *B. subtilis*). The positions of the conserved sequence motifs are indicated with the residues.

In conclusion, *M. tuberculosis* Rv2752c encodes a bifunctional β-lactamase/ribonuclease, which physically interacts with, and is negatively regulated by Rv2373c, a chaperone-like protein. The C-terminal 100 a.a. long tail of Rv2752c is essential for both the β-lactamase and ribonuclease activities. This is the first report of the characterization of a bifunctional β-lactamase and its regulation in a microorganism. These data offer important clues for understanding the regulatory mechanisms underlying the dual functions of Rv2752c, but also for clarifying its interaction with protein Rv2373c. These findings, derived from *M. tuberculosis*, will also advance the understanding of the structure and function of microbial β-lactamases and the mechanism of microbial lactam drug resistance.

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