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## **VapC-1 of nontypeable *Haemophilus influenzae* is a ribonuclease**

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## ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHi) are obligate parasites of the human upper respiratory tract that can exist as commensals or pathogens. Toxin-antitoxin (TA) loci are highly conserved gene pairs that encode both a toxin and antitoxin moiety. Seven TA gene families have been identified to date, and NTHi carry two alleles of the *vapBC* family. Here we have characterized the function of one of the NTHi alleles, *vapBC-1*. The gene pair is transcribed as an operon in two NTHi clinical isolates and promoter fusions display an inverse relationship to culture density. The antitoxin VapB-1 forms homo-multimers both *in vitro* and *in vivo*. The expression of the toxin VapC-1 conferred growth inhibition to an *E. coli* expression strain, and was successfully purified only when cloned in tandem with its cognate antitoxin. Using total RNA isolated from both *E. coli* and NTHi, we show for the first time that VapC-1 is a ribonuclease that is active on free RNA, but does not degrade DNA *in vitro*. Pre-incubation of the purified toxin and antitoxin together results in the formation of a protein complex that abrogates the activity of the toxin. We conclude that the NTHi *vapBC-1* gene pair functions as a classical TA locus and that the induction of VapC-1 ribonuclease activity leads to growth inhibition via the mechanism of mRNA cleavage.

## INTRODUCTION

Nontypeable *H. influenzae* (NTHi) are small facultative Gram-negative pleomorphic coccobacilli that lack the genes to produce and assemble a polysaccharide capsule. NTHi are fastidious and require exogenous heme or protoporphyrin IX for aerobic growth as well as nicotinamide adenine dinucleotide (NAD) when cultured in the absence of human cells. As these organisms are obligate parasites on the mucous membranes of humans, there are no natural animal or environmental reservoirs.

*H. influenzae* are acquired in the nasopharynx shortly after birth and can exist as commensals or pathogens. Mucosal infections associated with NTHi include otitis media, conjunctivitis, sinusitis, epiglottitis and pneumonia (24). NTHi is the leading cause of chronic bronchitis in adults, and is implicated in exacerbations of chronic obstructive pulmonary disease (COPD) (25). Otitis media (OM) is the second most prevalent infection of young children worldwide. Hearing loss is the most common sequelae of OM, with behavioral, educational and language development delays being additional consequences of early onset otitis media with effusion. The estimated annual direct medical costs from OM range from \$1.96 billion to \$4.1 billion (33).

Invasive NTHi infections include bacteremia and meningitis; traversal of epithelial or endothelial cells by the bacterium is required for these infections to occur. Prior to 1985, most invasive *H. influenzae* infections were due to encapsulated type b strains.

During that year, a polysaccharide conjugate vaccine against the type b polyribosylribitol phosphate capsule (Hib) was licensed and distributed in the United States. Disease caused by type b has been drastically curtailed since. However, the vaccine is not protective against NTHi, since these strains lack the capsular antigen. Efforts to identify NTHi vaccine candidates are ongoing and include strategies using immunogenic surface-exposed loops of the P2 and P4 outer membrane proteins (15, 27), the C-terminal fragment of the Hap autotransporter (22), conjugate vaccines with outer membrane protein P6 as a carrier (37), and oral vaccines composed of killed bacterial extracts (2).

First identified in pathogenic strains of the Gram-negative, strict anaerobe *Dichelobacter nodosus*, virulence-associated protein (*vap*) genes were found on a novel area of the chromosome that hybridized to nearly all virulent strains tested, but to only 23% of the avirulent strains studied (16). The chromosomes of the NTHi strains Rd KW20 (11), R2866 (28) and 86-028NP (14) contain homologues of *vapA*, *vapB*, *vapC*, and *vapD*, with one gene pair, *vapBC*, in duplicate.

The genetic organization of the NTHi *vap* genes is similar to toxin-antitoxin (TA) loci. Characteristic features of TA loci are that the toxin/antitoxin gene pair is an operon, consisting of an upstream antitoxin and a downstream toxin gene. The antitoxins prevent the effects of the toxins by forming tight complexes with them. The antitoxins are more labile than the toxins, and under conditions of stress are quickly

degraded by cellular proteases. This allows their cognate toxins to become active (13).

Seven TA gene families have been identified to date: *relBE*, *parDE*, *higBA*, *vapBC*, *mazEF*, *phd/doc*, and *ccdAB* (30). All except the *vapBC* and *mazEF* loci were originally discovered on plasmids. Although TA loci were first thought to be merely plasmid addiction modules, the discovery that these gene pairs were conserved in the chromosomes of numerous diverse prokaryotes (Gram-negative, Gram-positive as well as the Archaea) did not support that hypothesis and led to the notion that TA loci are important in the regulation of certain cellular functions. In a database mining effort that included 126 completely sequenced prokaryotic genomes, 671 TA loci were identified (30). Of these, 285 (42%) were *vapBC* homologues.

The *vapBC* alleles in NTHi have been shown to be expressed by proteomic studies (12, 17, 19). As NTHi carry two chromosomal *vapBC* operons (HI0321/HI0322 and HI0948/HI0947), for clarity we shall refer to these as *vapBC-1* and *vapBC-2*. Here we describe the functional characteristics of NTHi *vapBC-1* (HI0321/HI0322).

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** The bacterial strains and plasmids used in these studies are listed in Table 1. *E. coli* strains were grown in LB broth or agar  $\pm$  30 micrograms/ml kanamycin or 100 micrograms/ml ampicillin, as required. Induction of protein expression was with the addition of 1 mM IPTG for 2 hours. For the

protein-protein interaction studies, SU101 carrying pDD687 was grown overnight in LB broth containing 1 mM IPTG, then diluted and grown to log phase in LB + 1 mM IPTG prior to beta-galactosidase activity assays. *H. influenzae* strains were grown in BHI broth or agar supplemented with 10 micrograms/ml heme-histidine and beta-NAD (sBHI). All were cultured at 37°C in room air.

**Cloning of *vapB-1*, *vapC-1* and *cat*.** Each *vap* gene was cloned into the expression vectors pTrcHisA and pET24b as a single copy. As well, both were cloned in a tandem fashion in pET24b. The *cat* gene was cloned into pET24b. All primers used in this study were synthesized by Integrated DNA Technologies (Coralville, IA).

pTrcHisA cloning: *vapB-1* was amplified by PCR using the primers 321 forward 5'-GAGAGAATTCATATGCTTACTAAAGTG-3' and 321 reverse 5'-AACAAAGCTTTCATAAATTTCTCGC-3'. The primers included the engineered restriction sites *EcoRI* and *HindIII* (underlined) respectively, and resulted in pDD676. Similarly, *vapC-1* was amplified using the primers 322 forward 5'-GCGAGAATTCTTATGATTTATATGTTAG-3' and 322 reverse 5'-TCAGAAGCTTCTATTTTGTCCAATCTTGCC-3' with the same engineered sites, and resulted in pDD677. Both were ligated into *EcoRI/HindIII*-cut pTrcHisA and expressed in *E. coli* strain DH5 $\alpha$ .

pET24b cloning: *vapB-1* was amplified using the primers 2866B Sac forward 5'-GGAGGAGCTCTATGCTTACTAAAGTG-3' and 2866 B1 Xho reverse 5'-ATATCTCGAGTAAATTTCTCGCTCC-3', with engineered *SacI* and *XhoI* sites (underlined), respectively. Ligation of the amplicon into *SacI/XhoI*-cut pET24b resulted in VapB-1 being fused to the C-terminal polyhistidine tag in pDD690. Both *vapBC* genes

were cloned in tandem into pET24b using the primers 2866B Sac forward 5'-GGAGGAGCTCTATGCTTACTAAAGTG-3' and VapC Xho reverse 5'-GAATCTCGAGTTTTGTCCAATCTTGCC-3' and the amplicon was ligated into *SacI/XhoI*-cut pET24b, resulting in pDD686. This strategy resulted in only VapC-1 fused to the C-terminal polyhistidine tag. The *cat* gene from pACYC184 was amplified using the primers CAT Sac forward 5'-AGGAGAGCTCTATGGAGAAAAAATCACTGG-3' and CAT Xho reverse 5'-AAAACTCGAGCGCCCCGCCCTGCCACTC-3' and ligated to *SacI/XhoI*-cut pET24b, resulting in Cat being polyhistidine-tagged in pDD689. Each pET24b-based construct was expressed in *E. coli* BL21(DE3).

**Homodimerization assays.** For these assays, *vapB-1* was fused to the LexA DNA-binding domain (DBD) in the vector pSR658 (7), resulting in pDD687, and expressed in the reporter strain SU101. This strain has a chromosomal construct that consists of a *lacZ* reporter gene controlled by the strong *sulA* promoter, which contains a LexA operator sequence. When there is no fusion to the LexA DBD, the strain constitutively expresses a high level of beta-galactosidase. However, if a protein fused to the LexA DBD in pSR658 can homodimerize, this results in a competent LexA dimer that can bind to the LexA operator and repress transcription of *lacZ*. Expression of the LexA fusion in pSR658 is induced by IPTG, and since beta-galactosidase is a very long-lived enzyme, the reporter strain is routinely grown overnight in the inducer, so that any enzyme that was transcribed prior to induction of the LexA chimera has the opportunity



to degrade. This results in a more reliable and specific measurement of homodimerization.

Following overnight incubation in LB broth with 1 mM IPTG, the reporter strain carrying pDD687 was diluted and grown to log phase in LB broth with 1 mM IPTG. The amount of homodimerization was quantitated by beta-galactosidase activity assays and compared to the reporter strain carrying pSR658 (no fusion).

**Protein purification and antibodies.** Proteins were purified from induced cultures using the MagneHis protein purification system (Promega #V8500) according to the manufacturer's instructions. Briefly, *E. coli* strains DH5alpha or BL21(DE3) carrying the various fusions were grown to log phase in LB broth with appropriate antibiotics and induced for 2 hours with 1 mM IPTG. The cells were pelleted, frozen at  $-80^{\circ}\text{C}$ , and subjected to 3 freeze-thaw cycles prior to being processed using the MagneHis kit protocol for native purification. Protein concentration was quantitated using the micro BCA protein assay (Pierce #23235). Aliquots of purified protein were frozen at  $-80^{\circ}\text{C}$  and thawed when needed, after which the thawed aliquot was held at  $4^{\circ}\text{C}$  until used in assays.

Antibodies used to probe immunoblots were monoclonal Anti-Xpress-HRP (Invitrogen #R911-25) for pTrcHis fusions and monoclonal Anti-His (C-term)-HRP (Invitrogen #R931-25) for pET24b fusions. Immunoblots were developed using the

SuperSignal West Pico substrate (Pierce #34080). SDS-polyacrylamide gels were stained with Coomassie Blue and destained with 10% acetic acid.

**Total RNA isolation.** Cultures of *E. coli* K-12 (ATCC #10798) and *H. influenzae* strains R2866 and 86-028NP were grown to log phase and processed using the Promega SV total RNA isolation kit (Promega #Z3101) according to the manufacturer's instructions. The purified total RNA was then subjected to spectrophotometry at 260 and 280 nm to determine its purity and concentration. Aliquots were frozen at  $-80^{\circ}\text{C}$ , and each was thawed only once before use in reverse-transcriptase PCR or ribonuclease activity assays.

**Reverse-transcriptase PCR.** First-strand cDNA synthesis was performed using AMV reverse transcriptase according to the manufacturer's instructions (Promega #M5101). Briefly, aliquots of purified total RNA corresponding to 250 nanograms from NTHi strains R2866 or 86-028NP were incubated at  $70^{\circ}\text{C}$  for 5 minutes with the primer RT BC1 reverse 5'-CAATGCGTGACAAGCGATCC-3', which anneals to *vapC-1*. The mixtures were chilled on ice, centrifuged to collect, then incubated at  $37^{\circ}\text{C}$  for one hour with 1 microliter (10 Units) AMV reverse transcriptase, 4 microliters 5X AMV buffer, 1 microliter of 10 mM dNTPs, and nuclease-free water to a final volume of 20 microliters. Control reactions were identical but included no AMV reverse transcriptase. Following this incubation, a 2.5 microliter aliquot of each cDNA synthesis reaction was used as the template for conventional PCR with the primers RT BC1 forward 5'-

CAAAAGTGGTAACAGCCAAGC-3', which anneals to *vapB-1*. and RT BC1 reverse 5'-CAATGCGTGACAAGCGATCC-3', which anneals to *vapC-1*.

**Promoter::reporter gene fusion construction.** A 340 base pair sequence located upstream of the *vapBC-1* allele in strains R2866 and 86-028NP that included the first seven amino acids of *vapB-1* was amplified by PCR using the primers BC 1403 forward 5'-ACTAGAAATTCATCATTTACTCATTGACTTGC-3' and BC 1403 reverse 5'-GTTAGGATCCTGAAACACTTTAGTAAGC-3', which included engineered *EcoRI* and *BamHI* sites (underlined), respectively. These fragments were then ligated in frame with a promoterless *lacZ* reporter gene in the vector pMC1403 (5), creating pDD693 (R2866 *vapBC-1* promoter fusion) and pDD694 (86-028NP *vapBC-1* promoter fusion). Beta-galactosidase activity assays were performed in triplicate during lag phase and early, mid, and late logarithmic phases.

**Ribonuclease activity assays.** Aliquots of purified VapC-1, VapB-1 or Cat proteins were incubated at 37°C for 15 minutes with approximately 400 nanograms of purified total RNA from *E. coli* or *H. influenzae* in a buffer consisting of 10 mM HEPES (pH 7.4), 15 mM NaCl in a final volume of 10 microliters. Negative controls consisted of the MagneHis protein elution buffer alone. For some assays, VapB-1 and VapC-1 were preincubated together at room temperature for 30 minutes prior to the addition of total RNA substrate, after which the reactions were held at 37°C for 15 minutes. Each reaction was stopped by the addition of 2 microliters 6X loading buffer and separated on

1% TBE agarose gels with 0.8 microgram/ml ethidium bromide. All solutions used were nuclease-free or DEPC treated. Spot density was measured using the FluorChem IS-8900 program (Alpha Innotech, San Leandro, CA) with automatic background determination.

## RESULTS

**Both NTHi strains R2866 and 86-028NP express *vapBC-1*.** The genomes of the NTHi invasive blood isolate R2866 (28), and pediatric otitis media isolate 86-028NP (14) have been completely sequenced, and the *vapBC-1* allele is identical at the DNA level in both strains. To determine if *vapBC-1* was expressed, total RNA from each strain grown to log-phase in sBHI was subjected to reverse-transcriptase PCR using a forward primer that annealed to *vapB-1*, and a reverse primer located in *vapC-1*. The resulting 535 base pair product in the reactions that included AMV reverse transcriptase, but not in the control reactions without reverse transcriptase, showed that both R2866 and 86-028NP express *vapBC-1* and transcribe the gene pair as an operon (Figure 1, lanes 2 and 5, respectively).

**Expression of *vapBC-1* is inversely related to culture density.** Fragments that included the predicted promoter and transcription start sites of *vapBC-1* in R2866 and 86-028NP identified by a neural network program (available at <http://searchlauncher.bcm.tmc.edu/seq-search/gene-search.html>) which also included the first seven amino acids of *vapB-1* were fused in frame with a promoterless *lacZ* reporter gene in pMC1403 and transferred to *E. coli* strain DH5alpha. Since there was

a single nucleotide polymorphism between strains R2866 and 86-028NP at a site located 10 bases into the predicted transcript, with five additional polymorphisms scattered upstream, we fused both promoter regions to the reporter gene and analyzed the activity of each at culture densities corresponding to lag phase and early, mid, and late logarithmic phases (Figure 2). We found that there was relatively greater beta-galactosidase activity in the strain carrying the R2866 promoter fusion during lag phase than in the strain carrying the 86-028NP promoter fusion, an average of  $241 \pm 15$  versus  $171 \pm 10$  Miller units (Figure 2A and 2B, respectively). In both cases, the activity decreased with increasing culture density and approached identical levels during late growth phase ( $95 \pm 2$  versus  $100 \pm 15$  Miller units, respectively). The negative control, pMC1403 with no promoter fusion, did not deliver detectable beta-galactosidase activity at any point during the growth cycle (data not shown).

**Purified NTHi VapB-1 forms multimers *in vitro*.** VapB-1 is the putative antitoxin protein of the *vapBC-1* operon. We used the pTrcHisA vector (Invitrogen, Carlsbad, CA) to clone, overexpress, and natively purify VapB-1. This vector includes an N-terminal polyhistidine fusion that allows affinity purification using paramagnetic nickel beads, as well as an epitope tag (Xpress) for which a monoclonal HRP-linked antibody is commercially available. Figure 3A shows a Coomassie-stained 12% SDS-PAGE separation of a typical pTrc::VapB-1 purification from *E. coli* strain DH5alpha. Figure 3B is an identical immunoblot probed with the monoclonal anti-Xpress HRP-linked antibody. In this fusion, the calculated molecular mass of pTrc::VapB-1 is 13.2

kDa. Note that there is an enormous difference in sensitivity between Coomassie staining and chemiluminescent detection methods. Even after being heated in SDS-PAGE loading buffer with a reducing agent and separated on a gel, it is apparent that pTrc::VapB-1 is forming multimers.

**The VapB-1 protein homodimerizes *in vivo*.** Since we observed multimerization of the pTrc::VapB-1 purified protein on an immunoblot (Figure 3B), and because many TA antitoxins display homo-interactions (38), VapB-1 was fused to the LexA DNA-binding domain in a bacterial-based protein-protein interaction reporter system to quantitate its homodimerization (7). The LexA protein consists of two domains, one that recognizes and binds to a specific operator site on DNA, and one which functions as a dimerization domain. The LexA dimerization domain can be removed and replaced with another protein or fragment. Since the repressor is only active as a dimer, the homodimerization of the fused protein allows the chimeric LexA to bind to its operator site and repress transcription of a *lacZ* reporter gene. This interaction was quantitated by beta-galactosidase activity assays (Figure 4), and we found that the NTHi antitoxin VapB-1 interacted strongly with itself *in vivo*. The activity of the reporter strain SU101 expressing the control unfused LexA DNA-binding domain was a mean of  $1086 \pm 59$  Miller units, and the same strain expressing the LexA::VapB-1 fusion was a mean of  $123 \pm 10$  Miller units ( $n = 3$ ,  $P < 0.0001$ , two-tailed *t* test). This represents an 88% repression of the reporter gene in this system, evidence of strong VapB-1 homo-interaction.

**NTHi VapC-1 causes growth inhibition *in vivo*.** The toxin portion of the R2866 *vapBC-1* allele, *vapC-1*, was also cloned into the pTrcHisA vector as a N-terminal polyhistidine fusion. However, even in the absence of the gratuitous inducer IPTG, carriage of the plasmid containing the toxin conferred growth inhibition to the *E. coli* expression strain DH5alpha, making this construct unsuitable for protein purification. Figure 5 shows a representative growth curve of DH5alpha carrying either the pTrcHisA vector, the pTrc::*vapB-1* fusion, or the pTrc::*vapC-1* fusion in LB broth. We hypothesized that the observed attenuation of growth caused by pTrc::*vapC-1* was due to the type of promoter used in the vector, which is leaky enough to allow some transcription of a cloned gene even in the absence of the inducer. The growth inhibition resulting from unintended protein expression indicated to us not only that the R2866 *vapC-1* gene encoded a functional protein, but also that it was toxic to *E. coli* cells in very small quantities.

**The VapC-1 toxin was natively purified by tandem cloning with the VapB-1 antitoxin.** As in most cases, Nature provided the most elegant and simple solution to the biological challenge of purifying a toxin protein, and we took advantage of this by the tandem cloning of the R2866 *vapBC-1* TA operon into the expression vector pET24b (EMD Biosciences #69750-3), such that the T7 promoter drove the operon's transcription, but the C-terminal polyhistidine tag was fused to VapC-1 only, creating pDD686 (Figure 6A). The vector pET24b is highly regulated, since the T7 phage

promoter that controls transcription requires prior induction of its cognate RNA polymerase. Thus, when the T7 RNA polymerase is induced by IPTG in *E. coli* strain BL21(DE3), both *vapB-1* and *vapC-1* are transcribed, but only *vapC-1* is polyhistidine-tagged. This strategy ensured that each full-length VapC-1 toxin was paired with at least one full-length VapB-1 antitoxin, and resulted in the restoration of normal growth in the expression strain (data not shown). In this system, the apparent molecular mass of pET::VapB-1 is 10.5 kDa, and pET::VapC-1 is 16.6 kDa. Figure 6B shows a Coomassie-stained SDS-PAGE gel of a typical VapC-1 paramagnetic bead purification. Because we were purifying VapC-1 from a tandem cloning, and TA loci proteins are known to form tight heterocomplexes, we hypothesized that we might observe at least some VapB-1 co-purifying with VapC-1. Note that a band of the approximate size of pET::VapB-1 appears with His-tagged pET::VapC-1 purified from BL21(DE3) on the Coomassie stained gel (Figure 6B). In contrast to the results observed with pTrc::VapB-1, an immunoblot of pET::VapC-1 probed with an anti-C-terminal polyhistidine tag HRP-linked monoclonal antibody showed only a single band at approximately 17 kDa (Figure 6C). The fact that we were able to successfully purify a native His-tagged VapC-1 toxin from this tandem cloning is evidence that the presence of VapB-1 is necessary to prevent the VapC-1-induced growth inhibition of the expression strain.

**The VapC-1 toxin is a ribonuclease that acts on free RNA *in vitro*.** The VapC-1 toxin contains a motif known as a PilT N-terminus (PIN) domain that consists of about 100 amino acids with two nearly invariant aspartate residues. These residues



have been shown to be essential for metal ion coordination in other PIN-domain containing proteins that are ribonucleases (10). The PIN domain protein family displays similarity to the nuclease domains of *Taq* polymerase, T4 RNase H, and the 5'-3' flap endonucleases (3). Accordingly, we performed ribonuclease activity assays with purified VapC-1 to determine whether it was enzymatically active on purified total RNA *in vitro*. As a control to ensure that any activity observed with VapC-1 was specific, and not due to a ribonuclease that may have co-purified with the protein, the chloramphenicol acetyltransferase (*cat*) gene from pACYC184 was fused to the pET24b vector, overexpressed in the *E. coli* strain BL21(DE3), and natively purified in an identical fashion to VapC-1. The Cat protein is a known homotrimer that displays no ribonuclease activity.

Figure 7A shows that the VapC-1 toxin can efficiently degrade both *E. coli* K-12 and *H. influenzae* strain 86-028NP total RNA in a concentration-dependent manner *in vitro*. Note the natural 23S rRNA cleavage pattern of strain 86-028NP. That *Haemophilus* species display 23S fragmentation heterogeneity is a previously reported phenomenon (34). Figure 7B shows that the VapC-1 ribonuclease activity is specific, as incubating strain 86-028NP total RNA substrate with the same amount of the Cat protein, which had been cloned, overexpressed, and purified in an identical fashion as VapC-1, results in no significant degradation as quantitated by densitometry.

**The VapC-1 toxin cannot degrade double- or single-stranded DNA.** To determine whether VapC-1 had general nuclease activity, a 760 bp PCR product of the *cat* gene was used as the substrate in nuclease assays. These assays were identical to the ribonuclease activity assays, except that the substrate was linear double-stranded DNA. No significant difference was observed between assays containing either the elution buffer control or VapC-1, indicating that it displayed no DNase activity under these conditions (data not shown). A purified plasmid was tested as a circular double-stranded DNA substrate, but was not degraded by VapC-1. Finally, an aliquot of M13mp18 single-stranded DNA (New England Biolabs #N4040S) was used as a substrate, with identical results.

**The VapB-1 antitoxin inhibits VapC-1 ribonuclease activity.** Following translation, canonical toxin and antitoxin proteins interact to form non-toxic complexes, and we observed the rescue of growth inhibition when VapB-1 and VapC-1 were expressed together in *E. coli*. To investigate whether these complexes could be reconstituted *in vitro* with purified proteins, we pre-incubated various ratios of the purified VapB-1 antitoxin and the VapC-1 toxin prior to adding the total RNA substrate. For these assays, we cloned VapB-1 into the pET24b vector as a single gene and purified it in an identical manner as the VapC-1 protein, so that both proteins would be expressed from the same vector in the same expression strain. Figure 8 shows that VapB-1 has no intrinsic ribonuclease activity by itself, and that preincubation with a 4:1 ratio of VapB-1 to VapC-1 abrogates the ribonuclease activity of VapC-1. A 2:1 ratio of

VapB-1 to VapC-1 partially inhibits the ribonuclease activity of VapC-1. For these assays, we used both *E. coli* K-12 total RNA and NTHi strain R2866 total RNA as substrates. Note that strain R2866 displays a different natural 23S ribosomal RNA fragmentation pattern than that of strain 86-028NP.

## DISCUSSION

*E. coli* expresses the chromosomal TA loci *relBE* and *mazEF*. The RelE toxin has been identified as a global inhibitor of translation, and is induced under starvation conditions (6). RelE is a sequence-specific RNase, cleaving mRNA in the A site of the ribosome, but it is unable to degrade free RNA (32). The MazE antitoxin is degraded by the cellular protease ClpPA (1), and this results in the activation of the toxin MazF, which cleaves single-stranded RNA independent of ribosomes. This makes MazF functionally distinct from the RelE toxin (39). In this study, we show that NTHi VapC-1 displays MazF-like activity; that is, the toxin is active on free RNA *in vitro*. Interestingly, *H. influenzae* does not contain a *mazEF* homologue, whereas *E. coli*, on the other hand, does not contain any *vapBC* homologues. Further support for this apparent trend toward mutual exclusivity comes from a group that surveyed 23 separate genomes and found that 20 contained either a *mazEF* locus or a *vapBC* locus, but not both (40).

Although most organisms carry various TA loci in their genomes, the reason why these genes are so highly conserved as well as what their exact purpose might be remains controversial. Some investigators posit that free-living organisms carry more TA loci in their genomes than host-adapted organisms do because the former

encounters harsh environmental conditions, whereas adaptation to a host organism may require only a few TA operons to serve a similar function (30). One caveat to this view is that the prevalence of TA modules in a genome may not always correlate with their expression or activity. In *E. coli*, it was originally thought that the toxins RelE and MazF killed the cells, since investigators observed a dramatic loss of colony formation when these toxins were released from their antitoxins (13). It was later shown, however, that these toxins did not actually kill the cells, but instead contributed to a reversible bacteriostatic state in which the cells were unable to form a colony – they were viable but nonreplicative. Expression of the cognate antitoxins rescued these cells and allowed colony formation (6, 31). It has been suggested that induction of a reversible viable but nonreplicative state is important for cells to survive in a competitive or harsh environment, in nature or inside a host (24), and there is evidence that *H. influenzae* enters a bacteriostatic state in the middle ear during the course of otitis media with effusion (26). In addition to enhancing survival under suboptimal conditions, a strategy of reversible growth arrest could provide a mechanism for non-specific antibiotic resistance (nongrowing cells are not susceptible to the cidal action of most antibiotics) as well as decrease each cell's metabolic burden and energy requirements.

A recent competing hypothesis put forward is that the *mazEF* TA locus can induce a programmed cell death (PCD) cycle that mimics the PCD observed in multicellular organisms (8). This line of reasoning is supported by the observation that, while the MazE antitoxin can rescue MazF toxin overexpression in the short term, there

seems to be a “point of no return” after which no amount of MazE antitoxin will allow the culture to recover. However, there are three points to consider in this debate: first, the results of these *in vitro* PCD experiments seem to show that, even after 24 hours, there is a very small (1-5%) portion of the culture that does survive (18). This could be enough to maintain a “founder” population that could resume replication after conditions improved, particularly *in vivo* (20). Second, a confounding element of life on human mucosal surfaces is that many organisms, including *H. influenzae*, form biofilms in this environment, and growth in biofilms has been shown to modulate the metabolic characteristics of a number of organisms as compared to those grown in planktonic culture (21, 36). Further, even within an *in vitro* planktonic culture, there is an observable difference between the amount of bacterial cells that persist following antibiotic treatment, which depends upon whether the culture is in logarithmic or stationary phase at the time of antibiotic addition (35). Third, the aforementioned experiments were performed with one type of TA locus (*mazEF*) and the results observed regarding this particular module might not be generally applicable to the TA superfamily as a whole. Indeed, a recent study of one of the two *higBA* TA alleles carried in the superintegron of ChrII of *V. cholerae* suggested that the expression of the HigB toxin at endogenous levels for several hours was not bactericidal, but the function of the toxin was not elucidated (4). Therefore, at this point, it may be premature to accept either view as being entirely correct.

Our results indicate that the *vapBC-1* allele in two clinical isolates of NTHi is expressed as a functional TA locus with VapB-1 as the antitoxin and VapC-1 as the ribonuclease toxin. The gene pair is transcribed as an operon in both NTHi strains and each promoter appears to be more active during lag phase, with expression displaying an inverse relation to culture density. VapC-1 is active on free RNA purified from two different genera *in vitro*, but does not display general nuclease activity, as it did not degrade double- or single-stranded DNA under the same conditions. The homodimerization observed with VapB-1, coupled with the ratio of antitoxin to toxin protein required to abrogate VapC-1 activity, suggests that the nontoxic VapBC-1 complex consists of VapB-1 multimers interacting with VapC-1 moieties *in vivo*. This is reminiscent of the MazEF complex, which has been shown to form a heterohexamer (38). The degradation of the labile VapB-1 antitoxin under stress would allow the stable VapC-1 toxin to actively inhibit protein synthesis by cleaving mRNA. The entry of NTHi into a bacteriostatic state induced by suboptimal conditions could be involved in mucosal infections that recur following antibiotic treatment, such as otitis media and bronchitis. On the other hand, even if most of the infecting population were killed by toxin induction, a small surviving segment could be responsible for the re-seeding of tissues observed in chronic disease.

Interestingly, TA loci are conserved (often in multiple copies) in the genomes of many organisms that can cause persistent infections and/or persist in the environment: *M. tuberculosis*, *H. pylori*, *C. burnetii*, *L. interrogans*, *V. cholerae*, *S. enterica* serovar

*typhi* and *typhimurium*, as well as *H. influenzae* (9, 23, 29, 30). It is intriguing that a human-adapted organism such as *H. influenzae* would maintain two *vapBC* alleles on its very small (~2.0 Mb) chromosome, and suggests that both may be important for its lifestyle. Further studies are planned to investigate the *in vivo* function and synergy of the NTHi *vap* alleles.

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## FIGURE LEGENDS

### FIGURE 1 - NTHi strains R2866 and 86-028NP express *vapBC-1* as an operon.

Reverse-transcriptase (RT) PCR reactions with a forward primer in *vapB-1* and a reverse primer in *vapC-1* electrophoresed on a 0.8% agarose gel. Lanes 1 and 4: 1 kb DNA ladder; lanes 2 and 5: R2866 and 86-028NP with reverse transcriptase (+ RT); lanes 3 and 6: R2866 and 86-028NP without reverse transcriptase (– RT).

### FIGURE 2 – Expression of *vapBC-1* over the cell cycle.

- A. The beta-galactosidase activity of the R2866 *vapBC-1* promoter fusion (curve) is inversely related to culture density (bars).
- B. The 86-028NP *vapBC-1* promoter fusion (curve) displays lower initial beta-galactosidase activity than the R2866 fusion, but mirrors the trend toward decreasing expression with increasing culture density (bars).

### FIGURE 3 - VapB-1 forms multimers *in vitro*.

- A. Coomassie-stained 12% SDS-PAGE separation of a typical pTrc::VapB-1 purification (2 micrograms) from DH5alpha.
- B. An identical immunoblot probed with anti-Xpress-HRP monoclonal antibody. Note the multiple bands resulting from apparent homo-interactions.

### FIGURE 4 - VapB-1 homodimerizes *in vivo*

With no protein fused to the LexA DNA-binding domain (DBD), the repressor cannot form a dimer and transcription of the *lacZ* reporter gene is constitutive (shaded bar). However, when the LexA DBD is fused to VapB-1, competent dimers are formed and

the chimeras can bind to the LexA operator sequence, repressing transcription of the reporter gene (black bar). This level of repression indicates strong VapB-1 homo-interaction.

### **FIGURE 5 - VapC-1 causes growth inhibition *in vivo***

When grown in LB broth without IPTG, the pTrcHisA vector promoter allowed a small amount of transcription of pTrc::VapC-1, which conferred growth inhibition to the DH5alpha expression strain and made this construct unsuitable for protein isolation. No significant growth effects were observed with the pTrc::VapB-1 fusion or the vector alone.

### **FIGURE 6 - VapC-1 was successfully purified via tandem cloning into pET24b and expression in BL21(DE3)**

- A. The *vapBC-1* operon was fused to pET24b such that *vapB-1* was in frame with the vector's ATG start codon at the N-terminus, and *vapC-1* was in frame with the C-terminal polyhistidine tag, creating pDD686. Induction of the construct with IPTG resulted in no significant growth inhibition of the expression strain.
- B. Coomassie-stained 12% SDS-PAGE separation of a typical pET::VapC-1 purification (3.5 micrograms). Two bands are visible: one at the calculated molecular mass of pET::VapC-1 (16.6 kDa) and one at the size of pET::VapB-1 (10.5 kDa).
- C. Identical immunoblot probed with anti-His C-terminal HRP-linked monoclonal antibody. Note that only a single band is apparent.

### FIGURE 7 - VapC-1 is a ribonuclease toxin

- A. Total RNA from *E. coli* K-12 or *H. influenzae* strain 86-028NP was used as the substrate in ribonuclease activity assays with increasing amounts of the purified VapC-1 toxin. Lanes 1 & 4: MagneHis protein elution buffer control; lanes 2 & 5: 0.35 microgram VapC-1; lanes 3 & 6: 0.7 microgram VapC-1.
- B. The Cat protein was cloned into pET24b and purified in the same manner as VapC-1, as a control for any co-purifying ribonuclease activity. Lane 1: MagneHis protein elution buffer control; lane 2: 0.35 microgram Cat protein; lane 3: 0.35 microgram VapC-1. Densitometry indicated that the observed ribonuclease activity was specific to VapC-1.

### FIGURE 8 - VapB-1 forms nontoxic complexes with VapC-1 *in vitro*

The antitoxin VapB-1 was cloned into pET24b as a single gene and purified using the MagneHis native protein purification protocol. Varying amounts of purified VapB-1 was incubated with a constant amount of VapC-1 for 30 minutes prior to addition of the total RNA substrate in ribonuclease activity assays. A 4:1 ratio of VapB-1 to VapC-1 abrogates the ribonuclease activity of VapC-1. Lanes 1 & 6: MagneHis protein elution buffer control; lanes 2 & 7: 0.2 microgram VapB-1; lanes 3 & 8: 0.4 microgram VapB-1 + 0.1 microgram VapC-1 (4:1 ratio); lanes 4 & 9: 0.2 microgram VapB-1 + 0.1 microgram VapC-1 (2:1 ratio); lanes 5 & 10: 0.1 microgram VapC-1 alone. For these assays, *H. influenzae* strain R2866 total RNA was used. Note the natural 23S fragmentation pattern, which differs from that of strain 86-028NP.



TABLE 1 - Bacteria and plasmids used in this study.

<b>Bacteria</b>	<b>Description</b>	<b>Source</b>
<b><i>H. influenzae</i></b>		
86-028NP	Otitis media isolate from a pediatric patient.	R.S. Munson Jr.
R2866	Blood isolate from an immunocompetent child with meningitis immunized with the Hib vaccine.	A.L. Smith
<b><i>E. coli</i></b>		
BL21(DE3)	F <sup>-</sup> <i>ompT hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup>m<sub>B</sub><sup>-</sup>) gal dcm</i> (DE3)	EMD Bioscience
DH5alpha	F <sup>+</sup> Phi80Δ <i>lacZ</i> ΔM15 Δ( <i>lacZYA-argF</i> )U169 <i>deoR recA1 endA1 hsdR17(rK<sup>-</sup>mK<sup>+</sup>) phoA supE44 thi-1</i>	Lab collection
K-12	Feces isolate from a diphtheria convalescent	ATCC
SU101	<i>lexA71 ::Tn 5(Def)sulA 211 A (lacI<sup>+</sup>POZYA) 169/F lac<sup>+</sup>lacZA M15::Tn9</i>	M. Granger-Schnarr
<b>Plasmids</b>	<b>Description</b>	<b>Source</b>
pACYC184	Source of <i>cat</i> gene	Lab collection
pDD676	pTrcHisA with <i>vapB-1</i>	This work
pDD677	pTrcHisA with <i>vapC-1</i>	This work
pDD686	pET24b with <i>vapBC-1</i>	This work
pDD687	pSR658 with <i>vapB-1</i> fused to the LexA DBD	This work
pDD689	pET24b with <i>cat</i>	This work
pDD690	pET24b with <i>vapB-1</i>	This work
pDD693	pMC1403 with R2866 <i>vapBC-1</i> promoter fusion	This work

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pDD694	pMC1403 with 86-028NP <i>vapBC-1</i> promoter fusion	This work
pET24b	C-terminal His tag expression vector	EMD Bioscience
pMC1403	Promoter:: <i>lacZ</i> fusion vector	(5)
pSR658	LexA DNA-binding domain fusion vector	Lab collection
pTrcHisA	Expression vector with N-terminal polyhistidine tag	Invitrogen

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Figure 1

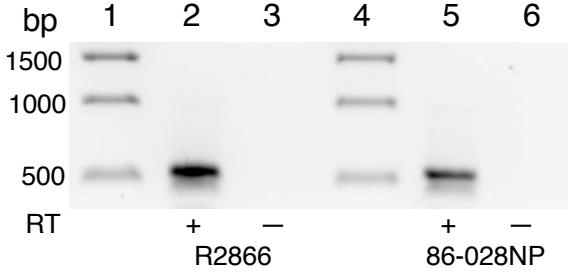


Figure 2

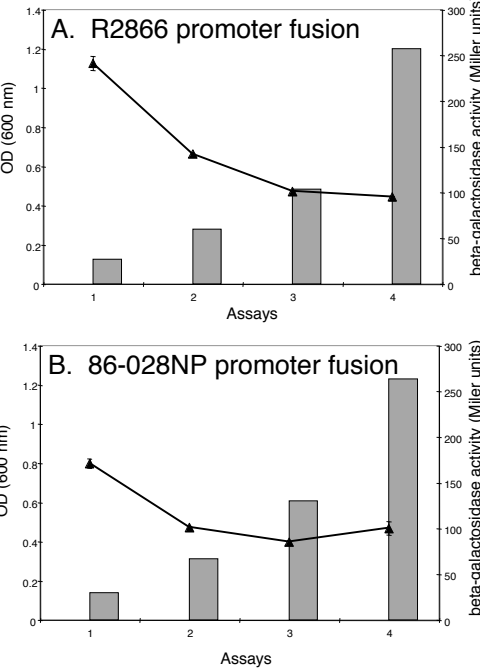


Figure 3

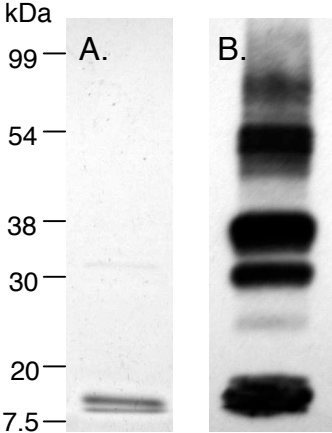


Figure 4

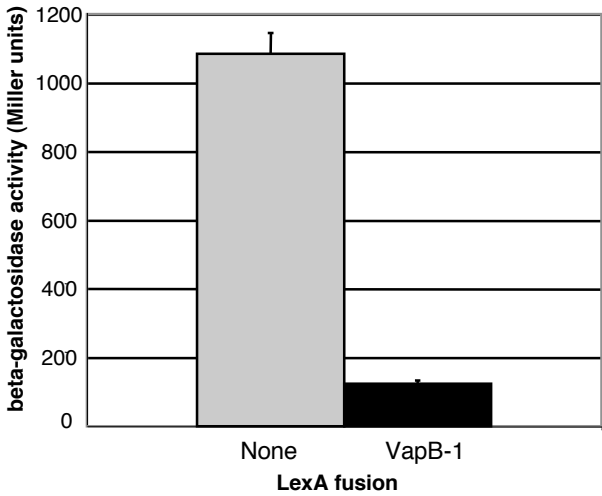


Figure 5

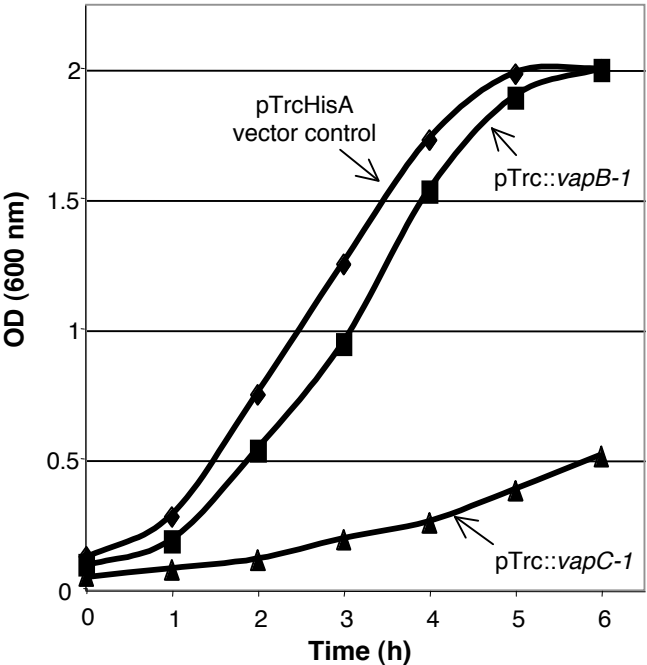


Figure 6

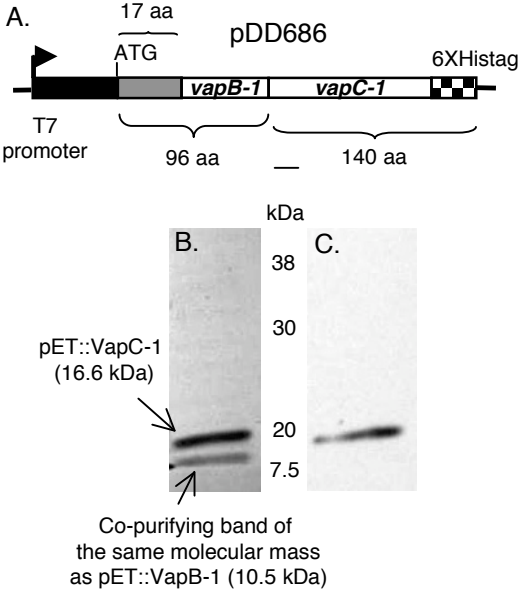




Figure 7

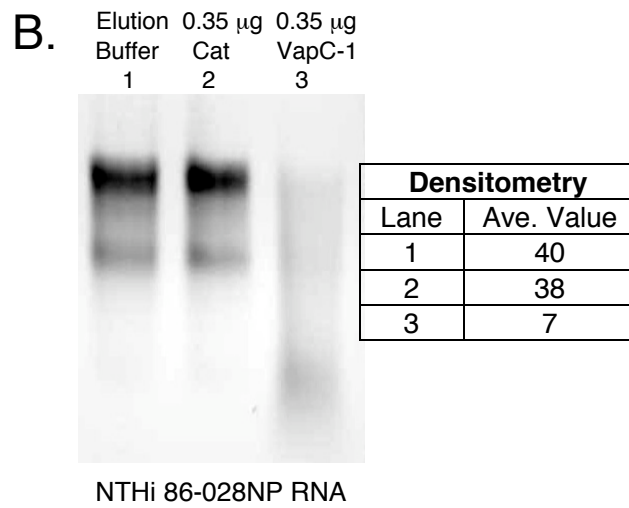
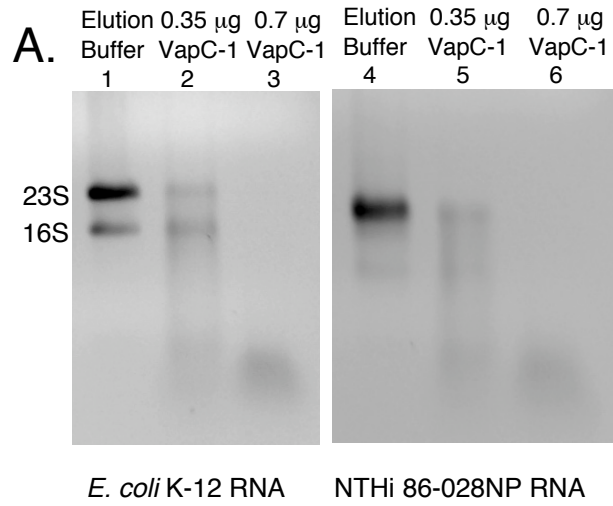


Figure 8

