The ToxAvaP A Toxin-Antitoxin Locus Contributes to the Survival of Nontypeable Haemophilus influenzae during Infection

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Abstract

Nontypeable Haemophilus influenzae (NTHi) is an opportunistic pathogen that is a common cause of acute and recurrent mucosal infections. One uncharacterized NTHi toxin-antitoxin (TA) module, NTHI1912-1913, is a host inhibition of growth (higBA) homologue. We hypothesized that this locus, which we designated toxAvaP A, contributed to NTHi survival during infection. We deleted toxAvaP A and determined that growth of the mutant in defined media was not different from the parent strain. We tested the mutant for persistence during long-term in vitro co-culture with primary human respiratory tissues, which revealed that the ΔtoxAvaP A mutant was attenuated for survival. We then performed challenge studies using the chinchilla model of otitis media and determined that mutant survival was also reduced in vivo. Following purification, the toxin exhibited ribonuclease activity on RNA in vitro, while the antitoxin did not. A microarray comparison of the transcriptome revealed that the tryptophan biosynthetic regulon was significantly repressed in the mutant compared to the parent strain. HPLC studies of conditioned medium confirmed that there was no significant difference in the concentration of tryptophan remaining in the supernatant, indicating that the uptake of tryptophan by the mutant was not affected. We conclude that the role of the NTHi toxAvaP A TA module in persistence following stress is multifactorial and includes effects on essential metabolic pathways.

Introduction

Nontypeable Haemophilus influenzae (NTHi) are pleomorphic Gram-negative bacteria that reside in the human upper respiratory tract as commensals. However, these organisms are also responsible for a number of mucosal diseases, including chronic bronchitis, exacerbations of chronic obstructive pulmonary disease, and acute and chronic otitis media (OM). Recurrent OM caused by NTHi is common, and those infections that recur less than two weeks after the completion of antimicrobial therapy have been shown to largely be due to the same strain of bacteria, suggesting a subpopulation of persister cells [1,2]. Toxin-antitoxin (TA) gene pairs have been found in nearly all bacterial genomes sequenced to date, and type II loci encode a protein toxin and antitoxin that form a nontoxic complex upon translation which auto-represses the cognate promoter [3]. Under stressful environmental conditions such as nutrient limitation, antibiotic therapy, or oxidative stress, the labile antitoxin is degraded by intracellular proteases and the more stable toxin is freed to facilitate growth arrest, often via mRNA degradation [4,5]. The ability to induce a state of dormancy increases microbial fitness by decreasing nutrient requirements and the metabolic burden. As well, this persister state facilitates nonspecific antibiotic tolerance in the microorganism, as most antimicrobials target essential cellular functions necessary for bacterial growth and replication [6].

TA modules have been divided into several families, one of which is the host inhibition of growth (higBA) gene pair [7]. Most TA operons are organized such that the antitoxin is transcribed first, followed by the toxin, and the higBA locus is unusual in that the toxin gene precedes the antitoxin. Originally identified on the Proteus vulgaris Rts1 plasmid [7], the higBA module has been found in the chromosomes of a number of pathogens, including Vibrio cholerae, Mycobacterium tuberculosis, Pseudomonas aeruginosa, Yersinia pestis, and Acinetobacter baumannii [8–12]. NTHi strains contain three TA gene pair families, and one of these is a higBA homologue. In previous work, we have shown that two of the TA loci in NTHi exert significant effects on the organism’s ability to sustain an infection, both in vitro and in vivo [13]. In this study, we investigated the hypothesis that the higBA homologue in the NTHi strain 86-028NP (NTHI1912-1913, designated toxAvaP A), also played a role in the survival of this human-adapted pathogen. We found that the ToxAvaP A locus enhanced the ability of NTHi to persist during in vitro and in vivo infections.

Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in these studies are listed in Table 1. E. coli strains were grown in LB broth or agar
with or without 30 µg/ml kanamycin or 100 µg/ml ampicillin, as required. NTHi strains were grown in brain heart infusion supplemented with 10 µg/ml heme-histidine and 10 µg/ml β-NAD (sBHI) broth or agar. E. coli strain BL21(DE3) was used to overexpress toxAvapA for protein purification prior to use in ribonuclease activity assays. NTHi transformants were selected on chocolate agar plates with 25 µg/ml spectinomycin, and were routinely cultured on 37°C with 5% CO2.

Construction of a ΔtoxAvapA Mutant

The ΔtoxAvapA mutation construct was assembled by amplifying genomic DNA (gDNA) from NTHi strain 86-028NP [14] using Phusion FLASH high fidelity polymerase (Thermo Fisher Scientific, Waltham, MA USA), which results in blunt-ended amplicons, and primers with engineered restriction sites (underlined) KpnToxFor (5′-CTCTACTGCGCATGTAATTTCGACG-3′) and ClaToxRev (5′-GATAATCGAATTCTAAATGCTGACG-3′). The 1000 bp PCR product was cut with KpnI and ClaI and fused with KpnI/ClaI-digested pBluescript SK+, and designated as pDD849. The second arm of the construct was made by amplifying 86-028NP gDNA using the primers 86APstFor (5′-GTGATTCTAAATGCTGACG-3′) and 86XbaVapARev (5′-GTGATTCTAAGAGATCTCAACCGACG-3′). The XbaI-digested 883 bp amplicon was ligated to pDD849 digested with Smal and XbaI, resulting in pDD851. A 1200 bp product which contained a spectinomycin resistance cassette was PCR-amplified from pDD872 using the primer set pUC4 For (5′-TTCGCTATTACGCCAGCTGG-3′) and pUC4 Rev (5′-GCCGATTCATTAATGCAGCTG-3′), and pUC4 Rev (5′-GATAATCGAATTCTAAATGCTGACG-3′), cut with EcoRI and ligated to pDD851 digested with EcoRI, generating pDD857. A 3083 bp fragment from pDD857 was amplified with KpnI ToxFor and 86XbaVapARev primers and used to transform NTHi strain 86-028NP with the spectinomycin resistance cassette. This work resulted in pDD857 pDD851 with the spectinomycin resistance cassette. This work

Growth Curves of the ΔtoxAvapA Mutant and the Parent Strain

The 86-028NP parent strain or the ΔtoxAvapA mutant were re-suspended from sBHI agar plates grown for 18 hours at 37°C in 5% CO2 into fresh defined media [16] at an OD600 of ~0.1, then 200 microliters of each re-suspension was placed in duodecuplicate into a sterile non-treated flat-bottomed 96 well plate (#351172, BD Biosciences, Bedford, MA, USA). Empty wells were filled with 200 microliters of sterile water to decrease evaporation, and the plate was covered with sterile gas permeable sealing film (#9123-6100, USA Scientific, Ocala, FL, USA). The plate was incubated for 15 hours with shaking at 35°C in a Multiskan FC spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and the OD530 was read hourly. Three biological replicates were performed and analyzed by the repeated measures analysis of variance (RMANOVA).

NTHi Invasion Assays in the EpiAirway Tissue Model

EpiAirway tissues (MatTek, Ashland, MA USA) were inoculated with either 10⁷ NTHi strain 86-028NP (n = 6) or ΔtoxAvapA mutant (n = 6) suspended in pre-warmed Dulbecco’s phosphate-buffered saline with calcium and magnesium (DPBS⁺) in a total volume of ≤25 microliters onto the apical surface. Tissues were incubated at 37°C in 5% CO₂ until harvest at day 2, 4, or 8 after infection. Tissues were maintained by washing the apical surface with 200 µl of DPBS⁺ and the basal media was changed using 1 ml of MatTek airway serum-free medium on a daily basis. On the day of harvest, inserts were washed with DPBS⁺ three times, and then 250 µl of RPMI 1640 supplemented with 100 µg/ml gentamicin (MP Biomedical, Solon, OH USA) was added to the apical side, with 1 ml added to the basal side to kill any external cell-associated bacteria. After incubation at 37°C in 5% CO₂ for one hour, each insert was washed three times with DPBS without calcium and magnesium (DPBS⁻) and 250 µl of sterile 1% saponin (Sigma-Aldrich, St. Louis, MO USA) in DPBS⁻ was added to the apical surface. The inserts incubated at 37°C with 5% CO₂ for 10 minutes. The tissue was then physically disrupted from the membrane and collected followed by the addition of 250 µl DPBS⁻ to the apical surface and the remaining tissue was collected. Total tissue collection was confirmed by microscopy of the insert. The volume of the collected tissue suspension was increased to 1 ml with DPBS⁻. Each sample was then vortexed vigorously to de-aggregate the cells and serially diluted using sterile PBS. Aliquots were drop-plated onto chocolate agar plates to enumerate viable internalized bacteria.

Table 1. Bacterial strains and plasmids used in this study.

| Strain         | Description                                                                 | Source         |
|----------------|------------------------------------------------------------------------------|----------------|
| DH5α           | F−Φ80lacZ M15Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 Δ− thi-1 gyrA96 relA1 | Lab collection |
| BL21(DE3)      | F−ompT hsdS37/6 (mB−) gal dcm (DE3)                                          | NEB            |
| 86-028NP       | Nontypeable Haemophilus influenzae strain isolated from the nasopharynx of a child being treated for chronic otitis media | (14)           |
| ΔtoxAvapA      | Strain 86-028NP with the toxAvapA locus deleted                               | This work      |
| Plasmid        | Description                                                                 | Source         |
| pET24b         | Bacterial vector for expressing polyhistidine-tagged proteins                | Novagen        |
| pBluescript SK+| Bacterial cloning vectors                                                   | Lab collection |
| pDD849         | pBluescript SK+ with the 5′ arm of the toxAvapA plasmid.                     | This work      |
| pDD851         | pDD849 with the 3′ arm of the toxAvapA plasmid.                              | This work      |
| pDD857         | pDD851 with the spectinomycin resistance cassette.                           | This work      |
| pDD912         | pET24b with the toxAvapA locus for protein expression.                       | This work      |

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Survival of NTHi in the Chinchilla Otitis Media Model

**Ethics Statement.** Guidelines published in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health were followed for all animal handling and husbandry. The protocol #A1110011 was approved by the Mercer University Institutional Animal Care and Use Committee (Animal Welfare Assurance Number: A3725-01). All surgery was performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

Adult female chinchillas (400–600 g) were purchased from a commercial supplier and allowed to acclimate in the vivarium for one week prior to bacterial challenge. On the day of challenge, each animal was examined by otoscopy for signs of middle ear infection. Both the wild-type and the ΔtoxAvapA mutant strain from frozen stocks were plated on chocolate agar and incubated at 37°C with 5% CO₂ for 18 h. Sterile DPBS with 0.1% gelatin (Sigma-Aldrich, St. Louis, MO USA) (DPBS-G) was inoculated with the bacteria to a concentration of 1.0×10⁶ CFU/ml and 100 μl of this suspension was loaded into 1 cc syringes. After chinchillas were anesthetized by isoflurane inhalation, 100 μl (~10⁵ CFU) of bacteria was injected into the superior bullae of each animal (n = 4 animals with 8 middle ears per challenge strain). On day 4 post-infection, both the wild-type and the ΔtoxAvapA mutant were grown in 2 ml of this media to a concentration of 1.0×10⁷ CFU/ml and 100 μl of this suspension was injected into the middle ear of each animal. Five milliliters of each culture was pelleted and the supernatants were passed through 3 kDa MWCO filters (Vivaspin 700, GE Healthcare, Pittsburgh, PA USA). HPLC was done on pooled filtrates representing three replicates of each strain using a Waters model 680 automated gradient controller, two model 510 pumps, a Rhodyne model 7725i manual injector (Waters, Milford, MA USA) and a Shimadzu model SPD-M10A photodiode array detector (Shimadzu Instrument Co., Columbia, MD USA). The column was a Sorbax Eclipse AAA column, 150 mm×4.6 mm i.d. reversed phase C-18 stainless steel column containing 3.5 μm size silica particles (Agilent Technologies, Santa Clara, CA USA). The solvents used were (A) 40 mM NH₄H₂PO₄ solution (10.4 pH) and (B) acetonitrile: methanol, 60:40. All solvents were HPLC grade. Run conditions were: 95% A:5% B for 5 min; 95% A:5% B at 40 min; and, finally, return to initial conditions after 45 min [17]. Flow rates were 0.75 ml/min. Authentic tryptophan (Sigma Aldrich, St. Louis, MO USA) was suspended in sterile distilled water at 5 mg/ml. Pre-column derivatization was done with o-phthalaldehyde/mercaptoethanol (OPA) (Sigma Aldrich, St. Louis, MO USA). Samples ranging from 5, 10, 25 or 50 μg of authentic tryptophan were injected into the HPLC system to determine elution times and spectral characteristics for this amino acid standard (and distinct from background peaks due to OPA alone, which were excluded). Subsequently, samples of conditioned media from NTHi wild-type and mutant were derivatized, injected into the HPLC and the elution times, spectral characteristics and differences in the amounts (milli-absorbance units) of the putative sample tryptophan peak were determined. To confirm the identity of the elution times, several samples of the conditioned media also were spiked with authentic tryptophan. Tryptophan-specific peaks were collected using a Pharmacia Frac-100 fraction collector (Amersham Biosciences, Piscataway, NJ USA), lyophilized in a FreeZone Plus 2.5 L lyophilizer...
(Labconco, Kansas City, MO USA) and resuspended in sterile distilled water. The lyophilized fractions were then assayed by thin layer chromatography (TLC) using 10 × 20 cm silica gel/TLC cards (Sigma Aldrich, St. Louis, MO USA) and eluted for 2 h with n-butanol-acetic acid/water 3:1:1 (by volume). The dried card was then examined by UV fluorescence at 254 nm and stained with ninhydrin (0.1 g ninhydrin, 100 ml n-butanol, 0.5 ml acetic acid) followed by gentle heating. Trypsin was added to the fractions confirmed by co-elution with the authentic standard.

ToxA and VapA Protein Expression and Purification

The toxAvapA operon from NTHi strain 86-028NP was cloned into pET24b by amplifying gDNA with the following primer pair, 86ToxRamFor (5'-GAGGATCCGGATC-3') and ASacRev (5'-GGCTAGCTACATTGGCATTG-3'). The PCR product was cut with BamHI and SacI and fused with SacI and BamHI-digested pET24b generating pDD912. Following DNA sequencing, the plasmid pDD912 was transformed into E. coli BL21 (DE3) strain and grown in 30 ml of LB broth supplemented with 30 μg/ml kanamycin with shaking in baffled flasks at 35°C. When the OD600 reached ~0.4, 1 mM IPTG was added and the culture was incubated for an additional 3 hours. Ten ml cell pellets were isolated, subjected to 3 freeze/thaw cycles, resuspended in 5 ml of BugBuster solution (1X BugBuster, 100 mM HEPES, 1X Halt EDTA-free protease inhibitor, 10 μl DNase) and rotated at room temperature for 30 minutes. The solution was then sonicated with 1.5 second pulses at 10% power for 12 cycles using a Branson Sonifier (Branson Ultrasonics Co. Ltd, Shanghai, P.R. China) equipped with a microtip. The resulting lysate contained the ToxAvapA protein complex. The MagneHis™ protein purification system (Promega Corp., Madison, WI USA) was used to bind polyhistidine-tagged VapA with the following modifications. The lysate with the ToxAVapA complex bound to Ni²⁺ magnetic beads was washed twice with wash buffer (100 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM imidazole). The bead pellet was then resuspended in 200 μl of denaturing buffer (100 mM HEPES (pH 7.5) and 8 M urea) and incubated at room temperature for 15 min on a rotator. The supernatant was collected and removed, representing purified denatured ToxA protein. The polyhistidine-tagged VapA protein was then recovered from the Ni²⁺ beads with denaturing elution buffer (100 mM HEPES, 8 M urea and 500 mM imidazole) according to the manufacturer’s protocol. Protein concentrations of both ToxA and VapA were determined by Bradford assay and protein isolation was confirmed by SDS-PAGE followed by staining with GelCode Blue (Thermo Scientific Inc., Pittsburgh, PA USA).

To re-nature the purified proteins, two hundred microliter aliquots of denatured ToxA or VapA were placed into individual Slide-A-Lyzer® Mini Dialysis 3,500 Da MWCO tubes (Thermo Scientific Inc., Pittsburgh, PA USA) and dialyzed for 18 hours at 4°C against 25 ml of either acidic refolding buffer (50 mM Na acetate (pH 4.0), 100 mM KCl, 200 mM arginine, 50 mM NDSB-201 (3-(1-Pyridin-1-yl)-1-propanesulfonate) and 50 mM NDSB-256 (Dimethylbenzylammonium propane sulfonate) for ToxA (pI 9.1), or alkaline refolding buffer (50 mM Tris (pH 8.0), 100 mM KCl, 200 mM arginine, 50 mM NDSB-201 (3-(1-Pyridin-1-yl)-1-propanesulfonate)) and 50 mM NDSB-256 (Dimethylbenzylammonium propane sulfonate) for VapA (pI 6.7) [18]. These buffers were then replaced with fresh dialysis buffer containing 10% glycerol, and dialyzed at 4°C for an additional 4 h. The re-natured proteins were concentrated using 3.0 kDa MWCO centrifugal filters (Vivaspin 300, GE Healthcare, Pittsburgh, PA USA) and assayed via the Bradford method to determine total protein concentration.

RNase Activity Assays

The RNaseAlert® substrate (IDT, Coralville, Iowa USA) was used to determine re-natured ToxA and VapA RNase activity. ToxA and VapA were diluted to 0.05 mg/ml in 1X RNaseAlert proprietary buffer and acclimated to room temperature for 30 min. Eighteen pmol of each protein dilution was added to 25 μl RNaseAlert substrate in triplicate wells of a black clear-bottomed 96-well plate (Corning #3340, Tewksbury, MA USA) and incubated at 37°C for 1 h. The negative control was an identical volume of protein renaturation buffer, and the positive

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Table 2. Number of viable gentamicin-resistant internalized bacteria that survived over time in the EpiAirway human tissue model (n = 6).

| Day | CFU/ml WT (±SD) | CFU/ml ΔtoxAvapA (±SD) | p value | % WT |
|-----|-----------------|------------------------|---------|-----|
| 2   | 8.9E+05 (1.6E+06) | 6.2E+04 (5.9E+04) | 0.047   | 7.0 |
| 4   | 7.3E+05 (4.2E+05) | 4.3E+05 (1.3E+05) | 0.038   | 58.9|
| 8   | 2.5E+06 (1.4E+06) | 9.5E+05 (6.8E+05) | 0.014   | 38.2|

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Figure 2. Viable wild-type and ΔtoxAvapA mutant CFU/ml recovered from chinchilla middle ears after 4 days of infection. Asterisks denote outliers; p = 0.01; n = 8 ears. doi:10.1371/journal.pone.0091523.g002

Figure 1. Growth curve of the ΔtoxAvapA mutant and parent strain in defined media. No significant difference was found between the growth of the parent and mutant strains (n = 3 in duodecuplicate). doi:10.1371/journal.pone.0091523.g001
control was 18 pmol bovine pancreatic RNase A (Thermo Scientific Fermentas, Pittsburgh, PA USA). Fluorescence (excitation 485 nm, emission 520 nm) was measured using a FLUOstar OPTIMA plate reader (BMG Labtech, Ortenberg, Germany).

ToxA Kinetic Studies
To determine the initial reaction progress, increasing amounts of the RNaseAlert substrate (5, 10, and 12.5 pmol) were incubated with 18 pmol of renatured ToxA in a final volume of 30 microliters. The reactions were incubated at 37°C in a MiniOpticon real-time thermocycler (Bio-Rad, Hercules, CA USA) and fluorescence was measured every 30 seconds for the first 6 minutes. The time zero measurement was subtracted from each well. A separate set of reactions using 5 pmol RNaseAlert substrate and 18 pmol renatured ToxA were incubated at 37°C in a MiniOpticon and fluorescence was measured every 30 seconds for 30.5 minutes to allow the reaction to approach completion.

Data Analysis
All data are presented as the mean ± standard deviation. The significance of any differences between means was determined using Student’s t test, analysis of variance (ANOVA) followed by the Tukey-Kramer test, or repeated measures ANOVA (RMANOVA). A p value of ≤0.05 was considered statistically significant.

Results and Discussion
Growth Dynamics in Defined Media
We deleted the entire toxAvapA operon in NTHi strain 86-028NP, and following confirmation of the deletion, we performed growth curves in defined media to determine whether there were any growth defects in the ΔtoxAvapA mutant as compared to the parent strain. We found that there were no significant differences by repeated measures ANOVA between the growth of the wild-type versus the mutant over a 15 hour time period in defined media (Figure 1). These data suggest that any observed differences in survival of the mutant in vitro or in vivo would not be attributable to a fundamental reduction in the organism’s ability to replicate over the growth cycle.

Co-culture in Primary Human Tissues
NTHi normally reside in the human upper respiratory tract in close association with the respiratory epithelium. To determine any effects of the toxAvapA deletion on the ability of NTHi to survive during long-term co-culture with primary human respiratory epithelial tissues, we used the EpiAirway™ model (MatTek, Ashland, MA USA). These 3-dimensional, highly differentiated tissues from normal human donors are metabolically and mitotically active and have the capacity to survive at the air-liquid interface for weeks. Further, the proprietary media supplied with the tissues is serum-free. This allowed us to perform long-term co-culture of these tissues with both the wild-type and the ΔtoxAvapA mutant. We inoculated tissues with 1.0 × 10⁶ CFU of each strain, and at days 2, 4, and 8 post-infection, we recovered the viable gentamicin-resistant (internalized) bacteria that persisted within the tissues over time (Table 2).

In all cases, the ΔtoxAvapA mutant was attenuated for survival compared to the parent strain. This trend was significant at all time points, but the observed effect of the toxAvapA deletion was most substantial at the first harvest (2 days post-inoculation), at which only 7.0% of the wild-type numbers were recovered. This percentage increased at day 4 to 38.9% but declined again by day 8 to 38.2%, indicating that the mutant was unable to survive at the levels of the wild-type strain over the entire experimental period. This is the first report, to our knowledge, of the contribution of toxAvapA to the survival of NTHi in primary human tissues.
Chinchilla Model of Otitis Media

Because we noted a significant difference in the ability of the ΔtoxAΔvapA mutant to survive during long-term co-culture in human tissues, we performed challenge studies using the chinchilla model of otitis media. For these assays, four animals (8 ears each) were inoculated through the superior bullae with either the wild-type strain or the ΔtoxAΔvapA mutant, with the same strain inoculated into both ears. Four days after infection, the bullae were opened and lavaged, and dilutions of the lavage fluid were dropped onto chocolate agar plates and incubated for 24 hours at 35°C in 5% CO₂. Figure 2 shows a boxplot of the difference between the recovery of the mutant versus the wild-type strain during in vivo infection. Asterisks denote outliers, defined as ≥1.5 × the interquartile range [19]. Similar to the in vitro results, the ΔtoxAΔvapA mutant displayed significantly diminished recovery from the chinchilla middle ear, indicating that this TA locus contributes to NTHi survival during infection.

Otoscopic images of the tympanic membrane of an animal before (A) and 4 days after inoculation with the ΔtoxAΔvapA mutant (B) are shown in Figure 3. Swelling and redness can be observed around the membrane, suggesting inflammation. An average of 1.5 × 10^3 viable CFU/ml was obtained from a lavage of this ear. To confirm a productive infection, the bulla of this animal was step-sectioned and mounted onto slides. Images of hematoxylin-eosin (H&E)-stained sections of the middle ears of three animals are shown in Figure 4. Note the characteristic goblet cell hyperplasia and edema observed during NTHi infection (ΔtoxAΔvapA, 4B and wild type, 4C) compared to the uninfected control animal (4A). This indicates that the ΔtoxAΔvapA mutant strain was capable of producing an infection, even though it was less able to survive in vivo than the wild-type parent.

Microarray Analysis

In order to identify any effects on the transcriptome of deleting the toxAvapA TA locus in NTHi, we performed microarray analysis on total RNA from both the wild-type and the mutant strain grown in defined media to an OD₆₀₀ of ~0.4. Interestingly, we found a significant (defined as greater than 2-fold) reduction in the transcription of the tryptophan biosynthesis regulon (Table 3). We confirmed this microarray data by qPCR of NTHI1763 (trpCF), a key gene in the tryptophan biosynthesis pathway. However, we did not observe significant differences between the wild-type and mutant strains in the transcription of the tryptophanase and tryptophan permease genes trnA and trnB (NTHI0396), the tryptophanyl-tRNA synthetase trpS (NTHI0755), the Trp operon repressor trpR (NTHI0996) or the high-affinity tryptophan-specific transport protein gene ntr (NTHI0396). This suggested that transcription of the biosynthetic regulon was specifically affected. To further investigate these results, we performed HPLC studies on conditioned medium of both the wild-type and mutant strains to determine the levels of tryptophan remaining in the media. All ≥2-fold differentially-regulated genes identified in the ΔtoxAΔvapA mutant and their fold changes are listed in Supplemental Table 1.

Table 3. Fold decrease in transcription of the tryptophan biosynthesis regulon in the NTHi ΔtoxAΔvapA mutant compared to the wild-type strain.

| Gene                  | Fold Decrease | Description                                                                 |
|-----------------------|---------------|-----------------------------------------------------------------------------|
| NTHI1701              | 2.591         | Tryptophan synthase alpha chain; TrpA                                       |
| NTHI1702              | 2.150         | Tryptophan synthase beta chain; TrpB                                        |
| NTHI1763              | 6.183         | Tryptophan biosynthesis protein; TrpCF                                      |
| NTHI1764              | 3.442         | Anthranilate phosphoribosyltransferase; TrpD                                |
| NTHI1767              | 3.574         | Glutaminase amidotransferase; TrpG                                          |
| NTHI1768              | 3.068         | Anthranilate synthase component 1; TrpE                                     |

Table 4. HPLC results of the relative amounts of tryptophan in derivatized samples of conditioned medium from the wild-type or mutant NTHi strains.

| Strain            | Micrograms of tryptophan in sample (±SD) | p value |
|-------------------|------------------------------------------|---------|
| 86-028NP          | 0.315 (0.003)                            | >0.05   |
| ΔtoxAΔvapA        | 0.365 (0.049)                            | (not significant) |

Figure 5. Ribonuclease activity assays using purified ToxA and VapA proteins. Eighteen pmol of ToxA, VapA and the positive control RNase A were incubated at 37°C for one hour with 5 pmol of RNaseAlert substrate. The fluorescence of the negative control (buffer alone) was subtracted from each value. (n=3, RFU = relative fluorescence units). doi:10.1371/journal.pone.0091523.g005
These results indicate that the observed decrease in the transcription of the tryptophan regulon in the ΔtoxAΔvapA mutant was not due to increased transport of this amino acid into the mutant strain. This is further supported by our findings that there were no significant differences in the transcription of genes active in the degradation or transport of tryptophan in the mutant as compared to the wild-type strain. Since HigA antitoxin homologues in other organisms have not been found to be involved in the regulation of genes other than their own operons [21], it is not clear why the NTHi ΔtoxAΔvapA mutation results in decreased tryptophan regulon transcription.

RNase Activity Assays

To determine if the HigB homologue in NTHi could cleave free RNA, we purified both the toxin (ToxA) and antitoxin (VapA) proteins and subjected each to ribonuclease activity assays. This assay consists of a commercially-available RNA substrate that has a quencher on one end and a fluorophore (FAM) on the other (RNaseAlert®, Integrated DNA Technologies, Cora lville, IA USA). The intact substrate is not fluorescent, but when cleaved it emits a bright green fluorescence. The probe is a single RNA moiety of a proprietary length and sequence. RNA cleavage is monitored by fluorescence intensity measured on the fluorescein channel (485 nm excitation, 520 nm emission) that increases over time. Figure 5 shows the results of 5 pmol of substrate incubated at 37°C for one hour with 18 pmol of purified ToxA and VapA. The negative control (protein renaturation buffer) was subtracted from each value, and the positive control was 18 pmol of commercially-available RNase A. To further characterize the ribonuclease activity of ToxA, kinetic studies were performed in which the concentration of the RNaseAlert substrate was increased while keeping the concentration of ToxA in each reaction at 18 pmol. Figure 6A illustrates the initial reaction progress in the first 6 minutes with increasing concentrations of substrate, while Figure 6B shows the reaction approaching completion at 30.5 minutes (5 pmol RNaseAlert; 18 pmol ToxA).

The HigB toxin from P. vulgaris has been identified as having ribonuclease activity on mRNA in the context of a ribosome at A-rich sites in E. coli [22], and here we show that ToxA from NTHi displays activity on RNA that is unbound in solution. This is consistent with our previous results, in which we have shown that two other TA loci toxins in NTHi, VapC-1 and VapD, are active on free RNA in vitro [13,23]. Other studies have found that induced ectopic expression of the HigB toxin in M. tuberculosis lead to growth arrest and cell death as well as cleavage of tmRNA and mRNA predominantly from genes regulated by the IdeR iron-dependent repressor and the zinc uptake repressor Zur [24]. However, the HigB cleavage sites identified in the M. tuberculosis tmRNA ssrA in that investigation were not particularly A-rich. This suggests that there may be a broader range of RNA targets of the HigB toxin homologues in different organisms than originally thought. If this is true, the observed repression of the tryptophan biosynthetic regulon in the ΔtoxAΔvapA strain might be due to the loss of the native function of ToxA on other targets, rather than directly on the regulon itself. Our data indicate that this loss does not influence growth in defined media, but significantly impacts NTHi survival during infection both in vitro and in vivo.

Conclusions

We show that deleting the toxAvapA locus results in attenuation of NTHi survival during infection, both in vitro and in vivo. We purified ToxA and identified it as having ribonuclease activity, suggesting that the mechanism by which the toxAvapA TA locus affects persistence is to facilitate a state of dormancy via mRNA degradation. Unexpectedly, we found by microarray analysis that deleting this locus also resulted in the repression of the tryptophan biosynthetic regulon in the mutant strain. No significant difference between the ability of the wild-type and mutant strains to take up amino acids into defined media, but significantly impacts NTHi survival during infection both in vitro and in vivo.

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Supporting Information

Table S1 All genes that displayed ≥2-fold change in transcription in the ΔtoxAΔvapA mutant.

Author Contributions

Conceived and designed the experiments: DES DAD. Performed the experiments: DR AAK DES DAD. Analyzed the data: DES DAD. Wrote the paper: DES AAK DAD.
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