A Novel, Molybdenum-Containing Methionine Sulfoxide Reductase Supports Survival of Haemophilus influenzae in an In vivo Model of Infection

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Haemophilus influenzae is a host adapted human mucosal pathogen involved in a variety of acute and chronic respiratory tract infections, including chronic obstructive pulmonary disease and asthma, all of which rely on its ability to efficiently establish continuing interactions with the host. Here we report the characterization of a novel molybdenum enzyme, TorZ/MtsZ that supports interactions of H. influenzae with host cells during growth in oxygen-limited environments. Strains lacking TorZ/MtsZ showed a reduced ability to survive in contact with epithelial cells as shown by immunofluorescence microscopy and adherence/invasion assays. This included a reduction in the ability of the strain to invade human epithelial cells, a trait that could be linked to the persistence of H. influenzae. The observation that in a murine model of H. influenzae infection, strains lacking TorZ/MtsZ were almost undetectable after 72 h of infection, while ~3.6 × 10³ CFU/mL of the wild type strain were measured under the same conditions is consistent with this view. To understand how TorZ/MtsZ mediates this effect we purified and characterized the enzyme, and were able to show that it is an S- and N-oxide reductase with a stereospecificity for S-sulfoxides. The enzyme converts two physiologically relevant sulfoxides, biotin sulfoxide and methionine sulfoxide (MetSO), with the kinetic parameters suggesting that MetSO is the natural substrate of this enzyme. TorZ/MtsZ was unable to repair sulfoxides in oxidized Calmodulin, suggesting that a role in cell metabolism/energy generation and not protein repair is the key function of this enzyme. Phylogenetic analyses showed that H. influenzae TorZ/MtsZ is only distantly related to the Escherichia coli TorZ TMAO reductase, but instead is a representative of a new, previously uncharacterized clade of molybdenum enzyme that is widely distributed within the Pasteurellaceae family of pathogenic bacteria. It is likely that MtsZ/TorZ has a similar role in supporting host/pathogen interactions in other members of the Pasteurellaceae, which includes both human and animal pathogens.

Keywords: molybdenum enzymes, Haemophilus influenzae, methionine sulfoxide reductase, host–pathogen interaction, DMSO reductase enzyme family
INTRODUCTION

An emerging aspect of bacterial pathogenesis that is receiving increased attention is the role of metabolic interactions between the host and pathogen (Rhee et al., 2011; Bliska and Van Der Velden, 2012; Grubmüller et al., 2014; Heroven and Dersch, 2014; Hofreuter, 2014; Othman et al., 2014). We have previously analyzed the metabolic properties of Haemophilus influenzae (HI), a host adapted human pathogen of the family Pasteurellaceae that causes or contributes to a diverse array of upper and lower respiratory tract infections (Eldere et al., 2014; Othman et al., 2014). As is typical for a host-adapted pathogen, about 60–80% of healthy children and a high percentage of adults are asymptomatic carriers of HI (Mukundan et al., 2007). At the same time HI is one of the most common pathogens contributing to chronic and acute otitis media, as well as diseases of the lower respiratory tract such as chronic obstructive pulmonary disease (COPD), asthma and pneumonia (Wood et al., 2010; Essilfie et al., 2011, 2015; Tay et al., 2015).

Our previous analyses indicated that strain- as well as niche-specific factors including varying oxygen tension influence the metabolite profile of HI strains and also alter gene expression profiles, especially in genes involved in central carbon metabolism and the respiratory chain (Othman et al., 2014). These results suggested that the energy generation processes in HI provide the adaptability required for specific niches, e.g., during infection of the middle-ear or in biofilms, which would be mostly anaerobic, or for colonization of the more aerobic environment of the nasopharynx and respiratory tract, while specific, strain related adaptations may confer the ability to cause disease in these particular body niches.

A gene showing particularly striking changes in expression between the HI RDKW20 (HIRD) laboratory reference strain (Fleischmann et al., 1995) and the non-typeable COPD clinical isolate strain HI 2019 (Campagnari et al., 1987) (HI2019) was the torZ gene, that encodes a putative trimethylamine-N-oxide (TMAO) reductase. In both HIRD and HI2019 strains expression levels of torZ were maximal under anaerobic conditions but in HI2019 the observed levels of expression were significantly higher than in HIRD (Othman et al., 2014). A gene encoding a distantly related, S- and N-oxide reducing enzyme, dmsA, did not show increased expression in the HI2019 strain compared to HIRD (Othman et al., 2014). The torZ-encoded enzyme belongs to the DMSO reductase family of mononuclear molybdenum enzymes and these enzymes have been shown to have important roles in a variety of pathogenic bacteria. For example, a gene knockout in a tetrathionate reductase family of mononuclear molybdenum enzyme that converts methionine sulfoxide (MetSO) as its preferred substrate, but appears to be unable to reduce S-oxides, while all combined S- and N-oxide reductases contain physiological role of TorYZ in E. coli is still unknown.

Here we report the characterization of the HITorZ enzyme and its effects on virulence and host–cell interactions. We were able to demonstrate that TorZ from HI is a novel type of molybdenum enzyme that converts methionine sulfoxide (MetSO) as its preferred substrate, but appears to be unable to repair MetSO damage in proteins. A torZ gene knockout showed reduced biofilm formation and in biofilm survival in vitro, as well as impaired host cell interactions and reduced survival in a murine model of infection, clearly indicating a role for TorZ in host–pathogen interactions.

MATERIALS AND METHODS

Growth of Bacterial Strains

HI 2019 wild-type (HI2019WT) (Campagnari et al., 1987) and derivatives of this strain were cultivated on supplemented brain heart infusion (sBHI) agar (Becton Dickinson) that contained 10 µg/mL hemin (Johnston, 2010) and 10 µg/mL β-NAD at 37°C with 5% CO₂. A chemically defined growth medium (sRPMI) was also used and contained 25 mM HEPES, pH 7.2.
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7.3, 0.8 mM sodium pyruvate, 0.08 mg/mL uracil, 0.17 mg/mL Haemophilus influenzae, 0.2 mM benzyl viologen, 0.3 mM sodium dithionite and one in 20 mM sodium phosphate buffer (pH 6.8) containing.

Weiner, 1979). Enzyme assays were performed anaerobically monitoring the oxidation of reduced benzyl viologen radical (kan) (100 µM E. coli; 10 µM Hl). S(-)-biotin sulfoxide and S(S)-biotin sulfoxide (R-BSO and S-BSO), and 20 mM TMAO. For kinetic assays the concentrations of the substrates were varied. The diastereomers S(R)-biotin sulfoxide and S(S)-biotin sulfoxide were prepared as described in Melville (1954) and Melville et al. (1954) and their isomeric purity (>95%) was confirmed by 1H NMR.

Specific enzyme activities are given as µmoles of substrate reduced per min (U) and mg of protein present, kinetic data were fitted using either Sigma Plot 12 (Systat) or Prism 6.0 (GraphPad).

**Construction and Complementation of a HI2019torZ Strain**

Two DNA fragments (~1200 bp each) covering the torZ gene were amplified from HI2019 genomic DNA and primer pairs (i) HI_torZ_extF and HI_torZ_intR and (ii) HI_torZ_extR and HI_torZ_intF (Table 1). The obtained fragments were digested with BamHI before being used in a three way ligation with pGEMT-easy (Promega) to create pGEM-torZ. The kanamycin (kan) resistance cassette was amplified from the pUC-4K plasmid (Vieira and Messing, 1982) using primers Kan BamHI-F and Kan BamHI-R (Table 1) followed by insertion into the pGEM-torZ BamHI site yielding pGEM-torZ:kan. The resulting plasmid was linearized using ScaI and transformed into competent HI2019 using the method described in Poje and Redfield (2003) generating HI2019torZ (genotype: HI2019 Kan torZ:kan) following selection on sBHI 20 µg/mL kan agar plates. The inactivation of torZ was confirmed by PCR.

To complement the HI2019torZ mutant, the torYZ operon (4450 bp) was amplified using primers HI2019torZcomp_Xma_F and HI2019torZcomp_Xma_R (Table 1) and cloned into p601.1-Sp2 (Johnston et al., 2007) using the Xmal site. The plasmid was

### Table 1 | Sequences of oligonucleotide primers used in this study.

| Primer name | Sequence |
|-------------|----------|
| Kan-BamHI-F | 5′-AAAAA GGA TCC GGA AGG CCA CGT TGT GTC-3′ |
| Kan-BamHI-R | 5′-AAAAA GGA TCC CTG AGG TCT GGC TCG TGA-3′ |
| HI_torZ_extF | 5′-TAACGCCACCTGTTAAGG-3′ |
| HI_torZ_extR | 5′-ATGGGAAAAATGATCGTAAA-3′ |
| HI_torZ_intF | 5′-AAAAAGATCCCGGTGGGAAAAACATCGTGGT-3′ |
| HI_torZ_intR | 5′-AAAAAGATCCCGGTGGGAAAAACATCGTGGT-3′ |
| HI2019torZcomp_Xma_F | 5′-AAACCCGGGGGACATATAAACGCAGG AGA-3′ |
| HI2019torZcomp_Xma_R | 5′-AAACCCGGGGGACATATAAACGCAGG AGA-3′ |
| Hl_bisc-SP_pPro_Bam_F | 5′-AAAGAGATCCAAAGAAGGCTGAAATGAAA |
| Hl_bisc-SP_pPro_Xba_R | 5′-AAATATGCTAATTACCGCAGTTAGG-3′ |
| Hl_torZtorY R | 5′-CCTTTATGCCCCACAAAACCACC -3′ |
| Hl_torZtorY F | 5′-GTCAAGGGCGATCTTCGTGATTGTTG-3′ |
| HI2019_Qp_gyrAF | 5′-GCGATCATTTACTGGACCGAG-3′ |
| HI2019_Qp_gyrAR | 5′-GCCAACACGGGCGATTTTAC-3′ |

1www.doubling-time.com, (accessed 23/07/2016)
linearized using BamHI and transformed as described above, and H12019ΔtorZ_comp selected on sBHI plates containing 20 µg/mL kanamycin and 50 µg/mL spectinomycin. Correct integration of the construct was confirmed by PCR.

**Construction of a TorZ Overexpression Plasmid**

A pProexHb (Invitrogen) based protein expression plasmid containing the torZ gene without the region coding for the signal peptide was constructed using primers HlibisC-SP_pPro_Bam_F and HlibisC_pPro_Xba_R (Table 1) and the restriction enzyme sites introduced during high fidelity PCR. The resulting ligation products were transformed into DH5α (Life Technologies) to obtain pProex HItorZ-SP, which was verified by DNA sequencing.

Optimal expression of the recombinant TorZ (rTorZ) protein was obtained using pProex HItorZ-SP in E. coli DH5α and LB medium supplemented with 1 mM sodium molybdate and 100 µg/mL ampicillin. Overexpression cultures (100 mL supplemented LB in a 250 mL shake flask) were inoculated from overnight cultures to an OD600 of 0.6–0.8, followed by incubation with shaking at 37°C, with shaking at 200 rpm until an OD600 of 0.6–0.8 was reached. IPTG was added to a final concentration of 100 µM and the cultures incubated at 30°C, 200 rpm overnight before harvesting of cells by centrifugation (3000 × g, 4°C, 10 min). Cell pellets were stored at −20°C.

**Purification of Recombinant TorZ**

Recombinant TorZ (rTorZ) was purified from 4 L of cell culture by a French Press (Aminco) at 15000 psi. Imidazole was added to the expression culture. The cell pellets were resuspended in 20 mM 50 g/mL ampicillin. Optimal expression of the recombinant TorZ (rTorZ) protein was obtained using pProex HItorZ-SP in E. coli DH5α and LB medium supplemented with 1 mM sodium molybdate and 100 µg/mL ampicillin. Overexpression cultures (100 mL supplemented LB in a 250 mL shake flask) were inoculated from overnight cultures to an OD600 of 0.6–0.8, followed by incubation with shaking at 37°C, with shaking at 200 rpm until an OD600 of 0.6–0.8 was reached. IPTG was added to a final concentration of 100 µM and the cultures incubated at 30°C, 200 rpm overnight before harvesting of cells by centrifugation (3000 × g, 4°C, 10 min). Cell pellets were stored at −20°C.

**Purification of E. coli MsrP for Calmodulin Assays**

The E. coli YedY protein has recently been proposed to be renamed to MsrP based on its ability to repair MetSO damage in periplasmic proteins, the two names refer to the same protein (Gennaris et al., 2015). E. coli strain JM109 λpir harboring the plasmid pMSYZ3 for expression of His6-Tagged MsrP and native YedZ was kindly provided by Prof. Joel Weiner (University of Alberta, Edmonton, AB, Canada). E. coli MsrP-6xHis was purified as described in Loschi et al. (2004).

**In vitro Repair of Oxidized Calmodulin (CaMox)**

Calmodulin (CaM) (Astralscientific, Cat.N. 05-0103-2) was decalcified by dissolving in 50 mM Hepes, pH 7.5, 10 mM EDTA (CaM conc.: 150 µM), followed by treatment with 50 mM H2O2 for 4 h at room temperature. H2O2 was removed by gel filtration through PD MiniTrap G-10 (GE Healthcare) (Grimaud et al., 2001; Tsvetkov et al., 2005). CaM repair assays used 4 µM of purified enzyme (rTorZ or MsrP) in 250 µL containing oxidized CaM, 4 µM, 10 mM benzyli viologen and an excess of sodium dithionite (2 mM). Samples were incubated anaerobically at 37°C for an hour. Control reactions used both proteins, but without benzyliviolgen and dithionite. Sodium dithionite was removed from samples by four consecutive dilutions with 50 mM Hepes, pH 7.5 and concentration using Amicon® Ultra-3K (Millipore) followed by SDS-PAGE (17.5%) analysis.

**Quantitative RT-PCR (qRT-PCR) and torYZ Cotranscription Analysis**

Quantitative RT-PCR (qRT-PCR) was performed essentially as described in (Othman et al., 2014) with three biological replicates for each culture condition. RNA was isolated from 2 mL of HI cultures (anaerobic: OD600 nm = 0.4, aerobic and microaerophilic: OD600 nm = 0.8) preserved in RNAProtect bacteria (Qiagen). gDNA was removed using the Turbo DNA-free™ Kit (Life Technologies), cDNA was synthesized using 500 ng of RNA for the combined biological replicate samples using Superscript III (Life Technologies) and random hexamer primers (Life Technologies). RNA concentrations were determined using the Quant-IT RNA kit (Life Technologies). For RNA isolation from co-cultures planktonic cells were preserved directly in RNAProtect. RNA from adherent and internalized HI was preserved in RNAprotect following harvesting and water lysis of the epithelial cells with subsequent collection of the bacteria by centrifugation. qRT-PCR reactions (10 µL) used diluted cDNA (1:100–1:1000) as template, SYBR green Master Mix (Applied Biosystems) and primers described previously (Othman et al., 2014). The gyrA gene was used as the reference gene and data analysis and normalization was performed as in (Kappler et al., 2005). Co-transcription of the torYZ genes was tested using cDNA derived from anaerobically grown cultures using primers HItorY-torZ F and HItorY-torZ R (Table 1) and GoTaq Master Mix green (Promega). Genomic DNA was used as the positive control.

**Biofilm and Biomass Quantification Assays**

*Haemophilus influenzae* (anaerobic, microaerophilic, and aerobic condition) were grown to an OD600 nm of 0.2–0.3 in sBHI at 37°C. Cultures were diluted to an OD600 nm of 0.05, before being distributed into 96-well microtiter plates (125 µL per well) (U-bottom, polystyrene, TechnoPlas) and incubation (24 h, 37°C)
with or without shaking. Anaerobic jars were used for anaerobic incubations. For biofilm detection, planktonic cells were removed by careful washing with water, bound cells detected using crystal violet as described in Schembri and Klemm (2001). For biomass quantification, planktonic cells were removed by washing with sterile water, and the bound cells were incubated for 10 min with 200 µL 0.1 mg/mL proteinase K in 1x PBS (Izano et al., 2009). The detached bacteria were mixed thoroughly by vigorous pipetting, serially diluted in 1x PBS and plated on sBHI agar to estimate the number of colony-forming units (CFU) per well. Statistical comparisons of mean CFU/well and absorbances were performed by one-way ANOVA using the Tukey post hoc method (Prism 7 software package, GraphPad). Additional analyses were carried out using two tailed t-tests. A p < 0.05 was considered statistically significant.

**HOCl Susceptibility Assay**

The assay was carried out essentially as described by Benoit et al. (2013). HI2019̵WT and derivative strains were grown overnight on BHI agar plates, the cell material was harvested using an inoculation loop and resuspended in 1X PBS to an OD of 1.0 in sample, and samples incubated at room temperature with shaking for 60 min prior to serial dilution and determination of CFU/ml as described above. Final HOCl concentrations in samples ranged from 0.05 to 0.5 mM. Statistical comparisons of mean CFU/ml were performed by two-way ANOVA (Prism 7 software package, GraphPad). Additional analyses were carried out using two tailed t-tests. A p < 0.05 was considered statistically significant.

**Tissue Culture, Adherence and Invasion Assays and Neutrophil Killing Assays**

Human bronchial epithelial 16HBE14 cells (Gruenert et al., 1988), kindly provided by Dr. Kirsten Spann (Queensland University of Technology), were propagated in MEM–GlutamaxTM (Gibco®, Life Technologies), supplemented with 10% fetal calf serum (Gibco®, Life Technologies, Cat.-No. 16000-044) (sMEM). The cells were seeded into individual wells of 24-well culture dishes (Greiner Bio-One, Cat.-No. 662160) at an approximate density of 2*10⁵ cells/well, incubated at 37°C with 5% CO₂ until reaching confluence, then used for infection studies. Bacterial adherence and invasion was determined using a standard gentamicin-survival assay (St Gene and Falkow, 1990), as described previously (Dhouib et al., 2015). Fresh overnight cultures of HI2019̵WT, HI2019-AmobA and HI2019-AmobA-comp were prepared on sBHI-agar plates. The bacteria were resuspended in 5 mL sMEM and then used in the same culture medium to 2*10⁵ bacteria/mL. Confluent 16HBE14 monolayers in 24-well culture dishes were washed once with fresh pre-warmed sMEM and infected using a multiplicity of infection (MOI) of 1:100 (epithelial cells: HI). The infected monolayers were incubated for 4 or 24 h at 37°C with 5% CO₂, washed three times with prewarmed sMEM before sMEM containing gentamicin (50 µg/mL) was added followed by incubation for 1 h at 37°C, 5% CO₂. The monolayers were washed three times with fresh sMEM and lysed by the addition of sterile 1% (w/v) saponin in 1x PBS (pH 7.4). The epithelial cell lysates were mixed thoroughly by vigorous pipetting and serially diluted in BHI broth. Dilutions (5 µL of 10⁶–10⁸ dilutions) were plated on sBHI agar and incubated overnight to estimate the numbers of colony-forming units (CFU) per well. Experiments determining bacterial adherence and invasion were carried out in the same way but omitting the gentamicin incubation.

Neutrophil killing assays were also carried out as in Dhouib et al. (2015). Human neutrophils were isolated and purified from venous blood using the PolyMorphPrep kit (Axis-Shield) as per the manufacturer’s instructions and seeded into 96-well plates at 2*10⁵ cells/well. HI strains grown overnight on fresh sBHI-agar plates were resuspended in RPMI medium containing 2% heat inactivated autologous human plasma, diluted to 2*10⁵ CFU/mL in the same medium and then added to neutrophils at an MOI of 1:10 (neutrophils: HI) (Walker et al., 2007). Plates were centrifuged at 500 × g for 10 min then incubated at 37°C with 5% CO₂ for 2 h. After incubation, neutrophils were lysed with water, the content of each well serially diluted in BHI and plated on sBHI-agar for overnight incubation and enumeration of CFU. E. coli DH5α (Life Technologies) was used as a positive control. Percent survival of bacteria was calculated as ([CFU/ml experimental well]/[CFU/ml initial inoculum])*100. Statistical analyses were carried out with Prism7 (GraphPad) using One Way ANOVA (α = 0.05) and SIDAK’s multiple comparison test.

**Immunofluorescence Staining**

16HBE14 cells were grown to confluence on glass coverslips (13 mm, Number#1, ProSciTech), placed in 24-well plates (Greiner Bio-One, Cat.-No. 662160) and then infected with HI strains as described for adherence and invasion assays. After 4 or 24 h of incubation at 37°C with 5% CO₂, planktonic cells were removed by washing three times with 1x PBS. Epithelial cells and bacteria were then fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100 in 1x PBS for 5 min, and blocked overnight at 4°C with blocking buffer (2% BSA, 0.02% sodium azide in 1x PBS). Immunofluorescence staining of HI was performed using the primary antibody 6E4 (200 µL of 1:100 dilution) kindly provided by Prof. Michael Jennings (Institute for Glycomics, Griffith University, Australia) (Erwin et al., 2006). After 3 h of incubation at room temperature, the wells were washed three times with 500 µL of blocking buffer before addition of 200 µL of a 1:100 dilution of the secondary antibody Anti-mouse IgG (whole molecule)-FITC antibody produced in goat (Sigma–Aldrich) and incubation for 2 h in the dark. Epithelial cells were stained with CellTrackerTM Orange CMTMR fluorescent dye (Life Technologies) (200 µL of 1 µg/mL solution) for 1 h at room temperature in the dark, coverslips were mounted onto slides using ProLong® Gold antifade reagent (Life Technologies) and images were acquired using an Axiophot 2 epifluorescence light microscope (Zeiss).

**Murine Infection Assays**

Experimental animal procedures were carried out in strict accordance with the recommendations in the NSW Animal
Research Regulation 2005, and the Australian code of practice for the care and use of animals for scientific purposes of the National Health. Protocols were approved by the Animal Care and Ethics Committees of the University of Newcastle and the University of Queensland. For HI pulmonary infection, a mouse model described previously by Morey et al. (2013) was used. HI strains were grown in sBHI for 16 h at 37°C with 5% CO₂. BALB/c female mice (5–6 weeks old) were inoculated intranasally with 30 µL of a bacterial suspension containing 10⁷ CFUs. Groups of 6 mice were euthanized and necropsied at 0, 24, 48, and 72 h. To quantify the bacterial recovery, lungs were aseptically removed, homogenized in 1 mL 1x PBS and serially diluted in the same buffer. Each dilution was plated onto sBHI plate incubated overnight at 37°C with 5% CO₂ and CFUs per lung were calculated (Essilfie et al., 2011, 2012, 2015). Statistical comparisons of mean CFU/lung were performed by one-way ANOVA using the Tukey post hoc method as integrated into the Prism 6 software package. Additional analyses were carried out using two tailed t-tests. A \( p < 0.05 \) was considered statistically significant.

Phylogenetic Analysis of TorZ-Related Protein Sequences

One thousand and five amino acid sequences of proteins related to Molybdenum containing S- and N-oxide reductases from (see Supplementary Data) were retrieved from GenBank using BLASTP searches with different sulfoxide reductases as the search models. To maximize coverage of this group of proteins, initially several data sets were compiled using E. coli TorA (acc. no. P33225), Rhodobacter capsulatus DorA (acc. no. 1E61), E. coli BisC (acc. no. P20099), E. coli TorZ (acc. no. P46923) and HI TorZ (acc. no. P44798) as input sequences for database searches. Criteria for similarity were a query coverage or 80% or better combined with low e-values for the respective search models. Sequence lists were then combined and duplicates removed. Sequences were aligned using ClustalW as incorporated into the MEGA6.0 software package (Tamura et al., 2011), phylogenetic analyses (Neighbor joining, UPGMA, Minimum Evolution) and bootstrapping (500 cycles) were also carried out in MEGA 6.0.

Ethics Statement

Experimental animal procedures were carried out in strict accordance with the recommendations in the NSW Animal Research Regulation 2005, and the Australian code of practice for the care and use of animals for scientific purposes of the National Health. Protocols were approved by the Animal Care and Ethics Committees of the University of Newcastle (A-2012-211) and the University of Queensland (UN/SCMB/335/13/NHMRC). Human blood for isolation of human primary neutrophils was specifically obtained for this study from healthy donor. All donors provided informed written consent. The procedure was approved by the University of Queensland Medical Research Ethics Committee (project number 2010000491).

RESULTS

The torYZ Operon Is Conserved in H. influenzae Strains and Is Expressed under Oxygen-Limiting Growth Conditions

Haemophilus influenzae is known for its high genetic variability, however, our analyses showed that the torYZ operon is conserved in 65/80 (81%) of the available complete and partial genomes of HI strains, although in some cases one of the two genes was annotated as a pseudogene (Supplementary Table S1), possibly due to sequencing errors. The torY gene encodes a 366 amino acid (aa), membrane-bound c-type cytochrome with five heme groups related to the E. coli TorC (TIGR02162) cytochrome that is the electron donor to the TorA TMAO reductase (Ansaldo et al., 1999; Gon et al., 2001). TorC is a member of the NapC/NirT tetraheme cytochromes (pfam03264) that donate electrons to soluble nitrate and nitrite reductases, and a misinterpretation of NapC/NirT function is likely the reason why HItorY is erroneously annotated as encoding a ‘nitrate reductase’ in several of the available genomes.

The torY gene is separated by 25 bp from torZ, which encodes the molybdenum-containing catalytic subunit of the TorYZ system (Figure 1). TorZ is an 825 aa protein that belongs to the DMSO reductase family of molybdenum enzymes. The reaction catalyzed by TorZ is linked to the cellular Q-pool and thus the HI respiratory chain via its interactions with TorY, that uses reduced quinones as electron donors for subsequent reduction of TorZ. TorZ activity can thus influence both the cellular redox balance and energy generation, in addition to potential beneficial effects derived from the substrate converted by TorZ (Figure 1).

As expected, the HI2019 torY and torZ genes form an operon, as shown by a PCR-based co-transcription assay (Figure 1), and in HI2019 the operon is followed by hairpin loop (−5.3 kcal/mol, TATATATAAAAAGAACCCATTTTTGAGG) that could serve as a potential transcription terminator. HI genomes generally also encode another Mo-containing sulfoxide reductase, the DmsABC protein that can catalyze reductions of similar compounds and has a catalytic subunit (DmsA) which has ~30% sequence identity to TorZ.

To investigate the physiological function of TorZ, we assessed torZ gene expression and the presence of sulfoxide reductase activity in HI2019 crude extracts. Both assays clearly suggest a role for TorZ during anaerobic growth of both HI2019 and HIRD, with increased gene expression and DMSO reductase activity with decreasing levels of oxygen (Figure 1). However, it should be noted that the enzyme activities from crude extracts (Figure 1) reflect the combined activities of TorZ and DmsABC, as both enzymes react with the same enzyme assay chemistry, and cannot be distinguished in a crude extract. Moreover, it is interesting to note that neither of the main substrates described for TorZ and DmsA, i.e., TMAO and DMSO, respectively, are likely to be present in the human respiratory tract at high concentrations, raising the question of what the physiological substrate for HItorZ is. Possible candidates substrates are MetSO or BSO, and these were therefore included in activity tests.
A HI2019\(^{ΔtorZ}\) Knockout Strain Is Not Susceptible to HOCl Induced Oxidative Stress But Shows Reduced Survival in Biofilms

A HI2019\(^{ΔtorZ}\) mutant was constructed to enable analyses of the physiological roles of TorZ. As expected, we clearly observed a loss of sulfoxide converting enzyme activities in HI2019\(^{ΔtorZ}\) (Figure 2A), with both DMSO and MetSO reductase activities reduced by \(\sim 91\%\), while biotin sulfoxide reductase activity was reduced by 96%. TMAO reductase activity was not affected, indicating either that TorZ does not reduce TMAO or that a second enzyme system is present in the crude extracts that can carry out this reaction. The enzyme activities were restored to wild-type levels in a strain complemented for the \(torZ\) mutation, HI2019\(^{ΔtorZ,comp}\) (Figure 2A). These data indicate that HI TorZ is the major S-oxide reductase in HI2019, and can convert a variety of substrates including DMSO and the two physiologically relevant compounds MetSO and biotin sulfoxide.

Despite the loss of the S-oxide reductase activity, on sRPMI-based medium supplemented with glucose the HI2019\(^{ΔtorZ}\) strain showed no growth defect, with growth rates showing no statistically significant different to the wild type (Supplementary Figure S1). In contrast, \textit{in vitro} biofilm formation experiments using microtiter plates showed a reduced ability of HI2019\(^{ΔtorZ}\) to form biofilms under all conditions tested, and analysis of the CFUs present in the biofilm also revealed reduced survival of HI2019\(^{ΔtorZ}\) in the biofilm (Figure 2B; Supplementary Figure S2). As TorZ is a periplasmic enzyme and appears to be able to repair oxidatively damaged methionine that can form during infection/interaction of HI with host cells, we also tested the susceptibility of the strain to hypochlorite that can form easily in the presence of ROS, however, survival of HI2019\(^{ΔtorZ}\) in the presence of HOCl was the same as for the wild type (Figure 2C).

Mutations in \(torZ\) Lead to a Reduced Ability of \textit{H. influenzae} to Interact with Host Cells

Analyses of gene expression for HI2019\(^{WT}\) in co-culture with 16HBE14 epithelial cells revealed significant levels of \(torZ\) expression both in bacteria present in the tissue culture medium (‘planktonic’) and those adherent to the 16HBE14 cells (Supplementary Figure S3). Together with the observed biofilm formation defect this led us to hypothesize that HITorZ might affect interactions of the bacteria with host cells.

In adhesion and invasion assays using the 16HBE14 bronchial epithelial cell line, total cell numbers (adherent and internalized cells, CFU/mL) were the same for HI2019\(^{WT}\) and HI2019\(^{ΔtorZ}\) after 4 h of incubation. However, after 24 h a statistically significant reduction in the CFU/mL for HI2019\(^{ΔtorZ}\) to \(\sim 33\%\) (\(p = < 0.0001\), One-Way ANOVA) of the wild-type level was observed (Figure 3), indicating that HI2019\(^{ΔtorZ}\) has reduced ability to either colonize epithelial cells during longer

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**FIGURE 1** | Structure and function of the HI2019 \(torYZ\) operon. (A) Schematic representation of the HI2019 \(torYZ\) gene region. The black bar indicates the position of the PCR product shown in (B). (B) PCR based co-transcription analysis of HI2019 \(torYZ\). ‘+’ = positive control containing gDNA; ‘−’ = no template control. C = cDNA (derived from anaerobic culture) as template. (C) Relative expression of the \(torZ\) gene in HI2019 cultures grown under aerobic (AE), microaerophilic (MA), and anaerobic (AN) growth conditions. Data were normalized using expression of the \(gyrA\) gene. (D) DMSO reductase activity in HI2019\(^{WT}\) cell-free extracts derived from cultures grown under aerobic (AE), microaerophilic (MA), and anaerobic (AN) growth conditions. DMSO reductase activity in cell-free extracts is a relative measure of the activity of Mo-containing S-oxide reductases under the conditions tested. Errors are given as standard deviations of the mean, at least three repeat assay were carried out per condition.
incubation or to survive in contact with them. As the inoculum for these experiments came from cells grown on complete growth medium where Hf2019^ΔtorZ shows no growth defect, it is possible that storage compounds may have contributed to growth of the mutant strain in the initial phases of the co-culture experiment. The reduction of adherence was also apparent in immunofluorescence stains of co-cultures (Figure 3). Invasion of 16HBE14 cells by Hf2019^ΔtorZ (determined using a gentamicin protection assay) was also reduced after both 4 and 24 h incubation where 20% (p = 0.0005, One-Way ANOVA) and 29% (p = < 0.0001, One-Way ANOVA) of the respective wild-type CFU/mL were observed for Hf2019^ΔtorZ (Figure 3). This suggests that the TorZ protein is either needed for intracellular survival or plays a role in the cell invasion process. Both of these functions are in keeping with a role in cellular energy generation via the respiratory chain.

Similar experiments were carried out using human primary neutrophils. After 2 h of incubation, Hf2019^WT had increased to ~128% of the originally used inoculum, while the level of Hf2019^ΔtorZ was approximately the same (97%, p = 0.0949, One-Way ANOVA) as at the start of the experiment. E. coli, used as a control, decreased to 30% of the original inoculum in the 2 h incubation period (p = 0.0005 vs. Hf2019^WT) (Figure 4).

**Loss of TorZ Leads to Reduced HI Survival in a Murine Model of Infection**

To determine if the *in vitro* survival defects were also observable *in vivo*, we then tested the ability of Hf2019^ΔtorZ to survive in a murine model of bacterial clearance. Mice were infected intranasally with 10^7 CFU and the number of remaining bacteria was assessed every 24 h up to 72 h relative to the wild type strain showing reduced survival of Hf2019^ΔtorZ relative to the wild type strain (Figure 4). After 24 h incubation the cell numbers...
of the mutant strain were already reduced by 85% compared to the respective wild type levels at 24 h, and this reduction in CFU/mL for HI2019ΔtorZ increased to 93 and 96% for the 48 and 72 h. In fact, at 72 h, four out of six mice infected with HI2019ΔtorZ were culture negative, while 6/6 mice infected with HI2019WT still reported on average 3.6 × 10³ CFU/mL (Figure 4). The HI2019ΔtorZ strain also appeared to cause a slower immune response in the mice with significant influx of immune cells only observed after 48 h, compared to 24 h for HI2019WT (Figure 4).

Taken together, our data indicate that the TorZ protein supports HI virulence, as a loss of the protein leads to reduced survival in a murine model of infection, and also reduced the ability of the bacteria to invade human cells. However, there is no information on the biological function of TorZ-like proteins in any bacterial species, and TMAO, the main substrate described for the E. coli TorZ (Gon et al., 2000), is not generally found in the respiratory tract. Therefore TMAO is unlikely to be the natural substrate for the HI enzyme, and a further characterization of HITorZ was required to determine whether the observed phenotypes were due to loss of oxidative damage repair through the TorZ substrate conversions or linked to the role of TorZ in the HI respiratory chain.

**Haemophilus influenzae TorZ Is a Periplasmic, S-isomer Specific Methionine Sulfoxide Reductase**

The HITorZ gene encodes a twin arginine signal peptide, with a predicted cleavage site at position 41 (aa sequence: AVA₀₋₄₁KE). This type of export signal targets the proteins for export via the membrane-bound TAT-system, all components of which are encoded in the HI2019 genome. Following cellular fractionation, ~60% of the MetSO reductase activity was recovered in the periplasm of HI2019WT, while no significant activity was present in HI2019ΔtorZ (Supplementary Figure S5). This clearly confirms that the observed activity was due to the presence of the functional, 87 kDaTorZ protein in the periplasm of H. influenzae.

The HI torZ gene without nucleotides 1–120 that encode the TorZ signal peptide was cloned into an expression plasmid, creating pProHITorZ-sp. Recombinant, soluble rTorZ protein was produced and purified with a yield of 5.6 mg of purified rTorZ from 4 L of expression culture (Figure 5A), and had a molybdenum content of 0.53 Mo atoms/TorZ molecule (or 0.53 moles Mo per mole TorZ) in the preparation as determined by ICP-OES. Based on gel filtration, the purified rTorZ was present as a monomer, and the optical absorbance spectra were similar to those of related Mo enzymes (Supplementary Figure S4) of...
the DMSO reductase family (Mcalpine et al., 1998), providing evidence that the Mo cofactor had been correctly inserted, and was able to interact with one of the proposed substrates, MetSO (Supplementary Figure S4, MetSO reduced spectrum).

Enzyme assays confirmed that rTorZ was able to reduce all four substrates tested previously, TMAO, DMSO, MetSO, and BSO (Figure 5B), showing that the enzyme is also an N-oxide reductase. We then tested the stereospecificity of rTorZ, as both MetSO and BSO exhibit diastereoisomerism due to the chirality of the S–O group that can be present in either an R or S form (Supplementary Figure S6). The substituted C-atom in the biotin bicyclic ring system alpha to the sulfoxide is always in a S configuration so only these two diastereoisomers (RS and SS) need to be considered. Racemic MetSO possesses four possible isomers due to chirality at the R-S(O)Me S-atom (R or S) and also the alpha C-atom of the amino acid (S or R) (Supplementary Figure S6). The RR/SS and RS/SR pairs are enantiomers.

rTorZ was able to reduce both racemic DL-MetSO (four isomers, Supplementary Figure S6) and L-MetSO (two isomers, both of the sulfoxide group) with similar rates, indicating that the enzyme is able to use both D- and L-MetSO as substrates. However, rTorZ reacted exclusively with S-BSO diastereoisomer (Figure 5B), while no activity with R-BSO was observed (data not shown). This suggests that the enzyme likely has a preference for S-sulfoxides over R-sulfoxides, and matches the observations made with crude extracts during the initial characterization of the HI2019 ΔtorZ strain (Figure 2). This stereospecificity can be rationalized on the basis of the structures of the two BSO isomers (Supplementary Figure S6). In the RS-BSO isomer the molecule adopts a distinct ‘U’-shaped conformation which creates a sterically congested environment for the O-atom of the sulfoxide that must ultimately coordinate to the Mo ion for catalytic reduction (de-oxygenation). Conversely, the extended conformation of SS-BSO creates a more accessible O-atom that can more easily coordinate to the active site of rTorZ.

Kinetic assays were used to clarify the natural substrate preference of rTorZ, and confirmed that the enzyme is a N- and S-oxide reductase, but is unlikely to be using the N-oxide TMAO as a natural substrate (Table 2; Figure 5C). While rTorZ had a high turnover rate with TMAO, this was only observed at physiologically irrelevant substrate concentrations ($K_M > 6$ mM). For the three S-oxides that were tested, turnover numbers differed significantly, between $183 \text{ s}^{-1}$ for SS-BSO and $\sim 85–90 \text{ s}^{-1}$ for DMSO and racemic-MetSO. The magnitude of the determined $K_M$ values was inversely related to the magnitude of the $k_{cat}$, with SS-BSO having a $K_M$ of 1.2 mM, racemic MetSO of 0.41 mM for the diastereomeric mixture (0.205 mM corrected for S/R form of the sulfoxide) and DMSO of 0.14 mM. It should be noted here that for the racemic MetSO, both enantiomers of the sulfoxide group would have been present in the mixture and we were unable to resolve these. Given that rTorZ is only able to convert S-sulfoxides as shown by our experiments, the $K_M$ reported here for racemic MetSO may be overestimating the actual $K_M$, as only the S-MetSO molecules present in the assay would have been converted by rTorZ.

These data indicate that rTorZ likely is a S-MetSO reductase, as, unlike DMSO, MetSO is a substrate that is present in the
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Haemophilus influenzae TorZ Appears to be Unable to Repair of MetSO–Damage to Proteins

Given its ability to reduce MetSO to Met, rTorZ might be involved in periplasmic protein repair following exposure to oxidative stress, and we tested this using an assay based on the Met-rich protein calmodulin and the MrsP MetSO reductase as a control (Gennaris et al., 2015). While MrsP was able to reduce calmodulin, under the same assay conditions, no reactivity of TorZ toward calmodulin was observed (Figure 5D). The assay used is very similar to the standard TorZ kinetic enzyme assay, except that calmodulin was added as the substrate, and thus should have been suitable for TorZ reactivity. While we cannot completely rule out that TorZ might be able to react with other oxidized proteins, this result strongly suggests that protein repair is not the primary enzymatic function of TorZ, and instead the observed phenotypes may be related to the function of TorZ in the HI respiratory chain.

Phylogenetic Analyses of H. influenzae rTorZ Confirm that it Is a Novel Type of Mo-containing S-oxide Reductase

The data collated above clearly highlight that HITorZ differs from other Mo enzymes in both the preferred catalyzed reaction and its effects on bacterial physiology. To determine whether rTorZ is a novel type of Mo enzyme or a variant of a known type, we analyzed the phylogenetic relationship of HITorZ to other characterized enzymes of the Dor/Tor type described in the literature (Supplementary Figure S7). This showed that HITorZ shares a common ancestor with E. coli BisC (Pierson and Campbell, 1990; Barber et al., 1992) and TorZ (Gon et al., 2000), but resides on a separate branch within that group, and does not group with the E. coli TorZ sequences (Supplementary Figure S7). This is also reflected in the sequence identities that were about 40–50% for all enzymes in the alignment relative to HITorZ (53% EcTorZ; 49% EcBisC; 42% EcTorA, and 48% EcDor).

TABLE 2 | Kinetic parameters of purified Hi rTorZ for different S- and N-oxide substrates.

| Substrate | $K_M$ (mM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_M$ (s$^{-1}$M$^{-1}$) |
|-----------|-----------|------------------|-------------------------------|
| TMAO      | 6.7 ± 2.0 | 434.7 ± 52.1     | 6.49 × 10$^4$                 |
| DMSO      | 0.14 ± 0.05 | 85.4 ± 9.1     | 6.5 × 10$^2$                 |
| DL-MetSO  | 0.41 ± 0.08 | 91.7 ± 4.4     | 2.24 × 10$^2$                 |
| S-BSO     | 1.52 ± 0.28 | 182.6 ± 10.9   | 1.2 × 10$^5$                 |

Errors are reported as standard deviations of the mean.
RcDorA), which is a moderate degree of conservation, reflecting the common phylogenetic origin of enzymes within this group. In keeping with previous published work, all S-oxide reductases in the alignment contained residues equivalent to Tyr114 and Trp116 (Rc DorA numbering), which were identified as crucial active site residues in R. capsulatus DorA (Mcewan et al., 2003). In the E. coli N-oxide reductase TorA Tyr114 is absent. The aspartate ligand to the Mo cofactor (Mcalpine et al., 1997) was also conserved in all sequences.

We then expanded the alignment to include a total of 1005 sequences of proteins of the Dor/Tor group of enzymes (see Supplementary Data, Figure 6) to obtain a global view of enzyme evolution within this group, and were able to identify three major phylogenetic groups of enzymes within the Dor/Tor group of Mo enzymes. Group I contained sequences related to E. coli TorA (Group 1A, also included sequences from Serratia sp.; Photobacterium sp.; Aeromonas sp.; Citrobacter sp., Pasteurella multocida, and Shewanella sp.) and R. capsulatus DorA (Group 1B, Burkholderia sp.; Rhodopseudomonas sp., Cupriavidus sp., and Rhodobacter sp.) respectively, while group III had five subgroups: (i) Group 3A: E. coli BisC sequences and homologs from S. enterica and Citrobacter sp.; (ii) Group 3B Klebsiella sp. and Enterobacter sp. sequences; (iii) Group 3C Cronobacter sp. sequences; (iv) Group 3D Vibrio sp. and Citrobacter sequences; and (v) Group 3E E. coli TorZ sequences. Whether the proteins in groups 3B–3D encode enzymes with E. coli BisC- or TorZ-like activities remains to be analyzed, however, as E. coli TorZ was originally described as a biotin sulfoxide reductase, we have tentatively called this group the biotin sulfoxide reductase group of sequences.

Group II (putative MetSO reductases) contained the sequences related to the HI TorZ protein which formed two large subgroups. Group 2A contained sequences from Yersinia sp., Serratia sp., Enterobacter sp., Photobacterium and Aeromonas sp., while Group 2B was dominated by sequences originating from various species of Pasteurellaceae, including HI, and in addition contained some sequences from Campylobacter sp. and Helicobacter sp.. These data clearly indicate that HITorZ protein is a unique protein within the Dor/Tor-type enzymes of the DMSO reductase family, and based on its apparent preference for MetSO as a substrate we propose that the HITorY and TorZ proteins be renamed to MtsY and MtsZ (Mts, Methioninesulfoxide) to clearly distinguish them from the E. coli TorY and TorZ.

![FIGURE 6](image-url) **FIGURE 6** | Phylogenetic analysis of HITorZ/MtsZ related protein sequences of the DMSO reductase enzyme family. HITorZ/MtsZ clearly forms a distinct group within this subgroup of DMSO reductase enzyme family enzymes as indicated by the branching pattern. The phylogenetic tree shown was constructed from a total of 1005 sequences retrieved from NCBI by BLAST searches (see Supplementary Data) using the neighbor-joining algorithm. The phylogenetic analyses used MEGA 6.0, bootstrapping used 500 replicates.
DISCUSSION

The DMSO reductase family of molybdenum enzymes contains a number of enzymes that are able to reduce N- and S-oxides, and in bacteria that can live freely or in association with a host such as E. coli, TMAO and DMSO are usually considered as the physiological substrates for these enzymes since the former is a nitrogen waste product found in a variety of animals, especially fish, while the latter is a component of the organo-sulfur cycle (Kappler and Schaefer, 2014). However, for an obligate host-adapted bacterium such as HI the nature of the physiological substrate for S-/N-oxide reductases is less obvious since this bacterium is unlikely to encounter DMSO and TMAO in its natural environment.

The HI MtsZ protein that we have characterized here is a novel, previously uncharacterized molybdenum enzyme of the DMSO reductase family that uses MetSO as its main substrate. Unlike the periplasmic DMSO and TMAO reductases (Jobbi-Nivol et al., 1996; Gon et al., 2000; McEwan et al., 2004) to which it is related, MtsZ is prevalent in pathogenic bacteria, especially of the Pasteurellaceae family of bacterial pathogens, where proteins related to HIMtsZ are found in every major group of this family (Figure 6).

Catalytically, purified MtsZ protein showed a clear preference for S-oxides rather than N-oxides based on measurements of $k_{cat}/K_M$ in combination with assessment of $K_M$ values (Table 2), with DMSO and MetSO being the only substrates tested with $K_M$ values in a physiologically relevant concentration range which is essential for cellular control of enzyme activity. MtsZ reacted specifically with sulfoxides in S-conformation, and thus has the same stereospecificity as the related R. capsulatus DorA DMSO reductase (Hanlon et al., 1998) and the BisC biotin sulfoxide reductases from E. coli and Salmonella (Denkel et al., 2013).

Interestingly, MtsZ appears to be specific for small molecule S-oxides such as free MetSO and DMSO rather than oxidized Met residues that form in many periplasmic proteins during oxidative stress and thus has a clearly different cellular function from the only other known Mo-containing enzymes (Gennaris et al., 2015). MsrP was recently described and appears to be specific for repair of MetSO residues in periplasmic protein repair. MsrP was characterized in E. coli, and belongs to an unrelated group of Mo-containing enzymes (Gennaris et al., 2015).

Based on what we know so far, there are two possible functions for HIMtsZ. First, the enzyme might support HI oxidative stress defenses by re-reducing oxidized S-oxides, e.g., by converting MetSO back to Met, which is the form in which it can be used by cells. Secondly, the MtsZ catalyzed reaction is connected to the respiratory chain via the pentaheme cytochrome MtsY and thus can also support respiration and redox balancing in HI under oxygen-limited conditions, as indicated by the enzyme activities and gene expression profiles (Figures 1 and 7).

In support of the first point, free methionine, the precursor of the natural substrate of MtsZ has been detected in human serum (Kermack et al., 2015). The sulfur group in methionine is easily oxidized and this oxidation can be associated with ROS or the action of enzymes such as myeloperoxidase (MPO) which is released by human neutrophils and catalyzes the production of hypochlorite (HOCl) from peroxide and chloride ions (Drozdz et al., 1988). While epithelial cells are not major producers of ROS it has been shown that they are able to produce ROS especially when stimulated by LPS, and that this may involve NOX or DUOX-type NADPH Oxidases (Boncompain et al., 2010; Uy et al., 2011; Rada et al., 2014) and our gene expression data from co-cultures clearly show high levels of mtsZ transcription in co-cultures. Converting molecules such as MetSO back to their reduced state could contribute to the formation of an extracellular oxidative stress buffer, as Met will react quickly with ROS or HOCl.

However, our data also show that MtsZ does not appear to be involved in repair of MetSO damage to proteins as indicated by a lack of increased HOCl sensitivity and inability to repair oxidized calmodulin, showing that the HI2019^mtsz^ mutant strain still contained a fully functional system for repair of HOCl induced oxidative damage.

Despite this, the MtsZ mutant strain showed clear defects in a number of assays, such as biofilm formation and survival,

**FIGURE 7** | Current model of HIMtsZ physiological function in HI2019^WT^ and HI2019^torZ^. MetSO, methionine sulfoxide; Met, methionine; MQ, oxidized menaquinone; MQH$_2$, reduced menaquinone. MQH$_2$ in bold (right panel) indicates accumulation of this compound due to lack of MtsZ activity.
adherence and invasion to host cells and survival in a murine model of infection. The pivotal role of MtsZ in survival of HI2019 in co-cultures with host cells and a mouse model suggests that these interactions might take place under oxygen-limitation, when mtsZ expression is high. In the co-culture experiments, the reduced ability of HI2019ΔtorZ to invade the human tissue cells could either be a follow on effect from a reduced number of adherent cells, reflect a loss of the ability of the strain to be taken up into the cells or a loss of the ability to survive intracellularly. All of these phenotypes are consistent with a role for the MetSO reductase activity of MtsZ in support of HI energy generation and redox balancing during growth under limiting oxygen conditions, including during contact with host cells.

A similar observation has been made for the BisC biotin sulfoxide reductase from S. enteria serovar Typhiimurium, where BisC activity was required for repair of biotin sulfoxide and, to a lesser extent, MetSO during growth inside macrophages (Denkel et al., 2013). The Salmonella ΔbisC strain had attenuated survival in a mouse model, with a stronger attenuation being observed in mice that had a more potent oxidative response (Denkel et al., 2013).

If free MetSO is the substrate of MtsZ, then MtsZ might also have a role in ensuring cellular methionine supplies. A possible side effect of oxidative stress can be an inactivation of the methionine biosynthesis through damage to the MetE protein, as has been observed in E. coli (Hondorp and Matthews, 2004; Leichert and Jakob, 2004). This inactivation of MetE renders the bacteria temporarily unable to synthesize methionine de novo, and in such a scenario MtsZ could also be involved in enabling scavenging of functional methionine from the extracellular environment. Interestingly, methionine biosynthesis genes including metE, have been shown to support virulence in another respiratory pathogen, Streptococcus pneumoniae (Basavanna et al., 2013).

In summary, our results clearly show that MtsZ is a previously uncharacterized type of molybdenum enzymes with a high degree of conservation in the Pasteurellaceae family of bacterial pathogens, and that the MtsZ protein and/or the associated enzymatic activities (Figure 2) are required for HI interactions with host cells both in vitro and in vivo. Given the striking effect of a loss of MtsZ on survival of HI in contact with host cells and its periplasmic location this protein might be a useful target of inhibitors for the prevention of HI infection. Our current model for MtsZ function is that this enzyme is required for optimal anaerobic energy generation and maintenance of a redox balance in HI, and this in turn compromises HI survival in complex environments such as biofilms, in contact with host cells or animal model tissues (Figure 7). By recovering oxidized methionine in the periplasm of the HI cells, MtsZ may also contribute to either scavenging of functional Met, or creation of a ‘redox buffer’ for prevention of damage to other cellular components in the HI extracellular space. Despite possessing a suitable catalytic activity for repair of proteins with oxidative damage, this does not appear to be a main role for MtsZ.

Future work on this system should include investigations of possible changes in host cell responses to exposure to an HI strain lacking TorZ, as well as further work on the mechanism of action of MtsZ, to fully understand how it supports virulence of HI.

AUTHOR CONTRIBUTIONS

UK coordinated the research, supervised all students and staff involved, UK, RD, and AM prepared the main manuscript drafts edited figures and contributed to data analyses. RD carried out the majority of experiments on co-cultures and enzyme characterization. DKS/MPO carried out the initial analyses of torZ expression and generation of the knockout mutant strains. AD, VL, MN, HW, and XL carried out the phylogenetic analyses, preparation of TorZ expression plasmids and optimized protein expression and purification. PB prepared purified sulfoxide substrates for enzyme assays. A-TE and PH carried out the mouse experiments an associated data analyses. All authors reviewed the manuscript and contributed to the drafting of sections relevant to their work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01743/full#supplementary-material

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