Characterization of the *Haemophilus influenzae* *tehB* gene and its role in virulence

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The *Haemophilus influenzae* ORF designated HI1275 in the Rd KW20 genomic sequence encodes a putative *S*-adenosyl methyltransferase with significant similarity to tellurite-resistance determinants (*tehB*) in other species. While the *H. influenzae tehB* can complement an *Escherichia coli tehB* mutation, thus restoring tellurite resistance, its role in *H. influenzae* is unknown. In a previous study defining the iron and haem modulon of *H. influenzae*, we showed that transcription of this gene in *H. influenzae* Rd KW20 increases during growth in iron- and haem-restricted media. Since iron and haem uptake genes, and other known virulence factors, constitute the majority of the iron- and haem-regulated gene set, we postulated that *tehB* may play a role in nutrient acquisition and/or the virulence of *H. influenzae*. A *tehB* mutant was constructed in the *H. influenzae* type b strain 10810 and was evaluated for growth defects in various supplemented media, as well as for its ability to cause infection in rat models of infection. Deletion of *tehB* leads to an increase in sensitivity both to tellurite and to the oxidizing agents cumene hydroperoxide, tert-butyl hydroperoxide and hydrogen peroxide. The *tehB* mutant additionally showed a significantly reduced ability to utilize free haem as well as several haem-containing moieties including haem–human serum albumin, haemoglobin and haemoglobin–haptoglobin. Examination of the regulation kinetics indicated that transcription of *tehB* was independent of both tellurite exposure and oxidative stress. Paired comparisons of the *tehB* mutant and the wild-type *H. influenzae* strain 10810 showed that *tehB* is required for wild-type levels of infection in rat models of *H. influenzae* invasive disease. To our knowledge this is the first report of a role for *tehB* in virulence in any bacterial species. These data demonstrate that *H. influenzae tehB* plays a role in both resistance to oxidative damage and haem uptake/utilization, protects *H. influenzae* from tellurite exposure, and is important for virulence of this organism in an animal model of invasive disease.

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INTRODUCTION

Haemophilus influenzae is a significant cause of otitis media (OM) in children and pneumonia in patients with predisposing conditions (Murphy, 2003; Turk, 1984; St Geme, 2000). Unlike most other bacterial species, *H. influenzae* has an absolute requirement for haem as it is unable to synthesize the protoporphyrin ring (Evans *et al.*, 1974). Thus, it has evolved a variety of mechanisms to obtain this essential nutrient from human sources (Stull, 1987; Morton & Williams, 1989, 1990; Morton *et al.*, 2006b, 2008). Expression of many of the *H. influenzae* iron/

haem (Fe/Hm) acquisition systems is regulated in response to environmental levels of iron and/or haem. We previously undertook a microarray-based study to define all of the genes that are similarly regulated in response to environmental iron and/or haem levels with a view to identifying co-regulated virulence determinants. That study identified more than 80 genes for which transcription was significantly upregulated in Fe/Hm-restricted conditions in the *H. influenzae* isolate Rd KW20 (Whitby *et al.*, 2006a). Many of the genes identified in that study are associated with iron and haem uptake, utilization and homeostasis, while others had defined roles in cellular metabolism or were uncharacterized (Whitby *et al.*, 2006a). One of the Fe/Hm-regulated genes identified in that study,

Abbreviations: Fe/Hm, iron/haem; OM, otitis media; ROS, reactive oxygen species; SAM, *S*-adenosylmethionine.

designated HI1275, encodes a putative protein with a high degree of similarity to a previously described S-adenosyl methyltransferase. In other bacterial species, homologues of this gene have been implicated in resistance to the toxic oxyanion of the chalcogen tellurium. This gene together with an operonic accessory gene constitutes the tellurite-resistance determinant (*teh*) (Walter & Taylor, 1989; Turner *et al.*, 1997; Taylor *et al.*, 1994). The ORF designated HI1275 in strain Rd KW20 will hereafter be called *tehB* based on its significant identity to the experimentally characterized *tehB* gene of *Escherichia coli* (Taylor *et al.*, 1994).

Tellurite is a naturally occurring compound, the environmental presence of which is increasing as a by-product of the electronics and mining industries. This increase in environmental tellurite levels has resulted in the identification of a number of bacteria, of both clinical and environmental importance, that possess a natural resistance to the oxide (Bradley, 1985; Summers & Jacoby, 1977; Tantalean *et al.*, 2003; Taylor, 1999). Tellurite has no known biological function and appears to exert its toxic effects through the production of reactive oxygen species (ROS) (Borsetti *et al.*, 2005; Calderon *et al.*, 2006, 2009), with superoxide being the predominant species formed. A recent paper by Calderon *et al.* (2009) details the effect of the ROS on cellular components and the disabling of metabolically important enzymes containing [4Fe-4S] catalytic clusters. Iron released from these clusters is able to cause further cellular damage via localized Fenton reaction with peroxides to create hydroxyl radicals, which rapidly damage structures including lipids and nucleic acids.

To date, five broadly distributed, genetically distinct tellurite-resistance determinants have been identified on both chromosomes and plasmids of bacteria (Zannoni *et al.*, 2008). In addition to the broadly distributed tellurite-resistance determinants, several unrelated potential resistance determinants distinct to various bacterial species have also been identified (Zannoni *et al.*, 2008). These latter resistance determinants have been suggested as conferring selective advantages distinct from tellurite resistance, since the presence of the determinant does not correlate with exposure of the organism to tellurite in its natural environment (Zannoni *et al.*, 2008). Indeed, it is now recognized that the so-called tellurite-resistance genes often confer other potentially more biologically relevant phenotypic characteristics and that tellurite resistance may be essentially an artefact (Zannoni *et al.*, 2008). For example, overproduction of the cysteine biosynthetic pathway can mediate a tellurite-resistance phenotype by conferring resistance to oxidative damage incurred upon tellurite exposure (Tantalean *et al.*, 2003). This may be a common mechanism shared by many of the tellurite-resistance determinants.

Since *H. influenzae* grows only in humans, it is unlikely to encounter tellurite in its natural environment. Therefore, it is similarly unlikely that tellurite resistance is a biologically relevant phenotype encoded by the *H. influenzae tehB* gene.

In addition, the significant upregulation of transcription of *tehB* in response to Fe/Hm restriction in strain Rd KW20 indicates that this gene may be preferentially transcribed in the Fe/Hm-restricted environment encountered in the human host. Thus, the lack of knowledge of the biologically relevant role of *tehB* combined with the transcriptional response of the gene to Fe/Hm levels led us to study this gene further. The present study aimed to determine the prevalence of *tehB* across the species *H. influenzae* and the impact of *tehB* on tellurite resistance as well as other various biologically relevant phenotypes, including iron and haem utilization and resistance to oxidative stress, and also to establish the potential role of *tehB* in virulence in an animal model of *H. influenzae* infection. The recently sequenced *H. influenzae* type b isolate 10810 was chosen as the genetic background to perform these studies due to its clinical significance, available genome sequence and utility in animal models of infection.

METHODS

Bacterial strains and growth conditions. *H. influenzae* type b (Hib) strain 10810 is a clinical isolate from a patient with meningitis and its genome has recently been sequenced at the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk>). The nontypable *H. influenzae* (NTHi) strain R2866 was isolated from the blood of an immunocompetent child with clinical signs of meningitis subsequent to acute OM (Nizet *et al.*, 1996). NTHi strain R2846 was originally isolated from the middle ear of a child with acute OM (originally designated strain 12: Barenkamp, 1992). NTHi strain 86-028NP is a minimally passaged clinical isolate from a patient who underwent tympanostomy and tube insertion for chronic OM (Suzuki & Bakletz, 1994). *H. influenzae* strain Rd KW20 was originally isolated over 60 years ago (Alexander & Leidy, 1951) and its genome was sequenced in 1995 (Fleischmann *et al.*, 1995). Isolates of *H. influenzae* were routinely maintained on chocolate agar with bacitracin at 37 °C. When necessary *H. influenzae* were grown on brain heart infusion (BHI) agar supplemented with 10 µg haem ml⁻¹ and 10 µg β-NAD ml⁻¹ (supplemented BHI; sBHI) and the appropriate antibiotic(s). Haem-deplete growth was performed in BHI broth supplemented with 10 µg β-NAD ml⁻¹ alone (haem-deplete BHI; hdBHI). *E. coli* TOP10 (Invitrogen) was used for cloning experiments and was routinely grown on LB agar supplemented with the appropriate antibiotics. Kanamycin was used at 25 µg ml⁻¹ for *H. influenzae* and 50 µg ml⁻¹ for *E. coli*; chloramphenicol was used at 1.5 µg ml⁻¹ for *H. influenzae* and 50 µg ml⁻¹ for *E. coli*.

Haem sources. Human haemoglobin, pooled human haptoglobin and human serum albumin, as well as bovine haemin, were purchased from Sigma. Stock haem solutions were prepared at 1 mg haem ml⁻¹ in 4% (v/v) triethanolamine as previously described (Poje & Redfield, 2003a). Haemoglobin was dissolved in water immediately before use. Haemoglobin-haptoglobin complexes were prepared as previously described (Morton *et al.*, 1999). Haem-albumin complexes were made by mixing 100 µg haem and 20 mg human serum albumin per ml of water as previously described (Stull, 1987).

DNA methodology. Restriction endonucleases were obtained from New England Biolabs and used as directed by the manufacturer. Genomic DNA was isolated using the DNeasy Tissue kit (Qiagen) as directed by the manufacturer. Plasmid DNA was isolated using the Wizard Plus Minipreps DNA purification system (Promega) according to the manufacturer's directions. Sequencing of double-stranded

DNA was performed by automated sequencing on an ABI Prism model 3700 DNA Analyser at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater, OK, USA. Oligonucleotides were synthesized by Eurofins MWG Operon.

Construction of *tehB* deletion mutants. Deletion mutants of *tehB* were constructed as follows. Two pairs of primers were designed for use in the PCR based on the available *H. influenzae* strain Rd KW20 genomic sequence (Fleischmann *et al.*, 1995) to amplify an 800 bp and a 1300 bp DNA fragment upstream (TehMut-US1 and TehMut-US2) and downstream (TehMut-DS1 and TehMut-DS2) of the *tehB* gene respectively (Table 1). The PCR product of the upstream region was successfully cloned into the TA-cloning vector pCR2.1-TOPO (Invitrogen), to yield plasmid pJS29. The PCR-amplified DNA fragment corresponding to the downstream region of *tehB* was digested with *NotI* and *Sall* and cloned directly into *NotI/Sall*-digested pJS36 to create pJS40. Plasmid pJS36 comprises a kanamycin-resistance cassette in PCR2.1-TOPO bounded by adjacent unique *NotI* and *Sall* sites on one side and *Ascl* and *FseI* sites on the other. Plasmids pJS29 and pJS40 were digested with *Ascl*, ligated and used as template in a PCR using the distal upstream and downstream primers to create a DNA fragment comprising the upstream/kanamycin marker/downstream DNAs fused together. This final PCR product was ligated into pCR2.1-TOPO to create the mutagenic construct, pJS47. Confirmation of the final construct was achieved by automated nucleotide sequencing across all ligated junctions. This construct was used to transform competent *H. influenzae* to kanamycin resistance, as previously described (Morton *et al.*, 2004a), thus resulting in deletion of the *tehB* gene. Correct insertion of the mutagenic construct was confirmed by PCR of the genomic DNA.

Complementation of the *tehB* insertion mutation. The *tehB* gene together with approximately 300 bp upstream and downstream was amplified by PCR using primers *tehB*-compF and *tehB*-compR, which incorporate *HindIII* sites at each end. The resulting product was digested with *HindIII* and ligated with *HindIII*-digested pASK5 to yield pJS153. The use of pASK5 allows for complementation of gene disruptions in *H. influenzae* by insertion of a gene in the nonessential

outer-membrane protein OmpP1 locus (Saeed-Kothe *et al.*, 2004). Plasmid pJS153 was transformed into *H. influenzae* made competent using MIV medium as described by Poje & Redfield (2003b) and selected on sBHI containing chloramphenicol. Chloramphenicol-resistant colonies were identified and the correct chromosomal rearrangements were confirmed by the molecular size of PCR products from PCRs with two different primer pairs. The first PCR utilized primers *tehB*-compF and *tehB*-compR, while the second PCR utilized primers OMP-P1-F and OMP-P1-R (Saeed-Kothe *et al.*, 2004) and gave a single band corresponding to insertion of pJS153 in the OmpP1 locus (data not shown). To generate control strains, competent *H. influenzae* were similarly transformed to chloramphenicol resistance using pASK5 and chromosomal rearrangements of chloramphenicol-resistant isolates were confirmed by sizing of PCR products on agarose gels. Primers used in the PCRs are listed in Table 1.

Growth studies with *H. influenzae*. Growth studies were performed using the Bioscreen C Microbiology Reader (Oy Growth Curves) as previously described (Whitby *et al.*, 2006b; Morton *et al.*, 2006a). Growth curves were performed in 300 µl volumes with five replicates for each growth condition in each individual experiment. Experiments were performed at least twice with each strain/condition examined. Optical density measurements were taken at 600 nm at 30 min intervals with the Bioscreen C set to incubate at 37 °C with constant shaking (machine setting 'low').

Transcriptional regulation by iron/haem, tellurite and oxidative stress. To determine the impact of Fe/Hm levels on transcription of *tehB* in NTHi strains R2846 and 86-028NP the transcriptional regulation experiment detailed by Whitby *et al.* (2006a) was repeated with these isolates. To determine the response of Hib strain 10810 to tellurite or oxidative stress, we examined the transcription of the gene before and after addition of a sublethal dose of tellurite or cumene hydroperoxide. For each perturbant, two flasks were inoculated with Hib strain 10810 as previously described (Whitby *et al.*, 2009) and allowed to grow at 37 °C with shaking. At 60 min, a sample (500 µl) was taken from each flask and mixed with 500 µl RNeasy lysis reagent (Qiagen) and immediately frozen and stored for subsequent Q-PCR analysis. Immediately following removal of the initial 500 µl sample,

Table 1. Oligonucleotide sequences of PCR primers

Name	Sequence (5'–3')*	Site†
TehMut-US1	ACGATTGAATTCGCGCTGAATGATAACTACATTTGG	<i>EcoRI</i>
TehMut-US2	ACGATTGGCGCGCCATGGGCGAATTACACAAAACCGATG	<i>Ascl</i>
TehMut-DS1	ATTCGATTGCGGCCGCCGCCGAATTTTCATTTTACGTGG	<i>NotI</i>
TehMut-DS2	ACGATTGTCGACATGGTTTGAGAGCGATTTACAACAATGG	<i>Sall</i>
<i>tehB</i> -compF	ATCAAAGCTTTGTTCATTCGACGGGGGGGACA	<i>HindIII</i>
<i>tehB</i> -compR	CGGGATAAGCTTGGTGATCATACTCGTCAAGTCTTCTCT	<i>HindIII</i>
QPCR-HI 16S rRNA-F	TCGTCAGCAAGAAAGCAAGCT	
QPCR-HI 16S rRNA-R	GCTGGCGGCAGGCTTAA	
QPCR- <i>tehB</i> -uF	CTGCAAAAATTATCTCGCCTTGT	
QPCR- <i>tehB</i> -uR	CCAAGAGGTCACATCATAGCCTAATA	
Q-PCR-pgdX-F	AACCAGGCGATCCGTTCA	
Q-PCR-pgdX-R	TTGGTGTGTGGTGCAAGGT	
Q-PCR-hkte-F	TTGAAGTCGGCGAGTTTGAA	
Q-PCR-hktE-R	TGCAATGCAGATTGTCTACA	
OMP-P1-F	CGTACGGACGTCAAGCGTATTCAGCAAGCG	
OMP-P1-R	CTCGCAAAGCTTGCCAAATGGCTTGTGAAATGCG	

*Bold type indicates the sequence of the incorporated restriction site.

†Restriction enzyme cleaving the incorporated site.

the perturbant was added to one of the two experimental flasks at a sublethal final concentration (4 μ M tellurite or 100 μ M cumene hydroperoxide). Samples for Q-PCR (500 μ l) were taken at 1, 2.5, 5, 10 and 20 min following addition of the perturbant from both flasks. In addition, 0.1 ml samples of culture were collected for 2 h after addition of the individual perturbants to examine the growth profiles of strain 10810 in each flask, and the bacterial count was quantified using the track-dilution procedure as previously described (Morton *et al.*, 2004b).

Quantitative real-time PCR (Q-PCR). Q-PCR was performed as previously described (VanWagoner *et al.*, 2004). Oligonucleotide primers targeting the *tehB* and the 16S rRNA gene were designed using Primer Express 2.0 (Applied Biosystems) (Table 1) and were tested to determine the amplification specificity, efficiency and linearity of the amplification with RNA concentration. RNA was purified using the RNeasy Mini kit (Qiagen) and used as template for cDNA synthesis as described previously (VanWagoner *et al.*, 2004). The resulting cDNA was used in Q-PCRs. A typical 25 μ l reaction contained 12.5 μ l SYBR Green Master Mix, 250 nM of each primer, and 5 μ l cDNA sample. Quantification reactions at each time point were performed in triplicate and normalized to concurrently run 16S rRNA levels from the same sample. Relative quantification of gene expression was determined using the $2^{-\Delta\Delta C_t}$ method of Livak & Schmittgen (2001), where $\Delta\Delta C_t = (C_{t,Target} - C_{t,16S})_{Time\ x} - (C_{t,Target} - C_{t,16S})_{Control}$. As controls, primers were also designed to target the peroxiredoxin gene (*pgdX*-HI0572) and the catalase gene (*hktE*-HI0928). Primers used in the Q-PCRs are detailed in Table 1.

Infection of animals, collection of blood samples and quantification of bacteraemia. The rat model for haematogenous meningitis following intraperitoneal infection with *H. influenzae* (Smith *et al.*, 1973) was used to compare the abilities of strains to cause bacteraemia in both 5-day-old and 30-day-old rats. The inoculum was prepared as previously described (Morton *et al.*, 2004b). Rats were inoculated with 100 c.f.u. in 100 μ l by intraperitoneal injection. To determine the actual infective dosage, aliquots of the inoculum were plated on chocolate agar containing bacitracin. At specified time intervals pups were examined for symptoms of infection, and blood specimens (50 μ l) were obtained. Bacteraemia was quantified using the track-dilution procedure as previously described (Morton *et al.*, 2004b). All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center.

Statistics. Statistical comparisons of growth between strains under the same growth conditions *in vitro* were made using the Kruskal-Wallis test. Statistical comparisons of bacteraemic titres in groups of rats infected with different bacterial strains on given days post-infection or over the entire time-course of infection also were assessed by the Kruskal-Wallis test. Analyses were performed using Analyse-It for Microsoft Excel v1.71 (Analyse-It Software Inc.). A *P*-value <0.05 was taken as statistically significant.

Percentages of bacteraemic rats infected with wild-type and mutant strains were compared with the Fisher exact test. Analyses were performed using SigmaStat Software and a *P*-value <0.05 was taken as statistically significant.

RESULTS

Distribution and conservation of *tehB* across the Pasteurellaceae

The *H. influenzae* Rd KW20 gene *tehB* is annotated as a tellurite-resistance protein based on homology with the

characterized *E. coli* *tehB* gene; comparison of TehB from Rd KW20 with that from *E. coli* K-12 MG1655 showed 55 % identity and 70 % similarity between the two proteins (Taylor *et al.*, 1994). Examination of the two gene products shows that the *E. coli* protein is truncated by comparison with the TehB of *H. influenzae*. The latter gene has an additional approximately 100 amino acids at the amino terminus of the protein. We sought to identify the presence of a *tehB* homologue in the 35 completed, or in progress, genome sequences representing the family *Pasteurellaceae* that are currently available (31 of these sequences are available in the current GenBank database and a further four at the Wellcome Sanger Institute: <http://www.sanger.ac.uk>). Examination of these genome sequences showed the presence of *tehB* in 18 of 18 isolates of *H. influenzae* (including Rd KW20), 4 of 4 isolates of *Actinobacillus pleuropneumoniae*, 2 of 2 isolates of *Actinobacillus minor*, 2 of 2 isolates of *Haemophilus parasuis* and single isolates of *Haemophilus parainfluenzae*, *Mannheimia haemolytica*, *Mannheimia succiniciproducens*, *Aggregatibacter actinomycetemcomitans*, *Aggregatibacter aphrophilus*, *Actinobacillus succinogenes* and *Pasteurella multocida*. Interestingly, each of the above proteins had the N-terminal extension found in *H. influenzae*. Two species within the *Pasteurellaceae* did not possess a TehB homologue, *Haemophilus ducreyi* (1 strain) and *Histophilus somni* (2 strains). The TehB proteins of all 17 *H. influenzae* strains examined exhibited >97 % identity with the TehB protein of strain Rd KW20; the *tehB* genes of four strains contained frame-shift mutations leading to truncation of the encoded protein and for these analyses were altered by the addition or removal of nucleotides to bring the gene into frame (it is unclear whether these frame-shift mutations are real or an artefact of sequencing). Among the members of the *Pasteurellaceae*, other than *H. influenzae*, that contained a *tehB* homologue, the encoded proteins all possessed >64 % identity to the Rd KW20 protein. Homology of the TehB proteins suggested that they are S-adenosylmethionine (SAM)-dependent, non-nucleic acid methyltransferases. These proteins differ from the nucleic-acid-directed methyltransferases by the possession of three distinct motifs which are lacking in the nucleic acid methyltransferases (Liu *et al.*, 2000).

In *E. coli*, *tehB* is operonic with the gene encoding the accessory protein TehA, which has been characterized as a membrane-associated efflux pump (Turner *et al.*, 1997). While there is not an operonic accessory gene to *tehB* in *H. influenzae*, the Rd KW20 gene HI0511, and its homologue in the Hib strain 10810, encodes a protein with 38 % identity and 55 % similarity to the *E. coli* TehA. The potential role of the TehA homologue in *H. influenzae* was not examined in this study.

Transcriptional regulation of *tehB* by iron and/or haem concentration

Previously we have demonstrated that *in vitro* transcription of *tehB* varies depending upon the iron and/or haem status

of the growth medium. Global transcriptional studies demonstrated that *tehB* was preferentially transcribed under Fe/Hm limitation in the strains Rd KW20 and NTHi R2866 while the Hib isolate 10810 showed only a modest increase in transcription (Whitby *et al.*, 2006a, 2009). Using the same primer pairs as used in the above-cited studies, two additional isolates, NTHi R2846 and NTHi 86-028NP, were examined by Q-PCR. For each isolate the change in *tehB* transcripts was determined between maximal derepression in a Fe/Hm-deplete culture and after Fe/Hm supplementation as described previously (Whitby *et al.*, 2006a, 2009). The Q-PCR results in Table 2 show that three of the five isolates displayed significantly increased levels of *tehB* transcripts during Fe/Hm starvation. The lack of increased transcripts in the other isolates however does not mean that the gene is not transcribed, but may reflect constitutive expression of this gene under both environmental conditions. Examination of the genetic sequences of *tehB* and its associated upstream region in each isolate indicates a high degree of similarity between isolates and thus the differences in apparent regulation are unlikely to be explained by primary structure alone. In addition, no clear Fur-binding site was observed in the promoter region of each *tehB* homologue (data not shown).

Role of *tehB* in resistance to tellurite and oxidation

Our expectation was that mutation of *tehB* would lead to a change in the relative resistance of *H. influenzae* to tellurite toxicity and perhaps increased sensitivity to other chemical oxidants. Because we planned to assess the change in resistance phenotype in growth curve assays, we initially determined whether the mutation affected growth in a non-specific manner. The outcome of a representative experiment to compare the growth of the wild-type Hib strain 10810 and the corresponding *tehB* mutant (HI2129) in our standard growth media is shown in Fig. 1. At a low

Fig. 1. Growth of *H. influenzae* strain 10810 and the corresponding *tehB* deletion mutant HI2129 in hdbHI supplemented with haem. ●, ▲, Wild-type strain with haem at 5 µg ml⁻¹ (●) and at 1 µg ml⁻¹ (▲); ○, △ *tehB* deletion mutant with haem at 5 µg ml⁻¹ (○) and at 1 µg ml⁻¹ (△). Values are mean ± SD for quintuplicate results from representative experiments. The Kruskal–Wallis test was used to compare growth of strains 10810 and HI2129 over the entire growth period at either concentration of haem; comparing the two strains at 5 µg ml⁻¹ $P=0.8258$, and at 1 µg ml⁻¹ $P<0.0001$.

concentration of haem (e.g. 1 µg ml⁻¹), the mutant grew poorly in comparison to the wild-type strain. However, at a non-growth-limiting concentration of haem for the wild-type strain (≥ 5 µg ml⁻¹), the *tehB* mutant grew comparably to the wild-type strain. Thus, the *tehB* mutant exhibited neither an inhibitory effect of haem to support growth relative to the wild-type nor an intrinsic impairment of growth in broth culture. These findings allowed us to conduct growth-based assays with 5 µg haem ml⁻¹, to compare the effects of tellurite and other perturbants on the two strains.

To assess the contribution of TehB to both tellurite resistance and resistance to several chemically distinct oxidants, paired comparisons of growth of the wild-type strain 10810 and its isogenic *tehB* mutant (HI2129) were made during growth with or without addition of potassium tellurite, hydrogen peroxide, tert-butyl peroxide or cumene hydroperoxide. The outcomes of these comparisons are shown in Fig. 2. Resistance to tellurite toxicity was greatly reduced in the mutant strain HI2129 compared to the wild-type strain. The wild-type was able to grow at concentrations as high as 8 µM tellurite, while the mutant was killed at concentrations above 0.5 µM. Fig. 2(a) shows representative growth curves of the wild-type and the mutant strain at 1 and 0.5 µM.

In addition, susceptibility to hydrogen peroxide and two well-characterized lipophilic organic oxidants (Olczak *et al.*, 2002; Wang *et al.*, 2006) was assessed. Sensitivity to

Table 2. Fold change of *tehB* transcripts following addition of iron and haem to an Fe/Hm-restricted culture

<i>H. influenzae</i> strain	Fold change*	
	Microarray	Q-PCR
Rd KW20	-3.34	-5.77
Hib 10810	-1.37	1.01
NTHi R2866	-2.01	-1.07
NTHi R2846	n/a	-2.38
NTHi 86-028NP	n/a	-2.65

*Fold change in transcripts at 20 min after addition of iron and haem to an Fe/Hm-restricted culture. Data for strains Rd KW20, 10810 and R2866 are derived from Whitby *et al.* (2006a, 2009). Microarray refers to results derived from a microarray analysis and Q-PCR data are derived from representative quantitative PCRs.

Fig. 2. Growth of the type b *H. influenzae* strain 10810 and the corresponding *tehB* deletion mutant HI2129 in hdBHI supplemented with 5 $\mu\text{g haem ml}^{-1}$ and additionally supplemented with one of four perturbants as specified. (a) $\blacktriangle$, $\bullet$, Wild-type strain with 1 μM potassium tellurite ($\blacktriangle$) and with 0.5 μM potassium tellurite ($\bullet$); $\triangle$, $\circ$, *tehB* deletion mutant with 1 μM potassium tellurite ($\triangle$) and with 0.5 μM potassium tellurite ($\circ$). (b) $\blacktriangle$, $\bullet$, Wild-type strain with 750 μM hydrogen peroxide ($\blacktriangle$) and with 500 μM hydrogen peroxide ($\bullet$); $\triangle$, $\circ$, *tehB* deletion mutant with 750 μM hydrogen peroxide ($\triangle$) and with 500 μM hydrogen peroxide ($\circ$) (the curve for the *tehB* mutant at 750 μM lies directly under that for the *tehB* mutant at 500 μM). (c) $\blacktriangle$, $\bullet$, Wild-type strain with 50 μM cumene hydroperoxide ($\blacktriangle$) and with 25 μM cumene hydroperoxide ($\bullet$); $\triangle$, $\circ$, *tehB* deletion mutant with 50 μM cumene hydroperoxide ($\triangle$) and with 25 μM cumene hydroperoxide ($\circ$). (d) $\blacktriangle$, $\bullet$, Wild-type strain with 500 μM tert-butyl peroxide ($\bullet$) and with 300 μM tert-butyl peroxide ($\blacktriangle$); $\diamond$, *tehB* deletion mutant with 200 μM tert-butyl peroxide. Values are mean $\pm$ SD for quintuplicate results from representative experiments.

challenge with these oxidants also significantly increased in the *tehB* mutant. Hydrogen peroxide at a concentration of 500 μM had no effect on the growth of the wild-type strain, whereas the *tehB* mutant was unable to grow at the same concentration (Fig. 2b). Qualitatively similar differences in susceptibility of the wild-type and the *tehB* mutant to the impairment of growth by the organic oxidants cumene hydroperoxide and tert-butyl hydroperoxide also were observed (Fig. 2c and d, respectively). The wild-type strain grew normally in broth culture with ≤ 50 μM cumene hydroperoxide, whereas the *tehB* mutant grew only slightly in medium containing this oxidant at 25 μM and not at all

at 50 μM (Fig. 2c). Similarly, addition of ≤ 300 μM tert-butyl hydroperoxide had no effect on growth of the wild-type strain while growth of the *tehB* mutant was prevented by tert-butyl hydroperoxide concentrations as low as 200 μM (Fig. 2d). These data clearly establish a role for TehB in resistance to oxidative damage caused by oxidants bearing no chemical relationship to tellurite.

We also attempted to correct the mutation by complementation as described in Methods. Repeated attempts to transform the Hib strain 10810 *tehB* mutant (HI2129) with the complementation construct failed to generate a com-

plemented strain. However, the same construct successfully inserted into a *tehB* mutant in the *H. influenzae* strain Rd KW20. Studies on the complemented Rd KW20 *tehB* mutant showed restoration of the tellurite-resistance phenotype of this isolate (Fig. 3), as well as restoration of oxidative resistance (data not shown). With respect to haem utilization, the Rd *tehB* mutant was marginally impaired in haem utilization and thus the effect of complementation was difficult to discern. This difference likely reflects the genetic differences between the pathogenic, clinically isolated, type b isolate 10810 and the multiply passaged Rd KW20 isolate, which was originally isolated over 60 years ago (Alexander & Leidy, 1951).

Transcriptional regulation of *tehB* by tellurite and oxidative stress

Since *tehB* was shown to be involved in resistance to both tellurite and various oxidants we sought to determine whether transcription of *tehB* was affected by exposure to either tellurite or oxidants. To assess potential transcriptional regulation of the *tehB* gene in response to exposure to sublethal concentrations of tellurite and the oxidant cumene hydroperoxide, Q-PCR was performed on mid-exponential-phase Hib strain 10810 both prior to and following addition of each perturbant and compared to cultures grown in parallel with no addition. Transcriptional profiles for *tehB*, as well as the control genes *pgdX* (encoding peroxiredoxin) and *hktE* (encoding catalase), both of which have been shown to be part of the *oxyR* regulon (Harrison *et al.*, 2007), are shown in Fig. 4(a–c). The *tehB* gene showed no transcriptional changes on the addition of cumene hydroperoxide and the profile resembled that of the control

Fig. 3. Growth of *H. influenzae* strain Rd and its corresponding *tehB* mutant and complemented *tehB* mutant in hdBHI supplemented with 5 µg haem ml⁻¹ and additionally supplemented with 4 µM tellurite. ●, Wild-type Rd; ▲, Rd *tehB* mutant; △, complemented Rd Δ *tehB* mutant. Values are mean $\pm$ SD for quintuplicate results from representative experiments.

flask (Fig. 4a). Conversely, the control genes *hktE* and *pgdX* showed significant changes in transcriptional level on addition of cumene hydroperoxide (Fig. 4b, c). None of the tested genes, *tehB*, *hktE* or *pgdX*, exhibited transcriptional changes in response to tellurite addition (Fig. 4). To ensure that neither tellurite nor peroxide caused significant cell death over the course of the experiment, viable counts were determined for each flask (Fig. 4d). Viability of strain 10810 was unaffected by addition of either cumene hydroperoxide or tellurite over the time-period during which transcript levels were determined. These data indicate that transcript levels of *tehB* are not affected by challenge with either oxidants or tellurite.

Role of *tehB* in iron and haem utilization

An unexpected finding of the preliminary experiments described above was the reduced ability of the *tehB* mutant, relative to its wild-type progenitor, to utilize low levels of exogenous haem to support aerobic growth (Fig. 1). We had anticipated that this mutant strain might be more susceptible to haem-induced oxidative damage and thus be characterized by a fine balance between meeting its obligate requirement for exogenous haem and its increased susceptibility to haem toxicity. In fact, the mutant strain had a significantly reduced ability to utilize growth-limiting concentrations of haem. This observation led us to evaluate whether utilization of other haem sources typically supporting aerobic growth of *H. influenzae* – haemoglobin, haemoglobin–haptoglobin, and haem–human serum albumin – was impaired in the *tehB* mutant. When haemoglobin, the haemoglobin–haptoglobin complex or haem–human serum albumin was provided as sole haem source a clear deficit in haem utilization was observed in the mutant strain (Fig. 5). At either 20 µg haemoglobin ml⁻¹ or 10 µg haemoglobin ml⁻¹ the *tehB* mutant strain grew significantly less well than the wild-type strain (Fig. 5a). Similarly with either 5 or 10 µg haemoglobin–haptoglobin ml⁻¹ (concentration given as haemoglobin equivalent) the *tehB* mutant showed an increased lag phase and lower maximum optical density compared to the wild-type strain (Fig. 5b). Of all the haem sources tested the most profound impact of the *tehB* mutation was on the utilization of haem–human serum albumin. The wild-type strain grew well in media supplemented with 50, 100 or 200 ng haem–human serum albumin ml⁻¹ (concentration given as haem equivalent) while the mutant strain was unable to use any concentration of haem–human serum albumin tested (Fig. 5c). These data indicate that *tehB* is likely to be important in utilization of physiologically relevant levels of haem sources likely to be encountered *in vivo*.

Importance of *tehB* for virulence in rat models of bacteraemia

An animal model of human invasive disease frequently used in *H. influenzae* virulence studies is the production of bacteraemia in infant rats challenged at 5 days of age (Seale

Fig. 4. (a–c) Transcriptional regulation of (a) *tehB*, (b) *hktE* and (c) *pgdX* in response to the addition of 4 μ M tellurite ($\blacktriangle$) or 200 μ M cumene hydroperoxide ($\square$), and in control flasks to which no addition was made ($\bullet$). (d) Viable counts across the experimental period in the flask supplemented with 4 μ M tellurite ($\blacktriangle$) the flask supplemented with 200 μ M cumene hydroperoxide ($\square$), and in the control flask ($\bullet$). The time scale for panel (d) shows zero time as the point of addition of each perturbant.

et al., 2006). Previously published findings from our laboratory indicate that the severity of bacteraemia occurring after challenge with *H. influenzae* invasive clinical isolates is reduced in weanling rats compared to infant animals. Mutant genes with subtle effects on virulence, such as those with overlapping/redundant functions (e.g. genes involved Fe/Hm acquisition), often exhibit a significantly reduced ability to produce and sustain bacteraemia in weanling rats compared to infant rats (Seale *et al.*, 2006; Morton *et al.*, 2007a, b, 2009). For this reason we assessed the impact on virulence of the *tehB* deletion in both infant rats (5 days old) and weanling rats (30 days old) (Fig. 6).

All infant rats (10/10 for each strain) challenged with either the wild-type 10810 strain or the *tehB* mutant developed bacteraemia by day 1 post-challenge and remained

bacteraemic for at least 8 days. No clearance of bacteraemia produced by either strain was observed in individual infant rats over the 8 day evaluation period. There was a trend toward reduced survival of infant rats challenged with the wild-type strain compared to the mutant strain (Fig. 6a). However, the increased lethality following injection of the wild-type strain was not statistically significant. Bacteraemic titres were 20–97 times higher in infant rats challenged with the wild-type 10810 strain than in rats receiving the *tehB* mutant strain (Fig. 6b). The lowest mean titre for the wild-type strain [$2.0 \times 10^5 \pm 1.2 \times 10^5$ c.f.u. (ml blood)⁻¹], which occurred on day 1 post-challenge, was 32 times higher than that observed in rat pups infected with the *tehB* mutant [$6.3 \times 10^3 \pm 5.0 \times 10^3$ c.f.u. (ml blood)⁻¹]. Maximal mean bacteraemic titre [$1.8 \times 10^6 \pm 3.0 \times 10^6$ c.f.u. (ml blood)⁻¹] for wild-type-infected infant rats occurred on day 3 post-challenge. This titre was 95 times higher than

Fig. 5. Growth of the type b *H. influenzae* strain 10810 and the corresponding *tehB* deletion mutant HI2129 in hdBHI supplemented with one of three haem sources. (a) ●, ▲, Wild-type strain with 20 µg haemoglobin ml⁻¹ (●) and with 10 µg haemoglobin ml⁻¹ (▲); ○, △, *tehB* deletion mutant with 20 µg haemoglobin ml⁻¹ (○) and with 10 µg haemoglobin ml⁻¹ (△). (b) ●, ▲, Wild-type strain with 10 µg haemoglobin-haptoglobin ml⁻¹ (●) and with 5 µg haemoglobin-haptoglobin ml⁻¹ (▲); ○, △, *tehB* deletion mutant with 10 µg haemoglobin-haptoglobin ml⁻¹ (○) and with 5 µg haemoglobin-haptoglobin ml⁻¹ (△) (concentrations of haemoglobin-haptoglobin are given as haemoglobin equivalent). (c) ●, Wild-type strain with 100 ng haem-human serum albumin ml⁻¹; ○, *tehB* deletion mutant with 100 ng haem-human serum albumin ml⁻¹. Values are mean ± SD for quintuplicate results from representative experiments. The Kruskal-Wallis test was used to compare growth of strains 10810 and HI2129 over the entire growth period at each concentration of all haem sources. At all concentrations of all haem sources growth of the mutant strain was statistically significantly different from growth of the wild-type strain ($P < 0.0001$ for all analyses).

rats were challenged with each of the two strains (Fig. 6c). All weanling rats (10/10) challenged with the wild-type strain exhibited bacteraemia within 24 h of challenge, and all but one animal remained bacteraemic through day 6 post-challenge. Wild-type bacteraemic titres ranged from 10^4 to 10^5 c.f.u. (ml blood)⁻¹ over this period (data not shown). In contrast, only 29% (4/14) of weanlings challenged with the same infective dosage of the *tehB* mutant were bacteraemic on day 1. Bacterial titres in these bacteraemic animals were ≤ 300 c.f.u. (ml blood)⁻¹ (data not shown). By day 4 post-challenge, 93% (13/14) of the weanling rats had completely cleared the mutant strain. The differences between the *tehB* mutant and the wild-type strain in establishment and persistence of bacteraemia were statistically highly significant on each day of the experiment (Fig. 6c).

DISCUSSION

SAM-dependent methyltransferases are intimately involved in many cellular processes in both prokaryotes and eukaryotes. These processes include such diverse functions as metabolism and maturation of novel tRNAs, control of protein trafficking, signal transduction, modification of DNA, protein and small molecules, as well as general biosynthesis. Based on sequence homology, it is probable that the *H. influenzae tehB* gene encodes a SAM-dependent methyltransferase involved in small-molecule modification. The functional importance of *tehB* is attested to by the fact that homologues of *tehB* exist in all of the *Pasteurellaceae* examined (with the exception of *H. ducreyi* and *Hist. somni*). Additional homology searches indicate that TehB homologues are widely distributed across many bacterial species (data not shown). In several organisms *tehB* has been implicated in the detoxification of tellurite via a presumed methylation-based pathway (Dyllick-Brenzinger

the mean bacterial titre of infant rats infected with the mutant strain [$1.9 \times 10^4 \pm 6.0 \times 10^3$ c.f.u. (ml blood)⁻¹]. These differences in bacteraemic titre were statistically highly significant. Taken together these data suggest that virulence of the mutant strain is reduced compared to the wild-type strain in the infant rat.

A more dramatic demonstration of the deleterious effect of the *tehB* deletion on virulence was observed when weanling

Fig. 6. Comparison of the severity of bacteraemia following challenge by the 10810 wild-type or *tehB* mutant strains in 5-day-old infant and 30-day-old weanling rats. (a) Survival of infant rats following intraperitoneal challenge with 100 c.f.u. of either strain ($n=10$ per strain at challenge). Although survival was lower for infant rats challenged with the wild-type strain, this difference did not reach statistical significance by day 8 ($P=0.11$, Fisher exact test). Open bars, wild-type; filled bars, *tehB*. (b) Bacteraemic titres in infant rats from the same animals as challenged in (a). Mean $\pm$ SD titre values [c.f.u. (ml blood)⁻¹] were significantly higher ($P<0.0009$, Kruskal–Wallis test) at each time point after challenge with the wild-type ($\bullet$) than after challenge with the *tehB* mutant ($\circ$). The overall difference between the bacteraemic titres of the wild-type and mutant strain for the 8 day test period also was highly significant ($P<0.0001$, Kruskal–Wallis test). The horizontal bars indicate the means for the wild-type (upper bars) and the *tehB* mutant (lower bars). (c) Clearance of bacteraemia in weanling rats challenged with either the 10810 wild-type or *tehB* mutant strain. The percentage of rats remaining bacteraemic was significantly higher at each time point for animals challenged with the wild-type strain ($n=10$) compared to the *tehB* mutant ($n=14$) (* $P<0.001$; ** $P<0.0001$; *** $P<0.00001$, Fisher exact test). Open bars, wild-type; filled bars, *tehB*.

et al., 2000; Liu *et al.*, 2000; Liu & Taylor, 1999). While methylated tellurium intermediates have yet to be identified, the loss of tellurite from solution is SAM dependent (Liu *et al.*, 2000). Studies on other tellurite-resistance determinants, distinct from *tehB*, from various bacteria have indicated a potentially broad range of phenotypic traits that are distinct from detoxification of tellurite; these have been extensively reviewed by Zannoni *et al.* (2008).

In this study we show that deletion of the *tehB* gene in *H. influenzae* strain 10810 results in several notable phenotypic changes. Growth studies show a clear impact of the *tehB* mutation on resistance to tellurite. Within the human host, which is the only natural environment for *H. influenzae*, it is unlikely that *H. influenzae* is exposed to tellurite. Thus, it is probable that tellurite resistance represents a secondary phenotypic trait of TehB.

Since it has been proposed that tellurite toxicity is primarily due to oxidative damage, the *tehB* mutant was further examined to determine its resistance to various oxidative agents. In each case, the *tehB* mutant exhibited increased sensitivity to the agents tested. These findings support the view that resistance to tellurite is likely to be mediated via resistance to resulting oxidative damage rather than detoxification of the metal oxide itself. Further evidence for this proposal is the observed presence of small black deposits following growth of *H. influenzae* in the presence of sublethal levels of tellurite (data not shown). These deposits have been reported with other organisms challenged with tellurite and are presumed to be insoluble tellurium (elemental) (Chasteen & Bentley, 2003; Amoozegar *et al.*, 2008). The deposits were observed in cultures of both the wild-type and the *tehB* deletion mutant strains and indicate that the reduction of tellurite to the insoluble metalloid proceeds in the absence of *tehB*. In a recent review of tellurite toxicity, Chasteen *et al.* (2009) propose a pathway(s) for the reductive detoxification of tellurite to tellurium involving the cooperative effect of multiple enzymes. Proposed pathway(s) include nitrate reductase, catalase, dihydrolipoamide dehydrogenase and other unspecified reductases (Chasteen *et al.*, 2009). As a byproduct of this process, reduced thiols and ROS are generated in the cell. The ROS can then interact with, and damage, other cellular components such as nucleic acids, proteins and lipids. As well as direct effects of ROS, the release of iron, from damaged Fe-S clusters, can further participate in oxidative damage via the Fenton and/or Haber-Weiss reaction(s), liberating hydroxyl radicals. Unchecked, these processes would lead to cellular damage and ultimately, cell death.

Transcription of the *tehB* gene in *H. influenzae* did not respond to challenge with oxidants. Despite its apparent role in protection against oxidative damage, *tehB* is not a member of the OxyR regulon. However, when the bacteria were grown in Fe/Hm-restricted media, transcription of *tehB* increased in several isolates. The observed regulation of *tehB* transcription by Fe/Hm, coupled with the reduced

ability of the *tehB* mutant to utilize various haem sources, supports an important role of *tehB* in haem acquisition or utilization. Increased expression of *tehB* during iron starvation has also been reported for *Act. pleuropneumoniae* and *P. multocida* (Deslandes *et al.*, 2007; Paustian *et al.*, 2001). For neither *Act. pleuropneumoniae*, *P. multocida* nor *H. influenzae* was there a clearly identifiable Fur sequence upstream of the *tehB* gene, and the precise mechanism of Fe/Hm-based transcriptional regulation of *tehB* requires further study.

The *tehB* mutant strain also exhibited a reduced ability to establish and/or maintain bacteraemia in both 5-day-old infant and 30-day-old weanling rats. The impact of the mutation with respect to bacteraemia in 30-day-old rats was greater than that in 5-day-old rats. We have noted similar differential impacts on virulence in 5-day-old and 30-day-old rats in studies of several proteins related to haem acquisition (Morton *et al.*, 2007a, b, 2009; Seale *et al.*, 2006). For example a mutant lacking the periplasmic haem-binding protein HbpA exhibited wild-type levels of virulence in 5-day-old rats but was significantly attenuated compared to the wild-type strain in 30-day-old rats (Morton *et al.*, 2009). Thus, the differential impact of the *tehB* mutation on virulence in 5- and 30-day-old rats may reflect the observed impact of the *tehB* mutation on haem utilization. However, at this point we cannot definitively state whether resistance to oxidative stress, reduced ability to acquire haem, or other, as yet unknown, phenotype(s) are responsible for the observed impact of *tehB* mutation on virulence.

The data reported herein indicate that *H. influenzae* requires a functional TehB to facilitate haem assimilation, resistance to oxidation, and virulence. Indeed, the widespread distribution and conservation observed for *tehB* across prokaryotes would suggest that the encoded function is likely to play an important role in many microbial species. It is important to reiterate that many of the bacterial species are highly unlikely to encounter tellurite in their natural environment. This suggests that the biologically relevant role of *tehB* is distinct from the phenotype of tellurite resistance which has been associated with this gene. Based on our findings it is possible that TehB acts in a regulatory capacity by methylation of currently unknown target(s) as an important step for the correct functioning of diverse pathways. Further studies will seek to identify the mechanism by which *tehB* transcription is affected by Fe/Hm status as well as to define the target molecule(s) of the encoded methyltransferase.

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