GROWTH AND TRANSLATION INHIBITION THROUGH SEQUENCE-SPECIFIC RNA BINDING BY A MYCOBACTERIUM TUBERCULOSIS VAPC TOXIN

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*Running head: VapC inhibits translation through RNA binding

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Background: Mycobacterium tuberculosis harbors a highly expanded number of toxin-antitoxin (TA) systems.

Result: The M. tuberculosis VapC-mt4 toxin blocks translation and arrests growth through RNA binding at a short recognition sequence.

Conclusion: M. tuberculosis VapC toxins have a function distinct from other characterized TA toxins.

Significance: TA systems may contribute to the slow growth and dormancy characteristic of M. tuberculosis during latent tuberculosis.

SUMMARY

The Mycobacterium tuberculosis genome harbors an unusually large number of toxin-antitoxin (TA) modules. Curiously, over half of these are VapBC (virulence associated protein) family members. Nonetheless, the cellular target, precise mode of action and physiological role of the VapC toxins in this important pathogen remain unclear. To better understand the function of this toxin family, we studied the features and biochemical properties of a prototype M. tuberculosis VapBC TA system, vapBC-mt4 (Rv0596c-Rv0595c). VapC-mt4 expression resulted in growth arrest—a hallmark of all TA toxins—in Escherichia coli, M. smegmatis and M. tuberculosis. Its expression led to translation inhibition accompanied by a gradual decrease in the steady-state levels of several mRNAs. VapC-mt4 exhibited sequence-specific endoribonuclease activity on mRNA templates at ACGC and AC(A/U)GC sequences. However, the cleavage activity of VapC-mt4 was comparatively weak relative to the TA toxin MazF-mt1 (Rv2801c). Unlike other TA toxins, translation inhibition and growth arrest preceded mRNA cleavage, suggesting that the RNA binding property of VapC-mt4, not RNA cleavage, initiates toxicity. In support of this hypothesis, expression of VapC-mt4 led to an increase in the recovery of total RNA with time, in contrast to TA toxins that inhibit translation via direct mRNA cleavage. Second, VapC-mt4 exhibited stable, sequence-specific RNA
binding in an electrophoretic mobility shift assay. Finally, VapC-mt4 inhibited protein synthesis in a cell free system without cleaving the corresponding mRNA. Therefore, the activity of VapC-mt4 is mechanistically distinct from other TA toxins since it appears to primarily inhibit translation through selective, stable binding to RNA.

*Mycobacterium tuberculosis* has the unique ability to persist for long periods of time in its host as a latent infection (1). This latent state, in which the bacteria are thought to be dormant with markedly altered physiology, is pivotal to the survival of the bacteria in the stressful environments it encounters. Importantly, *M. tuberculosis* cells in the latent state are generally refractory to antibiotics, most of which target processes occurring in actively replicating bacteria (2). Despite ongoing efforts to understand the latent state, the molecular switches that enable *M. tuberculosis* to slow or stop replication and enter a latent state remain unknown.

Toxin-Antitoxin (TA) systems (also referred to as addiction or suicide modules) have the potential to regulate *M. tuberculosis* replication and typically comprise an autoregulated operon encoding a labile antitoxin and a stable toxin. All characterized chromosomal TA toxins have been found to inhibit cell growth by targeted inhibition of an essential cellular process, such as protein synthesis or DNA replication. The activity of the toxin is regulated by its cognate antitoxin, which enables finely tuned control of TA module toxicity during relatively short periods of stress. However, TA toxin mediated growth arrest is only reversible up to a point of no return, after which prolonged activation of the toxin results in cell death (3-6).

Gerdes and coworkers initially reported that the genome of *M. tuberculosis* harbors a remarkably high number of TA systems (4) (more recently estimated at 88 putative TA systems (7)). They also uncovered a correlation between the number of chromosomal TA systems and growth rate, with TA loci being most abundant in organisms with slow growth rates (4). These observations suggest a possible role for TA systems in growth rate regulation. Despite these striking observations the physiological roles of TA systems are not well understood. In *E. coli*, activation of toxin-antitoxin systems is triggered by various stresses (8-11) and induces a state of cell growth arrest (5,6) with striking similarities to the slowly- or non-replicating state thought to occur in *M. tuberculosis* during the latent infection (12). As in *E. coli*, TA toxins in *M. tuberculosis* can be activated by selected stresses, including hypoxic conditions and macrophage infection (7). Overexpression of selected individual *M. tuberculosis* toxins in *E. coli*, *M. smegmatis* and *M. tuberculosis* also results in growth arrest (7,13,14). Consistent with their bacteriostatic effects, activation of *E. coli* TA toxins results in the generation of a persistent population of cells refractory to antibiotics (15-18). Likewise, overexpression or deletion, of individual *M. tuberculosis* RelE family toxins resulted in an increase (for overexpression) or decrease (for deletion) in persister recovery in antibiotic-treated *M. tuberculosis* cells. However, a *M. tuberculosis* RelE mutant did not exhibit a persistence defect in a mouse model (13), perhaps because only one of the three *M. tuberculosis* RelE genes was deleted. Because of the large number of TA modules in *M. tuberculosis*, the contribution of individual toxins might be marginal and measurable effects may require the concerted action of multiple toxins in vivo.

The chromosomal TA loci encoded in the genome of *M. tuberculosis* belong to five families, *mazEF, relBE, higBA, parDE* and *vapBC* (4,7). MazF toxins from many bacteria have been studied, including several from *M. tuberculosis* (19-21). In all cases, MazF acts as a sequence-specific endoribonuclease that cleaves mRNA independent of the ribosome thereby inhibiting translation. The *E. coli* RelE toxin also perturbs translation by interacting with the ribosome to enable RelE cleavage of mRNAs positioned in the ribosomal A site (22-25). The HigB toxin in the Rts1 plasmid from *Proteus vulgaris* functions in a manner analogous to RelE and inhibits protein synthesis by associating with the ribosome and cleaving mRNA at A-rich sequences (26,27). The ParE toxin in the RK2 plasmid, on the other hand, perturbs DNA replication by inhibiting the function of DNA gyrase (28). Therefore, although the mechanism of action is known for the other TA system families found in *M. tuberculosis*, the precise function of the VapC toxins remains unclear.

The *vapBC* (virulence associated protein) module is composed of the VapB antitoxin and the
VapC toxin. The VapC toxins present in *M. tuberculosis* are grouped together based on the presence of a PIN (PHIT N-terminal) domain containing a conserved quartet of acidic residues and a fifth invariant serine or threonine residue responsible for coordinating Mg\(^{2+}\) ion(s) in the catalytic center (29). The presence of a PIN domain suggests a putative role for the VapC toxins as Mg\(^{2+}\) dependent ribonucleases (29-31). The PIN domain containing protein, PAE2754 from *Pyrobaculum aerophilum* cleaves single-stranded DNA flap structures in a Mg\(^{2+}\) dependent fashion, while VapC-1 from nontypeable *Haemophilus influenzae* cleaves single-stranded RNA and the VapBC-mt5 toxin-antitoxin protein complex (Rv0626-Rv0627) from *M. tuberculosis* appeared to cleave dsRNA *in vitro* in the presence of Mg\(^{2+}\) (30,32,33). In contrast, no nuclease activity was detected for FitB (the PIN domain containing protein, PAE2754 from *Pyrobaculum aerophilum*) and another VapC. The VapBC-mt4 protein was shown to cleave fMet tRNA at a single site between the anticodon step and loop (36). These contradictory results suggest that not all VapCs function by cleaving RNA and that the specific mechanism of action may vary among different members of this family of PIN-domain proteins.

To better understand the function of the VapC toxin family, we performed detailed biochemical characterization of the VapC toxin from the *vapBC-mt4* (Rv0596c-Rv0595c) TA system. Our results demonstrate that VapC-mt4 targets RNA in a manner distinct from other TA toxins. Whereas other TA toxins, such as MazF, YafQ, HigB and ChpBK inhibit translation by cleaving mRNA, cleavage of mRNA is not the primary activity responsible for VapC-mt4 mediated translation inhibition. Instead, VapC-mt4 toxicity appears to result from the stable, selective binding of RNA containing an ACGC or AC(A/U)GC consensus sequence.

**EXPERIMENTAL PROCEDURES**

*Strains, Plasmids and Reagents-* The *E. coli* strains BL21(DE3)pLysE (F *ompT* hsdS\(^{-}\) [r\(_{6}^{+}\), m\(_{6}\)] dcm gal (DE3) pLysE (Cam\(^{8}\)) (Novagen) and BW25113Δ6 (F lac\(^{i}\) rrcB\(_{14}\) ΔlacZ\(_{M15}\) hsdR514 ΔaraBAD\(_{AH3}\) ΔrhaBAD\(_{LD78}\) ΔmazEF ΔchpBIK ΔrelBE ΔyefM-yoeB ΔdinJ-yafQ) (37) were used for all protein expression and toxicity studies. *E. coli* K-12 Mach1-T1 cells (F *ΔlacA1398 endA1 lacI* \(_{2}\) F\(_{\alpha}\) Pho80(lacZ)ΔM15 Δ lacY74 hsdR\(_{Km}\) m\(_{Km}\) ) (Invitrogen) were used for all cloning experiments. Plasmids used in this study include pINIII-A3 (38-40), pBAD18, pBAD33 (41), pMC1s (42), pET21c and pET28a (Novagen). The 23 VapBC and VapC ORFs were PCR amplified from *M. tuberculosis* H37Rv genomic DNA using primers with 5′Ndel-BamHI/EcoRI\(^{\prime}\) ends and cloned into the corresponding sites of pET21c to create pET21c-VapBC and pET21c-VapC. The pET21c-VapC plasmids were digested with 5′XbaI-EcoRI and cloned into the corresponding sites of pBAD33 to create pBAD33-VapC. Plasmid pET21c-VapC-mt4 was digested with 5′XbaI-EcoRI\(^{\prime}\) and cloned into pBAD18 to create pBAD18-VapC-mt4. Plasmid pET21c-VapC-mt4 was digested with 5′Ndel-EcoRI\(^{\prime}\) and cloned into the corresponding sites of pBAD33 to create pBAD33-VapC. Plasmid pET21c-VapB-mt4 was digested with 5′XbaI-HindIII\(^{\prime}\) and cloned into the corresponding sites of pBAD33 to create pBAD33-VapB-mt4. Plasmid pET21c-VapC-mt4 was digested with 5′XbaI-HindIII\(^{\prime}\) and cloned into the corresponding sites of pBAD33 to create pBAD33-VapB-mt4.

*Strains, Plasmids and Reagents-* The *E. coli* strains BL21(DE3)pLysE (F *ompT* hsdS\(^{-}\) [r\(_{6}^{+}\), m\(_{6}\)] dcm gal (DE3) pLysE (Cam\(^{8}\)) (Novagen) and BW25113Δ6 (F lac\(^{i}\) rrcB\(_{14}\) ΔlacZ\(_{M15}\) hsdR514 ΔaraBAD\(_{AH3}\) ΔrhaBAD\(_{LD78}\) ΔmazEF ΔchpBIK ΔrelBE ΔyefM-yoeB ΔdinJ-yafQ) (37) were used for all protein expression and toxicity studies. *E. coli* K-12 Mach1-T1 cells (F *ΔlacA1398 endA1 lacI* \(_{2}\) F\(_{\alpha}\) Pho80(lacZ)ΔM15 Δ lacY74 hsdR\(_{Km}\) m\(_{Km}\) ) (Invitro
M. tuberculosis liquid cultures were grown in 7H9 Middlebrook media supplemented with 0.05% Tween 80, 0.5% bovine albumin, 0.2% glucose and 0.85% NaCl (7H9-TW80-ADN). Spent culture supernatant was prepared from an early stationary phase M. tuberculosis H37Rv culture (OD600 between 1.1-1.2) by centrifugation (2000 x g, 10 min, 4°C) followed by filtration through a 0.22 µm filter (Millipore). 1 mM IPTG, 0.2% arabinose, 100 ng/ml anhydrotetacycline (M. smegmatis), 200 ng/ml anhydrotetracycline (M. tuberculosis), 50 µg/ml ampicillin, 20 µg/ml kanamycin (E. coli) and 25 µg/ml chloramphenicol were added to culture medium as required. The accuracy of DNA sequences in PCR products used for cloning was confirmed by DNA sequence analysis. Polyclonal antibodies used for Western analysis were produced in rabbits using purified His6-VapB-mt4 (Rv0596c) or His6-VapC-mt5 (Rv0627) as antigens (Pocono Rabbit Farm and Laboratory, Canadensis, PA).

Purification of recombinant protein - E. coli BL21(DE3)pLysE was transformed with pET28a-His6-VapB-mt4, pET28a-His6-VapC-mt4, or pET28a-His6-MazF-mt1 plasmids and grown in 1 L of M9 minimal media containing 0.2% glucose at 37°C until an OD600 between 0.6-0.8 was reached. IPTG was then added to a final concentration of 1 mM and the cultures were incubated for an additional 4 hours. The cultures were harvested by centrifugation (6,000 rpm, 10 min, 4°C) in a Sorvall SA-600 rotor and the cell pellet was resuspended in 25 ml of lysis buffer (50 mM NaH2PO4 (pH 8.0), 500 mM NaCl, 20 mM imidazole, 10 mM β-mercaptoethanol, 1 mM PMSF, 1 mg/ml lysozyme) and incubated for 30 min on ice. Triton-X100 was then added to a final concentration of 2%. Cells were lysed by sonication (Branson Digital Sonifier 250, 30% amplitude, 30 cycles, 10 sec ON, 30 sec OFF, 4°C) and the lysate was cleared by centrifugation (15,000 rpm, 10 min, 4°C). The supernatant was adsorbed on to 1 ml of pre-equilibrated Ni-NTA agarose resin (Qiagen) in 50 ml disposable centrifuge tube with mild agitation (1 hour, 4°C) and the resin was collected by centrifugation (1,000 rpm, 5 min, 4°C). The adsorbed resin was washed twice with 50 ml of Wash Buffer I (50 mM NaH2PO4 (pH 8.0), 500 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, 0.5% Triton X-100) with mild agitation (30 min, 4°C) and collected by centrifugation (1,000 rpm, 5 min, 4°C). The washed resin was resuspended in 50 ml of Wash Buffer I, loaded into a column, washed extensively (i.e. three times with 20 ml of Wash Buffer I, three times with 20 ml of Wash Buffer II (50 mM NaH2PO4 (pH 8.0), 500 mM NaCl, 43 mM imidazole, 5 mM β-mercaptoethanol, 0.45% Triton X-100), once with 10 ml of Wash Buffer III (50 mM NaH2PO4 (pH 8.0), 500 mM NaCl, 66 mM imidazole, 5 mM β-mercaptoethanol, 0.4% Triton X-100)) and eluted in 5 ml of elution buffer (50 mM NaH2PO4 (pH 8.0), 500 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol). The purified protein was dialyzed against 1 L of dialysis buffer (10 mM Tris (pH 8.0), 100 mM NaH2PO4, 10 mM β-mercaptoethanol) (>2 hours, 3x) and concentrated using a 3,000 NMWL Amicon Ultra-4 Centrifugal Filter (Millipore) as required. Glycerol was added to a final concentration of 5% and the purified protein was aliquoted and stored at -80°C.

Protein interaction - E. coli BL21(DE3)pLysE was transformed with pET28a or pET28a-His6-VapB/C-mt4 and pET21c-T7-VapB/C-mt4 and grown in 500 ml of M9 minimal media containing 0.2% glucose at 37°C until an OD600 between 0.6-0.8 was reached. IPTG was then added to a final concentration of 1 mM and the cultures were incubated for an additional 4 hours. The cultures were harvested by centrifugation (6,000 rpm, 10 min, 4°C) in a Sorvall SA-600 rotor and the cell pellet was resuspended in 25 ml of lysis buffer (50 mM NaH2PO4 (pH 8.0), 500 mM NaCl, 10 mM imidazole, 20 mM β-mercaptoethanol, 1 mM PMSF, 1 mg/ml lysozyme) and incubated for 30 min on ice. Cells were lysed by sonication (Branson Digital Sonifier 250, 30% amplitude, 30 cycles, 10 sec ON, 30 sec OFF, 4°C) and the lysate was cleared by centrifugation (15,000 rpm, 10 min, 4°C). The supernatant was adsorbed on to 500 µl of pre-equilibrated Ni-NTA agarose resin (Qiagen) and purified as recommended by Qiagen. The samples were separated on a 17.5% SDS-PAGE and visualized by Coomassie staining or Western analysis using a 1:300,000 dilution of the VapB-mt4 polyclonal antibody or 1:50,000 VapB-mt4 polyclonal antibody or 1:50,000 dilution of the VapC-mt5 polyclonal antibody.

Assess protein synthesis in vivo - E. coli BW25113Δ6 cells containing pBAD33-VapC-mt4 were grown in M9 minimal media containing 0.1% glycerol and 1 mM of all amino acids except cysteine and methionine at 37°C until an OD600
between 0.2-0.3 was reached. The culture was then split into equal portions and arabinose was added to one at a final concentration of 0.2%, while an equal volume of water was added to the other as a control. 500 µl aliquots were removed at 0, 10, 20, 30, 60, 120 and 180 min post induction and incubated with 30 µCi of [35S]methionine at 37°C. After 1 min of incorporation, the samples were chased with 0.3 mg of cold methionine at 37°C for 5 min. 50 µl of the culture was applied to a cellulose filter disc (Whatman). The filters were boiled for 30 min in 10% trichloracetic acid (TCA), washed three times with 10% TCA and once with acetone. The amount of radioactivity incorporated was determined using a liquid scintillation counter. Cell pellets were collected by centrifugation (3,000 rpm, 1 min, 4°C) and resuspended in equivalent volumes of TE buffer (pH 8.0) and 2x Laemml buffer (125 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue). Incorporation was visualized by autoradiography following electrophoresis on a 17.5% SDS-PAGE.

Assess DNA and RNA Synthesis in vivo- E. coli BW25113∆ cells containing pBAD18-VapC-mt4 were grown in M9 minimal media containing 0.1% glycerol at 37°C until an OD600 between 0.2-0.3 was reached. Then [methyl-3H]thymidine or [methyl-3H]uridine were added to a final concentration of 1 µCi/ml. The culture was then split into three equal portions and arabinose was added to one at a final concentration of 0.2%, chloramphenicol was added to another at a final concentration of 25 µg/ml and an equivalent volume of water was added to the other as a control. At 0, 10, 20, 30, 60, 120 and 180 min post induction 50 µl of culture was applied to a cellulose filter disc (Whatman). RNA and DNA were precipitated with 10% TCA. The filters were transferred to a fresh tube and re-extracted with an equal volume of chloroform. The organic and aqueous phases were separated by centrifugation (13,200 rpm, 15 min, 4°C). The aqueous phase was transferred to a fresh tube containing 1 ml of 100% ethanol and incubated at -70°C overnight. The RNA was pelleted by centrifugation (13,200 rpm, 30 min, 4°C), washed with 1 ml of 70% ethanol and incubated at -70°C for 30 min. The RNA was pelleted by centrifugation (13,200 rpm, 30 min, 4°C), dried at room temperature for 5 min and resuspended in 50 µl of Diethylpyrocarbonate (DEPC) treated water. The radiolabeled DNA fragments used for hybridization to the Northern blots were derived from PCR products for the open reading frames of the E. coli genes ompA (outer membrane porin protein A) and lpp (major outer membrane lipoprotein) and a 600 bp region immediately upstream of tufA (EF-Tu) using the Random Primed DNA Labeling Kit (Roche).

MS2 RNA cleavage assays- MS2 RNA cleavage assays were performed with 0.7 pmol of MS2 RNA (Roche), 0.5 µl of RNase Inhibitor (Roche) and 61.8 pmol of His6-VapC-mt4 or His6-MazF-mt1 in a 10 µl reaction containing 10 mM Tris (pH 7.8), 150 mM NaCl, 10 mM MgCl2, 61.8 pmol of His6-VapC-mt4 was preincubated with 123.6 or 247.2 pmol of His6-VapB-mt4 for 15 min at 37°C prior to starting the reaction. The reactions were performed at 37°C and time points were taken at 0, 1, 3, 6, 12 and 18 hours. The reactions were stopped by the addition of 10 µl of sequence loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). The samples were incubated at 95°C for 5 min prior to electrophoresis on an 8% polyacrylamide, 7M urea gel and stained with etidium bromide.
Synthetic RNA cleavage assays- The synthetic RNAs used were NWO1355 (+ACGC), 5'-UAAGAAGAAGACGCAAUUGUAUAAA-3'; NWO1430, 5'-AGGAAGAAGACGCAAUUGAA-3'; NWO1434, 5'-AGGAAGAAGACGAAUUGAA-3; and NWO1435, 5'-AGGAAGAAGACGUAAUUGAA-3. The RNAs were 5' end labeled with \([\gamma-^{32}P]\)ATP using T4 polynucleotide kinase (NEB). The cleavage reactions were performed with 0.2 pmol of radioactively labeled RNA, 61.8 pmol of His 6-VapC-mt4 and/or 247.2 pmol of His6-VapB-mt4 as described previously. RNA ladders were prepared by digesting 0.4 pmol of 5' end labeled RNA with 0.1 units of RNaseT1 (Sigma) in 20 mM Sodium Citrate (pH 5), 7M Urea, 1 mM EDTA for 15 min at 25 °C and by partial alkaline hydrolysis in 100 mM NaOH for 2 min at 75°C. The reactions were stopped by the addition of 10 µl of sequence loading buffer. The samples were incubated at 95°C for 5 min prior to electrophoresis on a 15% polyacrylamide, 7 M urea gel followed by autoradiography.

RNA binding assays- Thirty nucleotide synthetic RNAs were 5' end labeled with \([\gamma-^{32}P]\)ATP using T4 polynucleotide kinase (NEB). The RNAs used were NWO1353 (-ACGC), 5'-CGCAUGGCGUUCGUACUUAAAUAUGGAA-3' and NWO1355 (+ACGC), 5'- UAAGAAGAAGACGAAUUGAAUCAAA-3'. The 15 µl reactions contained 0.2 pmol of radioactively labeled RNA and 15.45, 30.9, 46.35 or 61.8 pmol of His6-VapC-mt4 in 10 mM Tris (pH 8.0), 10 mM KCl, 1 mM EDTA and 7.4% glycerol. Specific and nonspecific binding was shown using a 50 fold concentration (10 pmol) of unlabeled synthetic RNA. The binding reactions were incubated on ice for 20 min. For electrophoretic mobility shift assays (EMSAs) the samples were separated on a 15% native acrylamide (19:1) gel in 1x TBE (Tris-Borate-EDTA) buffer and run at 110 V at 4°C until the dye front was ¾ down the gel. The RNA was transferred to nitrocellulose using a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad) at 0.2 Amps for 30 min in 0.5x TBE and visualized by autoradiography.

In vivo primer extension analysis- Total RNA was extracted as described previously. Primer extension reactions were carried out at 47°C for 1 hour in a 20 µl reaction containing 0.7 pmol of 5’ end-labeled primer, 1 mM dNTPs, 0.5 µl RNase Inhibitor (Roche) and 0.5 µl AMV Reverse Transcriptase (NEB) in 50 mM Tris (pH 8.3), 75 mM Potassium Acetate, 8 mM Magnesium Acetate, 10 mM DTT. The primers were 5’ end labeled with [\(\gamma-^{32}P\)]ATP using T4 polynucleotide kinase (NEB). Cleavage products in tufA, ompA and lpp were detected using primers NWO1169, 5'-GGCGTTCCGTACATCATCCTGTCG-3' (Figure 5A and B); NWO938, 5'-GGTATCAAAGACGTTGTAACTCAGC-3' (Figure 5C); and NWO1121, 5'-GTGAGTGAAATATGGCGCACATTGTGCTG-3' (Figure 5D).

In vitro primer extension analysis- The MS2 RNA was incubated as described above with 61.8 pmol of His6-VapC-mt4, 247.2 pmol of His6-VapB-mt4, or 61.8 pmol of His6-VapC-mt4 plus 247.2 pmol of His6-VapB-mt4. Primer extensions were carried out using radioactively labeled primers as described previously. The primers used were NWO1459, 5'-CTCACAAGCTCGCAGCGAAC-3' (Figure 7A); J1, 5'-CATCAAGTGTAGTGCGGTCTG-3' (Figure 7B); NWO1461, 5'-GACTGGCTCCTACCTGTAGGTAAC-3' (Figure 7C); G, 5'-CATTAATCGACCAAGCGCCTT-3' (Figure 7D); NWO1462, 5'-CAAGCAACTCGCAGCGCAC-3' (Figure 7E); E, 5'-CTCTTTATGTATTGATCTTC-3' (Figure 7F); NWO1463, 5'-CTTAAGGGACGAATTGCTCACAA-3' (Figure 7G); D, 5'-CCCTCCCGCTCTGAGAGCGG-3' (Figures 7H-I); B4, 5'-GTTCCTCGGTGGAGCGGACAGCCTCTC-3' (Figure 7J); and B3, 5'-GTAACGGGGTGGGTGTGCT-3' (Figure 7K).

The DNA sequencing ladders for the in vivo primer extension reactions were prepared with a Sequenase Version 2.0 DNA Sequencing Kit (USB) as recommended by USB. The DNA sequencing ladders for the in vitro primer extension reactions were also prepared using the Sequenase Version 2.0 DNA Sequencing Kit (USB) with minor modifications. The primer was annealed to the MS2 RNA in a 10 µl reaction containing 1 pmol primer, 1.4 pmol of MS2 RNA (Roche) and 1.4 µl of RNase Inhibitor (Roche) in 50 mM Tris (pH 8.3), 75 mM Potassium Acetate, 8 mM Magnesium Acetate, 10 mM DTT. The DNA sequencing reaction was performed as recommended by manufacturer using 1 U of AMV Reverse Transcriptase (NEB) in place of Sequenase DNA polymerase. The primer extension and sequencing reactions were stopped by the addition of 12 µl or 6 µl of sequence loading buffer, respectively. The samples were...
incubated at 95°C for 5 min prior to electrophoresis on a 6% polyacrylamide, 7 M urea gel followed by autoradiography.

In Vitro Protein Synthesis- Prokaryotic cell-free protein synthesis was carried out using the E. coli T7 S30 Extract System for Circular DNA (Promega). The PinPoint Xa Control vector supplied with the kit was used as template. The 25 µl reaction mixture contained 154 pmol of His6-VapC-mt4, 616 pmol of His6-VapB-mt4 or 154 pmol of His6-VapC-mt4 plus 616 pmol of His6-VapB-mt4, respectively. The samples were preincubated for 15 min at 37°C prior to the addition of 0.24 µg of the PinPoint Xa Control vector after which the samples were incubated for an additional 1 hour at 37°C. Samples were separated on a 17.5% SDS-PAGE and visualized by autoradiography.

Reverse Transcriptase PCR- Total RNA was extracted from the E. coli T7 S30 Extract System with saturated phenol (pH 4.3). The samples were treated with 1U of RQ1 RNase-Free DNase (Promega) in 40 mM Tris (pH 8.0), 10 mM MgSO4, 1 mM CaCl2 for 30 min at 37°C. The RQ1 RNase-Free DNase was removed by extraction with saturated phenol (pH 4.3) followed by ethanol precipitation. Reverse transcription was carried out on equal volumes of RNA from each sample using primer NWO1443, 5’- TTACGCCCGCCCTGCCAC-3’. The reverse transcriptase was then heat inactivated for 5 min at 95°C and all samples were treated with DNase-free RNase for 10 min at 37°C. The samples were then used as templates for PCR using primers NWO1442, 5’- ATGAAACTGAAGGTAACAGTCAACGGC-3’ and NWO1443, 5’- TTACGCCCGCCCTGCCAC C-3’ to amplify the CAT fusion transcript. PCR reactions were then run on 1% agarose gels and stained with EtBr.

Ribosome Profile Analysis- Polysomes were isolated as described previously (43-45). Briefly, E. coli BW25113Δ6 cells containing pBAD18-VapC-mt4 were grown in M9 minimal media supplemented with 0.1% glycerol at 37°C until an OD600 between 0.3-0.4 was reached. The culture was then split into three equal portions and arabinose was added to one at a final concentration of 0.2%, while an equal volume of water was added to the others as a control. After 60 min chloramphenicol was added to a different culture at a final concentration of 0.1 mg/ml, while an equal volume of water was added to the others as a control. After 3 min, the cells were poured over an equal volume of ice, harvested by centrifugation (14,500 rpm, 5 min, 4°C) in a Sorvall SA-600 rotor and the cell pellet was resuspended in polysome profile buffer (20 mM Tris (pH 7.5), 100 mM NH4Cl, 10 mM MgCl2, 5 mM DTT). Lysozyme was added to a final concentration of 1 mg/ml and the cells were frozen in a dry ice/ethanol bath and thawed in a 5°C water bath three times. 600 µg of total RNA was layered onto a 5-40% (wt/vol) continuous sucrose gradient in polysome profile buffer and centrifuged (35,000 rpm, 3.5 hours, 4°C) in a Beckman SW41 rotor. Gradients were fractionated and analyzed with continuous monitoring at 254 nm. Fractions corresponding to the polysomes, 70S, 50S, 30S and tRNAs/RNAs not associated with ribosomes or ribosomal subunits were concentrated by centrifugation (45,000 rpm, 20 hours, 4°C) in a Thermo Fisher Scientific T647.5 rotor and subjected to Western analysis using a 1:50,000 dilution of the VapC-mt5 (Rv0627) polyclonal antibody.

tRNAfMet cleavage activity assay- Purified tRNAfMet from E. coli was purchased from Chemical Block (Moscow, Russia). M. tuberculosis tRNAfMet was synthesized in vivo following the method described by Sisido et al. with minor modifications. Briefly, a synthetic DNA oligonucleotide containing the T7 RNA polymerase promoter and the 5′ end of the M. tuberculosis tRNAfMet gene was annealed to a second oligonucleotide corresponding to the 3′ end of the M. tuberculosis tRNAfMet gene. The annealed oligos were then extended using Taq DNA polymerase to create dsDNA containing the entire tRNAfMet gene preceded by the T7 promoter. The product was then run on a 2% agarose gel to confirm its size and purity using a QIAquick gel extraction kit (Qiagen). The sequence of the product was confirmed by automated DNA sequence analysis. 200 ng of M. tuberculosis tRNAfMet dsDNA was then transcribed in vitro using the RiboMAX Large Scale RNA Production System (Promega) as recommended by the manufacturer. The transcription reaction was run on a 9% polyacrylamide, 7 M urea gel followed by staining with EtBr to confirm the size and purity of the tRNAfMet transcript.

The tRNAfMet transcript was purified from the gel as follows. The RNA band was excised from the gel and incubated for 18 hours at 37°C in
elution buffer (1 mM EDTA, 0.5 M Ammonium Acetate, 10 mM Magnesium Acetate, and 0.1% SDS). The eluate was then collected and the gel pieces were washed in fresh elution buffer. The elution and wash was combined and the RNA was collected by ethanol precipitation.

Cleavage of \textit{E. coli} and \textit{M. tuberculosis} tRNA\textsuperscript{fMet} by VapC-mt4 was performed \textit{in vitro} following the assay described by Winther and Gerdes (47). 20 pmol es of \textit{E. coli} tRNA\textsuperscript{fMet} was incubated with 2, 25 or 50 pmoles of VapC-mt4 or 2 pmol of \textit{M. tuberculosis} tRNA\textsuperscript{fMet} was incubated with 0.2, 2.5, or 5 pmoles of VapC-mt4 at 37\(^\circ\)C for 15 min in 10 mM HEPES (pH 7.5), 15 mM Potassium Chloride, 3 mM Magnesium Chloride and 10% glycerol. The products of these reactions were run on a 9% polyacrylamide, 7M urea gel and visualized by staining the \textit{E. coli} tRNA\textsuperscript{fMet} with EtBr and the \textit{M. tuberculosis} tRNA\textsuperscript{fMet} with SYBR Gold (Invitrogen).

\section*{RESULTS}

First, we determined if expression of any of the 23 VapC toxins originally annotated in the genome of \textit{M. tuberculosis} (4) caused growth arrest in \textit{E. coli}. This was accomplished by individually expressing each of the 23 VapC toxins using the arabinose-inducible pBAD33 plasmid in an \textit{E. coli} BW25113\(\Delta\)6 strain that lacks the loci for the six well-characterized \textit{E. coli} chromosomal TA modules (\textit{mazEF, chpBIK, relBE, hipBA, yefM-yoeB} and \textit{dinJ-yafQ}) (Figure S1). Use of the BW25113\(\Delta\)6 strain in all experiments precluded the activation of these TA toxins as a result of the stress imparted by VapC expression. Expression of four VapC toxins, VapC-mt4 (Rv0595c), VapC-mt11 (Rv1561), VapC-mt19 (Rv2548) and VapC-mt20 (Rv2549c), inhibited cell growth in \textit{E. coli}. From this group we elected to focus on the \textit{vapBC-mt4} operon as a representative of this class of TA systems.

The \textit{vapBC-mt4} module exhibits the features of a typical TA system: the open reading frames overlap or have minimal distance between them (\textit{vapB-mt4} and \textit{vapC-mt4} overlap by four base pairs) (Figure S2), both genes encode relatively small proteins (VapB-mt4 is 85 amino acids and 9.7 kDa; VapC-mt4 is 130 amino acids and 14.1 kDa), the proteins have opposing isoelectric points (VapB-mt4 pI is 7.70; VapC-mt4 pI is 4.86) and the proteins form a stable protein complex. This was demonstrated by co-expressing VapB-mt4 with a T7-tag fused to its amino terminus and VapC-mt4 with a His\(_6\)-tag attached to its amino terminus. After the resulting cell extract was passed over a Ni-NTA column we were able to co-purify T7-VapB-mt4 (lacking the His\(_6\)-tag) with His\(_6\)-VapC-mt4, demonstrating that VapB-mt4 and VapC-mt4 interact and form a stable protein complex (Figure S4A). Complex formation was confirmed by performing the reciprocal experiment using His\(_6\)-VapC-mt4 and T7-VapB-mt4 (Figure S4B).

\textit{VapC-mt4} expression leads to growth arrest in \textit{E. coli}, \textit{M. smegmatis} and \textit{M. tuberculosis} that can be rescued by co-expression with its cognate antitoxin \textit{VapB-mt4}. We then showed that the vapBC-mt4 module constitutes a functional TA system as induction of the VapC-mt4 toxin in \textit{E. coli} resulted in growth arrest that could be rescued by co-expression of the VapB-mt4 antitoxin from an independent plasmid (Figure 1A). We then demonstrated that the VapBC-mt4 TA module is active in mycobacteria. Expression of VapC-mt4 using the anhydrotetracycline-inducible pMC1s vector in \textit{M. smegmatis} (a rapid-growing nonpathogenic mycobacterium) or \textit{M. tuberculosis} inhibited cell growth, whereas coexpression of VapB-mt4 and VapC-mt4 from the same plasmid relieved the growth defect (Figure S3 and Figure 1B). These results demonstrate that \textit{vapBC-mt4} constitutes a functional TA system where VapB-mt4 is the cognate antitoxin for VapC-mt4 and validate the use of \textit{E. coli} for the characterization of VapC-mt4 activity.

\textit{VapC-mt4} inhibits protein synthesis. We next sought to identify the cellular process perturbed by VapC-mt4. To determine if VapC-mt4 expression affected translation, transcription or DNA replication we measured the incorporation of radioactive precursors for protein ([\textsuperscript{35}S]methionine), RNA ([\textsuperscript{3}H]uridine) and DNA ([\textsuperscript{3}H]thymidine) synthesis. Expression of VapC-mt4 reduced [\textsuperscript{35}S]methionine incorporation 76\% within 20 min and by as much as 94\% after 180 min relative to the control (Figure 2A and B). Curiously, VapC-mt4 expression decreased the incorporation of [\textsuperscript{3}H]thymidine after 60 min and increased the incorporation of [\textsuperscript{3}H]uridine after 60 min (Figure S5). As VapC-mt4 decreased [\textsuperscript{35}S]methionine incorporation to the greatest extent, we hypothesized that inhibition of protein synthesis was directly responsible for the growth
defect. The effects on [3H]thymidine and [3H]uridine incorporation appear to be indirect effects of inhibiting translation since treatment with chloramphenicol (which also arrests growth through translation inhibition) in the absence of toxin expression exhibited the same trends noted for VapC-mt4 expression (Figure S5).

**VapC-mt4 arrests translation elongation.** To learn more about the mechanism of VapC-mt4-mediated translation inhibition, we compared the ribosome profiles obtained from cells overexpressing VapC-mt4 (Figure S6C) to those from untreated (Figure S6A), or chloramphenicol treated (Figure S6B), cells. Expression of VapC-mt4 resulted in a marked increase in the recovery of polysomes, mirroring the polysome stabilization seen for chloramphenicol-treated cells. The accumulation of polysomes, which represent stalled translation elongation complexes, indicates that VapC-mt4 expression inhibits protein synthesis by arresting translation elongation.

To determine if VapC-mt4 increased the recovery of polysomes through interactions with translated mRNAs or rRNA in ribosomes we performed Western analysis on the fractions spanning the sucrose gradient. However, we were unable to detect VapC-mt4 in the polysome fraction containing translated mRNA and the 70S, 50S or 30S fractions containing rRNA. We did however detect VapC-mt4 in the fraction containing tRNA and RNAs not associated with ribosomes or ribosomal subunits (Figure S6C).

**VapC-mt4 does not cleave tRNA^fMet.** In contrast to *M. tuberculosis*, there is only one VapC toxin in *Shigella flexneri* and *Salmonella enterica*, derived from a single plasmid- and chromosomally-encoded vapB-vapC TA module, respectively. Each of these VapC toxins has been recently shown to efficiently and specifically cleave initiator tRNA at a single, identical position between the anticodon stem and loop (36). Using comparable experimental conditions, we did not detect any cleavage of *E. coli* or *M. tuberculosis* tRNA^fMet^ by VapC-mt4 (Figure S7, lanes 2-4). Therefore, unlike the VapC family members in *Shigella* and *Salmonella*, VapC-mt4 does not inhibit protein synthesis by cleaving initiator tRNA.

**Mutation of conserved PIN domain residues abolishes VapC-mt4 toxicity.** Sequence and structural similarity suggests that a conserved quartet of acidic residues and a fifth invariant serine or threonine residue compose the active site of PIN domain proteins (Figure S8A). In order to determine if these residues are required for VapC-mt4 toxicity we created D9A, E40A, D98A, T114A and D116A mutations in VapC-mt4 and examined the effect of mutating each of the residues individually. Unlike wild-type VapC-mt4, expression of the mutants did not inhibit growth on plates (Figure S8B). Despite the loss of toxicity the VapC-mt4 mutants retained the ability to form a protein complex with the VapB-mt4 antitoxin suggesting that the structure of the protein was retained (Figure S8C). Together, these results indicate a role for these residues in VapC-mt4 toxicity.

**VapC-mt4 expression leads to a gradual decrease in the steady-state levels of several mRNAs.** A common means by which TA toxins inhibit protein synthesis is by degrading the population of cellular mRNA using their sequence-specific endoribonuclease activity. The presence of a PIN domain, a motif associated with ribonuclease activity, suggested that the VapC-mt4 toxin might also perturb translation by a similar mechanism. We analyzed the effect of VapC-mt4 expression on the stability of several transcripts in vivo. Northern analysis for three *E. coli* mRNAs: tufA, ompA and lpp revealed that VapC-mt4 expression resulted in a gradual decrease in the steady-state levels of each transcript (Figure 2C). Degradation products were detected below the full-length tufA mRNA and a marked decrease in the level of the lpp transcript was detected as early as 20 min after VapC-mt4 induction. While these results are consistent with the function of VapC-mt4 as a ribonuclease, degradation of the three transcripts was not complete even 100 min after VapC-mt4 induction (Figure 2B-C).

**VapC-mt4 recognizes mRNA at ACGC and ACAGC sequences in vivo.** Because VapC-mt4 exhibited ribonuclease activity in vivo we sought to determine the RNA sequence recognized by VapC-mt4. We isolated total RNA from *E. coli* BW25113 Δ6 cells expressing VapC-mt4 and performed primer extension analysis on the three mRNAs (tufA, ompA and lpp) subjected to Northern analysis (Figure 2). We initially used a primer ~150 nucleotides downstream of the 5' end of the translation start site for each mRNA and then performed additional primer extension experiments to enable nearly complete coverage of the 1225 nt ompA and 318 nt lpp mRNAs. In total,
we detected four VapC-mt4 specific cleavage sites in the mRNAs revealing a preference for mRNA cleavage at ACGC and ACAGC sequences (Figure 3E).

**VapC-mt4 binds and cleaves MS2 RNA in vitro.** To ensure that the effect on the cellular mRNAs was directly due to VapC-mt4, we tested the RNA binding and cleavage activity of purified recombinant VapC-mt4 using bacteriophage MS2 RNA as the substrate. First, VapC-mt4 was added to a fixed amount of MS2 RNA and the reaction was monitored over time (Figure 4A). For comparison, we performed a parallel experiment using MazF-mt1 (Rv2801c), a MazF ortholog from *M. tuberculosis* that cleaves single-stranded RNA at UAC sequences (Figure 4B) (14). While the addition of purified VapC-mt4 resulted in the gradual cleavage of the MS2 RNA over time, there was a marked difference in the ribonuclease activity of VapC-mt4 and MazF-mt1. MazF-mt1 cut the MS2 RNA faster and more extensively than VapC-mt4. This inefficient cleavage of MS2 RNA by VapC-mt4 in vitro was consistent with the slow and incomplete cleavage of cellular transcripts in vivo by VapC-mt4. We also demonstrated that cleavage of the MS2 RNA was specifically due to VapC-mt4 and not a contaminating ribonuclease since pre-incubation of VapC-mt4 with the purified VapB-mt4 antitoxin prior to the addition of the MS2 RNA prevented RNA cleavage (Figure 4A, lanes 7-8).

**VapC-mt4 specifically recognizes MS2 RNA at ACGC and AC(A/U)GC sequences in vitro.** Due to the marked difference in the cleavage activity of VapC-mt4 and MazF-mt1, we wanted to determine if additional sequences were required for VapC-mt4 RNA binding and cleavage. Towards this end, we performed in vitro primer extension analysis using primers that allowed nearly complete coverage of the 3569 nt MS2 RNA. In doing so we identified twelve RNA recognition sites in the MS2 RNA that were cleaved by VapC-mt4 (Figure 5). Importantly, all twelve cleavage products were generated by VapC-mt4 as cleavage was inhibited in the presence of the cognate VapB-mt4 antitoxin.

The combined results of the in vivo (Figure 3) and in vitro (Figure 5) experiments revealed that VapC-mt4 specifically recognized RNA at sites containing the ACGC or AC(A/U)GC consensus sequence (Figure 3E and Fig. 5L). Further analysis of the in vitro primer extension results illuminated two notable trends. First, consistent with the mRNA cleavage activity we documented in vivo, all VapC-mt4 cut sites in MS2 mapped to single stranded regions based on modeling of the MS2 RNA using RNAfold (data not shown). Second, there was strict conservation of the GC sequence at the 3’ end of the VapC-mt4 recognition sequences. In fact, substitution of either the G or C within an internal ACGC cleavage site in a 20 nt RNA substrate precluded VapC-mt4 cleavage (Figure S9).

**mRNA cleavage is not required for VapC-mt4 mediated translation inhibition.** While VapC-mt4 possessed sequence-specific ribonuclease activity, there was a drastic difference between the cleavage activity of MazF-mt1 and VapC-mt4. These results in combination with the marked time lag from the start of translation inhibition (20 min) to the point where significant (≥50%) mRNA cleavage was observed (40-100 min) raised the possibility that mRNA cleavage was a secondary, downstream effect and was not directly responsible for the translational defect (Figure 2C). To reconcile these discrepancies, we further examined the effect of VapC-mt4 on protein synthesis in vitro using an S30 extract cell free transcription/translation system. We tested the effect of adding purified recombinant VapC-mt4 alone or both VapB-mt4 and VapC-mt4 on the transcription/translation of the chloramphenicol acetyltransferase (CAT) gene fused to the PinPoint peptide and β-lactamase encoded by the PinPoint Xa vector (Figure 6A). Purified VapC-mt4 inhibited the synthesis of the PinPoint-CAT fusion protein, which was reversed by pre-incubating VapC-mt4 with the VapB-mt4 antitoxin. Surprisingly, the addition of purified VapC-mt4 affected the expression of β-lactamase to a lesser extent and resulted in the appearance of a band corresponding to the full-length CAT protein lacking the N-terminal PinPoint peptide.

Since VapC-mt4 effectively inhibited expression of the PinPoint-CAT fusion protein we assessed the state of the corresponding transcript by reverse transcriptase PCR. While purified VapC-mt4 inhibited the synthesis of the PinPoint-CAT fusion protein, the PinPoint-CAT fusion transcript could still be detected in the sample in which translation was inhibited by VapC-mt4 (Figure 6C, lane 3). Therefore, cleavage of the PinPoint-CAT fusion transcript was not coincident with translation inhibition, suggesting that mRNA...
cleavage is not required for protein synthesis inhibition by VapC-mt4.

In support of a model in which VapC-mt4 toxicity is not mediated by RNA cleavage, we also observed that total RNA levels increased with VapC-mt4 expression over time (Figure S5B and Figure S10A). In contrast, a parallel experiment using the UAC-specific endoribonuclease MazF-mt1 revealed a decrease in total RNA levels following induction (Figure S10B). The MazF-mt1 data is also representative of the trend consistently observed upon induction of TA toxins that enlist RNA cleavage as their primary mode of translation inhibition (Woychik and Inouye laboratories, data not shown).

VapC-mt4 preferentially binds ssRNA containing the ACGC sequence. The inhibition of protein synthesis in the absence of mRNA cleavage raised the possibility that VapC-mt4 toxicity is primarily mediated by RNA binding. Subsequent cleavage of the mRNA may be a secondary effect of the prolonged association of VapC-mt4 with the mRNA. Electrophoretic mobility shifts assays (EMSAs) using purified recombinant VapC-mt4 and a synthetic 30 nt RNA containing an ACGC sequence were performed to assess the RNA binding activity of VapC-mt4. RNA binding experiments were performed in the presence of EDTA, a chelating agent that sequesters Mg$^{2+}$, to preclude RNA cleavage by VapC-mt4. VapC-mt4 was able to shift the mobility of the RNA containing the ACGC sequence (Figure 7A, lanes 2-5). The specificity of VapC-mt4 for the RNA containing the ACGC sequence was assessed by competition with 50 fold molar excess of unlabeled RNA. An unlabeled RNA containing the ACGC sequence (+ACGC) was sufficient to outcompete the labeled RNA containing the ACGC sequence, resulting in a loss of the VapC-mt4/RNA complex (Figure 7A, lane 6). However, 50 fold excess of unlabeled RNA lacking the ACGC sequence (-ACGC) did not disrupt the VapC-mt4/RNA complex (Figure 7A, lane 7). While all visible RNA was present in a VapC-mt4/RNA complex (Figure 7A, lane 5) less than 2% of the RNA was cleaved by VapC-mt4 in the presence of Mg$^{2+}$ (Figure 7B, lane 5) despite using identical amounts of RNA and protein. The inefficient cleavage of the 30 nt RNA by VapC-mt4 in vitro is consistent with the slow and incomplete cleavage of cellular transcripts in vivo by VapC-mt4. This marked difference between the RNA binding versus cleavage activities of VapC-mt4 indicated that a higher proportion of the RNA was stably bound by the toxin instead of being cleaved.

**DISCUSSION**

Our initial experiments showing that VapBC-mt4 overexpression caused growth arrest associated with decreased translation in vivo led us to pursue a mechanism of action comparable to previously reported TA toxins, mRNA cleavage. We were able to detect weak ribonuclease activity associated with VapC-mt4 in vivo and identified the consensus recognition sequence, ACGC or AC(A/U)GC, in the RNA products that were cleaved by VapC-mt4. This weak ribonuclease activity was confirmed by in vitro studies comparing the kinetics of RNA cleavage of VapC-mt4 to MazF-mt1 (Figure 6).

An unexpected finding of our work, however, was the large time lag between the point of translation inhibition (20 min post induction, Figure 2A-B) versus when a significant ($\geq$50%) decrease in the steady state levels of the mRNA transcripts was observed (40-100 min post induction, Figure 2C). These findings suggested that mRNA cleavage might not be the primary mechanism of translation inhibition and growth arrest. In this context, we discovered that translation inhibition by VapC-mt4 in a coupled transcription/translation assay was not the consequence of cleaving the RNA transcript (Figure 6). Using a synthetic 30 nt RNA containing an internal ACGC sequence we found that while the RNA was readily bound by VapC-mt4 only a small percentage (~2%) of the RNA was cleaved (Figure 7). These results support a model of VapC-mt4 mediated translation inhibition that has two novel features: RNA binding at ACGC or AC(A/U)GC sequences and inhibition of translation as the result of RNA binding rather than RNA cleavage.

This model of translational regulation is distinct from the sequence-specific endoribonuclease activity of the extensively characterized MazF family of toxins. In *M. tuberculosis*, there are at least seven MazF-mt toxins, three of which have been characterized in detail. MazF-mt1 cuts mRNA at UAC sequences (14), MazF-mt3 at UUCCU and CUCCU and MazF-mt7 at UCGCU (48). In each case the
MazF-mt toxins recognize and cleave at a unique recognition sequence. Similarly, if each of the *M. tuberculosis* VapC toxins possesses a unique RNA recognition profile for RNA binding the individual VapBC TA systems could regulate translation by selectively targeting distinct subsets of transcripts to yield different physiological consequences.

Although our data clearly indicate that mRNA cleavage is not the primary mechanism by which VapC-mt4 causes translation inhibition and growth arrest, the extent to which the RNA binding model is applicable to this family of TA modules is not known. The recently characterized examples in *Shigella* and *Salmonella* exhibit tRNA<sup>fMet</sup> cleavage activity. However, we demonstrated that VapC-mt4 does not cleave tRNA<sup>fMet</sup>.

All VapC toxin family members contain a PIN domain. PIN domain containing proteins were originally suggested to possess nuclease activity based on the properties and structural features of PIN domain proteins in eubacteria, archaeabacteria and eukaryotes. The first PIN domain structure (for the protein PAE2754 from the archaeabacterium *Pyrobaculum aerophilum*) exhibited distant structural homology to several Mg<sup>2+</sup>-dependent nucleases (30). The four conserved acidic residues in PAE2754 are clustered in a surface pocket facing into a tunnel with a diameter only wide enough to accommodate single-stranded (ss) nucleic acids. Additional PIN domain protein structures reported since then revealed that all PIN domains are structurally similar and thus have some homology to nucleases (PDB ID: ly82) (34,49-53).

Consistent with our model for VapC-mt4, the x-ray crystal structure of the VapC ortholog, FitB, has a high degree of structural homology to PAE2754 but no detectable nuclease activity (34). Our data suggest that some PIN domain proteins, including members of the VapC-mt family, may function as RNA binding proteins rather than high efficiency ssRNA endoribonucleases.

While our data strongly support a novel mechanism of VapC-mt4 translation arrest by RNA binding, a number of questions remain. One is the extent of RNAs targeted by this toxin *in vivo*. We have demonstrated that VapC-mt4 is able to specifically bind to RNA at sites containing the consensus sequence. It is possible that VapC-mt4 may also interact with other cellular RNAs with the same features to inhibit translation. Although we did not detect any significant loss of cellular tRNAs containing VapC-mt4 recognition sequences by reverse transcriptase PCR (data not shown) we cannot exclude the possibility that VapC-mt4 and other VapC-mt toxins act by targeting tRNAs other than tRNA<sup>fMet</sup> (or rRNA) as well as mRNA. A second question is the mechanism by which binding inhibits translation. Though our Western blot experiments did not identify VapC-mt4 in stabilized polysomes, ribosome stalling resulting from VapC-mt4 binding remains a leading possible mechanism. Answers to these questions will require further investigation of VapC-mt4 and other members of this large TA family.

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**FOOTNOTES**

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The abbreviations used are: CAT, chloramphenicol acetyltransferase; PilT, pili protein; PIN, PilT N-terminus; TA, toxin-antitoxin.

**FIGURE LEGENDS**

Fig. 1. Expression of VapC-mt4 leads to growth arrest. *A.* Growth profile for VapB-mt4 and VapC-mt4 uninduced (□), VapB-mt4 and VapC-mt4 induced (●) or pINIII and VapC-mt4 induced (●) in *E. coli* BW25113Δ6 containing either pINIII or pINIII-VapB-mt4 and pBAD33-VapC-mt4 grown at 37°C in M9 minimal medium containing 0.1% glycerol. Both 0.2% arabinose and 1mM IPTG were added to the induced samples. An empty pINIII vector was used to assess VapC-mt4 toxicity since leaky expression of VapB-mt4 from pINIII-VapB-mt4 abrogated VapC-mt4 toxicity. Data represent the average ± SD of three independent experiments. *B.* Growth profile for pMC1s (□), pMC1s-VapBC-mt4 (●) or pMC1s-VapC-mt4 (●) in *M. tuberculosis* H37Rv grown at 37°C in 7H9-TW80-ADN medium containing 30% spent culture supernatant and 200 ng/ml anhydrotetracycline. Cultures were inoculated with single colonies. Additional anhydrotetracycline was added every 48 hours to maintain the anhydrotetracycline concentration between 12.5-200 ng/ml. Data represent the average ± SD of three independent experiments.

Fig. 2. Expression of VapC-mt4 leads to translational arrest and RNA cleavage. *A.* [³⁵S]methionine incorporation into *E. coli* BW25113Δ6 cells with or without VapC-mt4 expression. Equivalent amounts of cell lysate were subjected to SDS-PAGE, followed by autoradiography. Numbers indicate the time (min) after VapC-mt4 induction. Data shown are representative of three independent experiments. *B.* Quantification of [³⁵S]methionine incorporation into *E. coli* BW25113Δ6 cells with (●) or without (□) VapC-mt4 expression. Data represent the average ± SD of three independent experiments. Time points correspond to those in Figure 2A. *C.* Northern analysis for tufA, ompA and lpp mRNAs extracted from *E. coli* BW25113Δ6 cells with or without expression of VapC-mt4. VapC-mt4 expression results in a gradual decrease in the steady state levels of tufA, ompA and lpp mRNAs. Numbers above lanes indicate the time (min) after VapC-mt4 induction. Numbers below lanes indicate the band intensity relative to the zero time point.
Fig. 3. VapC-mt4 mediated sequence-specific RNA cleavage. Primer extension analysis for A-B. tufA, C. ompA and D. lpp mRNAs extracted from E. coli BW25113Δ6 cells following induction of VapC-mt4. Numbers indicate the time (min) after VapC-mt4 induction. Cleavage sites/products are indicated by black arrows on the right side of gels and in the relevant RNA sequences/corresponding amino acid sequences below each gel. Lanes G, A, T, C correspond to DNA sequencing ladders prepared using the same primers used in the primer extension reactions. E. Alignment of sequences containing VapC-mt4 cleavage sites. The ACGC consensus sequence is in bold.

Fig. 4. VapC-mt4 mediated MS2 RNA cleavage. Equal molar amounts of A. His6-VapC-mt4 or B. His6-MazF-mt1 were added to MS2 RNA and RNA cleavage was monitored over time. Pre-incubation of His6-VapC-mt4 with His6-VapB-mt4 before the addition of the MS2 RNA prevented RNA cleavage. Data shown is representative of three independent experiments.

Fig. 5. VapC-mt4 mediated sequence-specific MS2 RNA cleavage. (A–K.) Primer extension analysis for MS2 RNA identified twelve VapC-mt4 cleavage sites. Lanes labeled “-” represent control reactions to which no proteins were added. In lanes labeled “B” purified His6-VapB-mt4 was incubated with the MS2 RNA. In lanes labeled “C” the addition of His6-VapC-mt4 resulted in the cleavage of the MS2 RNA. In lanes labeled “BC” pre-incubation of His6-VapC-mt4 with His6-VapB-mt4 prior to the addition of the MS2 RNA prevented RNA cleavage. Cleavage sites/products are indicated by black arrows on the right side of gels and in the relevant RNA sequences below each gel. Apparent secondary structure is indicated by white arrows on the right side of gels. Lanes G, A, T, C correspond to DNA sequencing ladders prepared by reverse transcription using the same primers used in the primer extension reactions. Data shown is representative of two independent experiments. L. Alignment of sequences containing VapC-mt4 cleavage sites. The ACGC consensus sequence is in bold.

Fig. 6. mRNA Cleavage is not required for VapC-mt4 mediated translation inhibition. A. Purified His6-VapC-mt4 inhibits coupled in vitro transcription/translation of the PinPoint-CAT fusion protein (Lane 3). His6-VapB-mt4 can rescue the inhibition and reconstitute translation (Lane 5). B. Schematic representation of the open reading frames encoded in the PinPoint Xa Control vector. The black rectangle denotes the PinPoint peptide. The white and grey arrows correspond to the ORFs encoding the 27.5 kDa CAT protein and the 31.5 kDa β-lactamase, respectively. The 39.5 kDa PinPoint-CAT fusion protein and β-lactamase are the major proteins expressed using the PinPoint Xa Control vector. The bent arrow shows the location of the T7 promoter. Thin arrows correspond to primers used for RT-PCR. C. The RNA was extracted from the coupled in vitro transcription/translation system and RT-PCR was performed to assess the state of the mRNA encoding the PinPoint-CAT fusion protein. The mRNA encoding the PinPoint-CAT fusion protein was not cleaved by His6-VapC-mt4. Data shown is representative of two independent experiments.

Fig. 7. VapC-mt4 selectively binds and cleaves ssRNA containing the ACGC sequence. A. Electrophoretic mobility shift assays (EMSAs) were performed in the presence of EDTA, a chelating agent that sequesters Mg$^{2+}$, to avoid RNA cleavage. His6-VapC-mt4 was able to bind and retard migration of a radioactively labeled synthetic 30 nucleotide RNA containing the ACGC consensus site (Lanes 2–5). Sequence specificity was assessed by competition with 50 fold excess of unlabeled RNA containing (Lane 6) or lacking (Lane 7) the ACGC consensus site. Data shown are representative of three independent experiments. B. RNA cleavage of the same radioactively labeled synthetic 30 nucleotide RNA containing the ACGC consensus site was performed in the presence of Mg$^{2+}$, a cation required for VapC-mt4 mediated RNA cleavage. The lane labeled “-” represents a control reaction to which no proteins were added (Lane 3). In lane “B” purified His6-VapB-mt4 was incubated with the RNA (Lane 4). In lane “C” the addition of His6-VapC-mt4 resulted in the cleavage of ~2% of the RNA (Lane 5). In lane “BC” pre-incubation of His6-VapC-mt4 with His6-VapB-mt4 prior to the addition of RNA prevented RNA cleavage (Lane 6). The cleavage site/product is indicated by a black arrow on the right side of the gel and in the relevant RNA sequence below the gel. T1 and AH denote RNA digested with RNase T1.
and partially hydrolyzed with alkali, respectively. Numbers indicate the position of G nucleotides. FL indicates the position of full length RNA. Data shown are representative of two independent experiments.
Figure 1

A

B

Time (hours)

OD\textsubscript{600}

Time (days)

OD\textsubscript{600}
**Figure 2**

A. Comparison of [³⁵S]Methionine Incorporation (%) between -VapC-mt4 and +VapC-mt4 conditions over time (min).

B. Graph showing the percentage of [³⁵S]Methionine Incorporation (%) over time (min) for -VapC-mt4 (black circles) and +VapC-mt4 (white squares). Error bars indicate standard deviation.

C. Gel blots showing expression levels of tufA, ompA, and lpp genes under -VapC-mt4 (left) and +VapC-mt4 (right) conditions at various time points (min).
Figure 3

(A) 5' GGG CAC GCC GAC UAU 3'
(B) 5' CAC UAC GCA CAC GUA 3'
(C) 5' AAA CAG CGU GCU GCA 3'
(D) 5' AGC AAC GCU AAA AUC 3'

E

|          | tufA | tufA | ompA | lpp |
|----------|------|------|------|-----|
|          | G G C A C | G C C G A | G A A A C | G C A A |
Figure 4

(A) VapC-mt4

| Time (hours) | 0 | 1 | 3 | 6 | 12 | 18 | 18 | 18 |
|--------------|---|---|---|---|----|----|----|----|
| VapB-mt4     |   |   |   |   |    |    | 1:2| 1:4|
| MS2 RNA      |   |   |   |   |    |    |    |    |

(B) MazF-mt1

| Time (hours) | 0 | 1 | 3 | 6 | 12 | 18 |
|--------------|---|---|---|---|----|----|
| MS2 RNA      |   |   |   |   |    |    |
Figure 7

A

B
Growth and translation inhibition through sequence specific RNA binding by a mycobacterium tuberculosis VAPC toxin
Jared D. Sharp, Jonathan W. Cruz, Sahadevan Raman, Masayori Inouye, Robert N. Husson and Nancy A. Woychik

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