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Transcription of Genes Encoding Iron and Heme Acquisition Proteins of *Haemophilus influenzae* during Acute Otitis Media

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Unencapsulated *Haemophilus influenzae* is the second most common etiologic agent of otitis media in children. *H. influenzae* requires heme for aerobic growth in vitro and is able to utilize hemoglobin and complexes of heme-hemopexin, heme-albumin, and hemoglobin-haptoglobin and ferritransferrin as sources of iron and heme in vitro. Several of the acquisition mechanisms have been characterized and been shown to be heme repressible in vitro. However, little is known about the expression of heme and/or iron acquisition mechanisms during infections in the middle ear. This study was performed to determine if the genes encoding heme and iron acquisition proteins are transcribed during in vivo growth and to compare these findings with those for samples grown in vitro. Reverse transcriptase PCR (RT-PCR) was used to analyze total RNA fractions derived from in vitro- and in vivo-grown *H. influenzae*. Genes encoding the transferrin-binding proteins TbpA and TbpB, the 100-kDa hemopexin-binding protein HxuA, and the hemoglobin-binding protein HgpA were transcribed during otitis media. Twelve middle ear fluid samples were analyzed by blind RT-PCR to determine the transcriptional status of these genes in *H. influenzae* during otitis media. Five isolates had transcripts corresponding to *tbpA*, *tbpB*, and *hxuA*. The presence of *hgpA* transcripts was variable, depending on the presence of *hgpA* in the genome of the *H. influenzae* isolate. Samples without *H. influenzae* gene transcripts contained other etiologic agents commonly causing otitis media. These data demonstrate that *H. influenzae* iron and/or heme acquisition genes are transcribed during otitis media and suggest that the microenvironment during acute otitis media starves *H. influenzae* of heme.

Vaccines based upon the type b capsular polysaccharide have reduced the incidence of invasive disease caused by *Haemophilus influenzae* strains with the type b capsule (28, 31). Although adults have presumably acquired immunity, type b strains still predominate in blood isolates (21). Unencapsulated, nontypeable, *H. influenzae* isolates are unaffected by the available vaccines and continue to be a significant cause of neonatal sepsis, pneumonia in adults, and otitis media in children (8, 40, 41, 43). Otitis media is the cause of 33% of visits by children to health care centers (40). By 3 years of age, 80% of children have had at least one episode and 40% have had three or more episodes. Of these cases approximately 20% are attributable to *H. influenzae*, and 90% of the isolates are unencapsulated (5, 13).

H. influenzae has an absolute requirement for an exogenous source of heme during aerobic growth (7). In vivo heme is intracellular, in the form of hemoglobin or heme-containing proteins such as cytochromes. Thus, its availability to invading microorganisms is strictly limited (2, 22). Hemoglobin released from erythrocytes is avidly bound by the serum protein haptoglobin, and the resulting complex is cleared by hepatocytes (2, 33). Free heme is bound by the serum proteins hemopexin and albumin and is also cleared by hepatocytes (2). Heme, hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, and heme-albumin complexes are all capable of satisfying the heme requirement of *H. influenzae*, and ferritransferrin satisfies the iron requirement (35, 39). *H. influenzae* expresses outer membrane proteins which bind the potential iron and heme sources. We have recently characterized protein HgpA, which binds hemoglobin and hemoglobin-haptoglobin (20). Proteins which bind hemoglobin-haptoglobin (HhuA), hemopexin (HxuA), transferrin (TbpA and TbpB), and heme have also been identified (6, 14, 23, 27, 36). While HhuA and HxuA are expressed under heme-replete conditions, the hemoglobin-binding protein and transferrin-binding proteins are repressible by high levels of heme (6, 12, 20, 27, 30). These proteins are readily expressed in vitro, but their in vivo expression is not well studied. Holland et al. detected circulating antibodies to the transferrin-binding proteins in sera from convalescent patients with invasive type b disease, and they showed the binding of transferrin to organisms isolated without subculture from the peritoneal cavities of rats with experimental infection (18). However, there are no data on the regulation of expression of these proteins in vivo.

Currently it is difficult to determine the level of gene regulation or expression of an individual protein in vivo. This is a major barrier to investigating proteins that are not constitutively expressed. To overcome this problem, several protocols that utilize reverse transcriptase PCR (RT-PCR) have been developed (1, 19, 42). The high degree of sensitivity of this technique allows for the identification of specific mRNA in samples of low bacterial count including clinical samples, which may provide information regarding bacterial physiology at the time of infection (34). The objective of this study was to apply the technique of RT-PCR to middle ear aspirates from children diagnosed with otitis media to investigate the in vivo transcription of *H. influenzae* genes encoding iron and heme acquisition-related proteins.

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TABLE 1. Bacterial strains

Strain	Characteristic	Source
H. influenzae		
HI689	Type b	J. Musser
HI1699	NT[a]	This study
HI1700	NT	This study
HI1701	NT	This study
HI1702	NT	This study
HI1703	NT	This study
B. catarrhalis		
NH212		This study
NH216		This study
NH217		This study
S. pneumoniae		
NH213		This study
NH215		This study
S. pyogenes		
NH214		This study

[a] NT, nontypeable.

MATERIALS AND METHODS

Study population. From February to September 1994, children aged 6 months to 8 years, seen for acute otitis media at the pediatric clinic of the University of Texas Medical Branch at Galveston, were enrolled prospectively in a drug study trial comparing the efficacy of azithromycin with that of cefaclor. The study was approved by the Institutional Review Board. The subjects were enrolled after parental or guardian permission was obtained.

Collection of middle ear fluid. Tympanocentesis was performed under sterile conditions to obtain middle ear fluid prior to beginning the antimicrobial therapy and at any time during a 4-week follow-up period to check for signs of clinical nonresponse, relapse, or recurrence. A portion of the fluid was processed immediately by the clinical microbiology laboratory to culture common middle ear pathogens. The remainder of the fluid was diluted in phosphate-buffered saline and immediately frozen at −70°C for subsequent analysis by RT-PCR.

Bacterial strains and growth conditions. All bacterial strains used in this study are listed in Table 1. *H. influenzae* type b strain HI689 has been described previously (30). Bacterial strains were routinely maintained on brain heart infusion (BHI) agar (Difco, Detroit, Mich.) supplemented with 10 μg of both hemin and β-NAD per ml. For experiments in heme-replete medium, *H. influenzae* was grown at 37°C in BHI broth (Difco) supplemented with 10 μg of β-NAD and 10 μg of hemin per ml (supplemented BHI; sBHI). Heme-restricted growth of *H. influenzae* took place in BHI supplemented with 10 μg of β-NAD and 0.1 μg of hemin per ml (hemin-restricted BHI; hrBHI). Viability counts were performed on chocolate agar plates (BBL, Becton Dickinson, Cockeysville, Md.).

DNA isolation. Bacterial genomic DNA was isolated with the DNA Now reagent (Biogentex, Seabrook, Tex.) as directed by the manufacturer. DNA concentrations were assessed spectrophotometrically with a Shimadzu UV-1201S spectrophotometer with a DNA/protein program pack (Kyoto, Japan).

Isolation of total RNA. In vitro-grown *H. influenzae* organisms were pelleted and resuspended to an optical density at 605 nm (OD_{605}) of 1.0. One milliliter of the sample was centrifuged, and the pellet was treated with Trizol reagent (Gibco-BRL, Gaithersburg, Md.). The organic and aqueous phases were separated, and the DNA was isolated by ethanol precipitation from the organic phase. Total RNA was precipitated from the aqueous phase and resuspended in RNAase-free water (U.S. Biochemicals, Cleveland, Ohio). Contaminating DNA was digested by incubation with 2 U of RNAase-free DNAase (Gibco-BRL) for 30 min at 37°C; DNAase was heat inactivated at 65°C for 10 min. The RNA was then used as a template in RT-PCR. Total RNA was extracted from the clinical samples by mixing the sample 1:4 with Trizol reagent and was processed as described above. The RNA concentration was determined spectrophotometrically.

Primer design. The primers used in this study are listed in Table 2 and were designed by using the sequences available in the GenBank database. Primers were designed to have similar melting temperatures and to show little or no homology with other genes contained within the GenBank database, which includes the entire genomic sequence of *H. influenzae* Rd (11). Oligonucleotide primers were synthesized by the Molecular Biology Resource Facility, University of Oklahoma Health Sciences Center, Oklahoma City, Okla.

RT-PCR. For in vitro-grown bacteria, several independent RT-PCRs were performed. Due to the limited quantity of clinical samples, each was analyzed once and subjected to a negative control lacking RT. To ensure comparable results between samples for which the same primer pair was used, a bulk mixture containing all the reaction components was prepared and aliquoted into separate tubes prior to the addition of the template. An aliquot, 2 μg or less, of the total RNA from in vitro-grown organisms or the entire preparation from the in vivo clinical sample, in 12.5 μl, was mixed with 1 μl of random primer (1 μg/ml) (Amersham Life Science, Arlington Heights, Ill.), heat denatured at 75°C for 5 min, and cooled on ice. To the cooled mixture was added 4 μl of First Strand synthesis buffer (Gibco-BRL), 0.5 μl of RNAase inhibitor (10 U/μl) (Gibco-BRL), 1 μl of a 10 mM concentration of each dNTP, and 1 μl of Superscript II RT (200 U/μl) (Gibco-BRL). The reaction mixture was incubated at 42°C for 1 h. For the clinical samples, 4 μl was used as the template source in the PCR. For the in vitro-derived RNA, 1-μl samples, or dilutions thereof, were analyzed by PCR. PCR was performed with the RapidCycler Thermocycler (Idaho Technology Inc., Idaho Falls, Idaho) in a cocktail containing a 1 μM concentration of each specific primer, a 100 μM concentration of each dNTP, 3 mM Mg-buffer, and 2 U of *Taq* polymerase (U.S. Biochemical). Samples in 50-μl capillary tubes were subjected to an initial 5-min denaturing step at 94°C. The samples were cycled 30 times with an annealing step of 55°C for 10 s, extension at 72°C for 1 min, and a denaturing step at 94°C for 10 s. Products were analyzed by loading 20 μl of the PCR mixture into adjacent wells of a 1% agarose gel. Controls consisting of reaction mixtures with no template and reaction mixtures with the RNA preparation without the cDNA synthesis step were tested with each primer pair.

Southern blotting. DNA was digested with *Eco*RI restriction enzyme, separated on agarose gels (0.8% [wt/vol] agarose) in TBE buffer (0.045 M Tris-borate, 0.001 M EDTA), and transferred to Magnagraph nylon membranes (Micron Separations, Inc., Westbrook, Mass.) by the method of Southern as described by Sambrook et al. (37). The enhanced chemiluminescence random prime labeling kit (Amersham) was used as directed by the manufacturer to label an *hgpA* gene DNA probe. The hybridization temperature was 60°C, and stringency washes were with 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0])–0.1% (wt/vol) sodium dodecyl sulfate for 15 min and 0.5× SSC–0.1% (wt/vol) sodium dodecyl sulfate for 15 min, both at 60°C. Hybridization was detected with enhanced chemiluminescence nucleic acid detection reagents (Amersham) as directed by the manufacturer. Blots were subsequently exposed to X-ray film (Fuji Photo Film Co., Tokyo, Japan).

RESULTS

Amplification of gene fragments from *H. influenzae* genomic DNA. Genomic DNA isolated from *H. influenzae* type b strain HI689 was used to optimize the PCR. To analyze several genes at one time, the primers were designed with similar melting temperatures. A single product of the predicted size (Table 2) was observed repeatedly for each primer pair (Fig. 1).

RT-PCR of *H. influenzae* grown in vitro. Serial dilutions of mid-logarithmic-phase *H. influenzae* HI689 cultures grown in sBHI and hrBHI were plated onto chocolate agar to determine CFU per ml. The OD_{605} of the culture was determined, and approximately 1 OD unit of cells was pelleted, resuspended in Trizol reagent, and stored overnight at −20°C. After the viable counts were determined the Trizol-suspended cells were processed to isolate total RNA and DNA, which were resuspended to a concentration equivalent to 10^7 CFU/ml. Following DNAase treatment, cDNA was prepared from RNA preparations representing total RNA derived from 10^5 bacteria

TABLE 2. Primer sequences used in RT-PCR

Gene	Sense primer (5′ to 3′)	Antisense primer (5′ to 3′)	Size of amplicon (bp)
*hin*dIIIM gene	GTGCATTAGGAGGAACATCTTCAGC	AATGTAGTACCACTACCAGCAAAGG	663
tbpA	TTCCCTACGGTTGGTATGC	CTGATGCTGCTGCGTAGC	477
tbpB	GGTCTCCGTACTCCCAGT	CACAACGGCAGCTCTTGC	456
hxuA	ATTGATGGGGGGGTATCCC	CCAGTTGGAGATATACCCTC	435
hgpA	GCTTCACAAATATCCTTAG	GAATTCCAAACTGAACTGAC	505

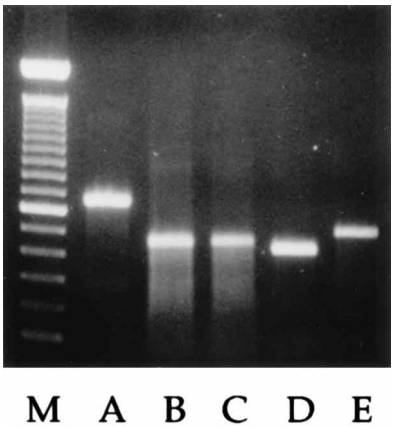

FIG. 1. Agarose gel (1%) electrophoresis showing the PCR amplicons derived from *H. influenzae* HI689 genomic DNA with primer pairs directed to the *hin*dIIIM gene (lane A), *tbp*A (lane B), *tbp*B (lane C), *hxu*A (lane D), and *hgpA* (lane E). Lane M contains a 100-bp molecular ladder, the 600-bp band being the most intense.

and was suspended in a final volume of 20 μl. One microliter of the cDNA was used as the template in the individual PCRs under the conditions previously optimized with the genomic DNA. To ensure that there was an equal amount of cDNA in each sample, PCR with the *hin*dIIIM (32) gene primer pair was performed. Previous studies confirmed this gene to be constitutively expressed under the growth conditions employed in this study (unpublished observation). The results demonstrated an equal quantity of each product (data not shown). By comparison with the quantity of amplicons derived from the genomic DNA (data not shown), there was a significantly lower quantity of products from the RT-PCR, indicating that the reaction had not reached a saturation of product and was still within the exponential phase of amplification. RT-PCR products derived from reaction mixtures with primers in *tbpA*, *tbpB*, *hxuA*, and *hgpA* were analyzed similarly. These reaction mixtures were separated on agarose gels alongside a product derived from a genomic DNA template (Fig. 2). This approach consistently resulted in greater amounts of amplified product from the heme-restricted samples, with the exception of the *hin*dIIIM gene PCR. By comparison with the products of genomic PCR, the RT-PCR products were in the exponential phase of amplification.

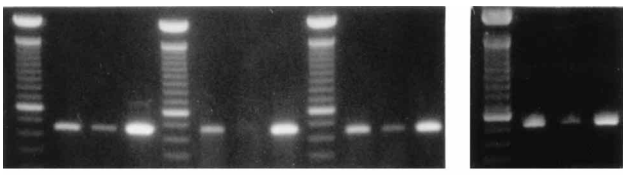

FIG. 2. RT-PCR analysis of *H. influenzae* HI689 after heme-supplemented (hs) or heme-restricted (hr) growth compared to genomic PCR analysis with the same primer pair. Amplicons from the *tbpA* primers in lanes A, B, and C result from RT-PCR after hr growth, RT-PCR after hs growth, and genomic PCR, respectively. Amplicons from the *tbpB* primers in lanes D, E, and F result from RT-PCR after hr growth, RT-PCR after hs growth, and genomic PCR, respectively. Amplicons from the *hxuA* primers in lanes G, H, and I result from RT-PCR after hr growth, RT-PCR after hs growth, and genomic PCR, respectively. Amplicons derived from *hgpA* primers in lanes J, K, and L result from RT-PCR after hr growth, RT-PCR after hs growth, and genomic PCR, respectively. Lanes M contain a 100-bp molecular ladder, the most intense band being the 600-bp band.

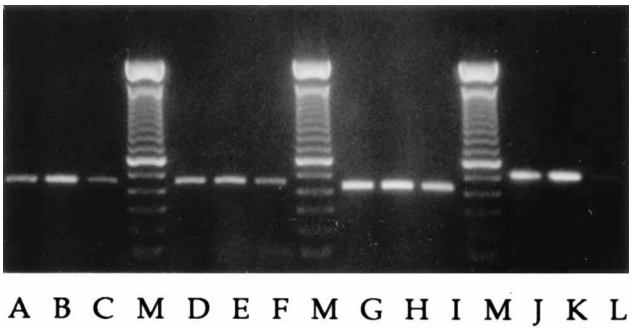

FIG. 3. Agarose gel (1%) electrophoresis of RT-PCR amplicons derived from 1,000 CFU equivalents of RNA from the CS1701 clinical sample and from the heme-supplemented (hs) and heme-restricted (hr) growth of *H. influenzae* HI689. Lanes: A, CS1701; B and C, hr and hs growth, respectively, of HI689 amplified with the *tbpA* primers; D, CS1701; E and F, hr and hs growth, respectively, of HI689 amplified with the *tbpB* primers; G, CS1701; H and I, hr and hs growth, respectively, of HI689 amplified with the *hxuA* primers; J, CS1701; K and L, hr and hs growth, respectively, of HI689 amplified with the *hgpA* primers; M, 100-bp molecular ladder, the most intense band being the 600-bp band.

To ensure there was no residual DNA contamination of isolated RNA, a 2-μl sample of the DNAase-treated total RNA extract was analyzed in the PCR mixture without prior cDNA synthesis. There was no detectable product in any of these reaction mixtures (data not shown).

RT-PCR of clinical samples. The method that used RT-PCR to determine gene transcription in vitro was used to analyze gene transcription during acute otitis media. The investigator performed these studies in the blind with respect to the *H. influenzae*-positive cultures. The number of bacteria within the sample was estimated to prevent saturation of the PCR by too high a template concentration. The quantity of PCR-amplified product from total RNA purified from clinical samples was compared to the quantity of product from DNA prepared from organisms grown in vitro. By using the *hin*dIIIM gene primer pair, a standard set of serially diluted genomic DNA across the range of 10^5 to 1 CFU equivalents were amplified by PCR. The DNA isolated from the clinical isolates was amplified with the same primers. A comparison of the intensities of the clinical samples with the standards suggested that five of the clinical samples had detectable *H. influenzae* genomic DNA with bacterial counts in the range of 100 to 1,000 cells per ml (data not shown). To determine if the genes of interest were being transcribed in the clinical samples, an aliquot of each containing the same quantity of total RNA as that used in the *hin*dIIIM gene analysis was used in the RT-PCR. The products were separated on agarose gels beside RT-PCR products derived from 1,000 CFU equivalents of RNA from organisms grown in sBHI and hrBHI. Figure 3 shows the results of RT-PCR for one clinical sample together with the products derived from HI689 grown in heme-replete and heme-depleted media. The size of the amplified DNA derived from the clinical samples were the same as that of amplified DNA derived from genomic PCR, and the intensity was comparable to that of the hrBHI-derived RT-PCR product. All samples generating an RT-PCR product with the *H. influenzae* primers were later confirmed as culture positive for *H. influenzae*. RT-PCR with the clinical samples containing *H. influenzae* yielded bands corresponding to *tbpA*, *tbpB*, and *hxuA*. However *hgpA* showed variable patterns of detection. To determine if the samples not yielding a product with the *hgpA* primers lacked detectable expression of the gene or if they reflected the absence of the gene in the genome, the cultured isolate was examined by PCR with the *hgpA* primers and by Southern blotting with the *hgpA* gene as

TABLE 3. Comparison of RT-PCR and genomic PCR results

Template source strain	Result[a] with primer pair for:								Culture result
	tbpA		*tbpB*		*hxuA*		*hgpA*		
	CS[b]	G[c]	CS	G	CS	G	CS	G	
HI1699	+	+	+	+	+	+	−	−	*H. influenzae*
HI1700	+	+	+	+	+	+	+	+	*H. influenzae*
HI1701	+	+	+	+	+	+	+	+	*H. influenzae*
HI1702	+	+	+	+	+	+	+	+	*H. influenzae*
HI1703	+	+	+	+	+	+	−	−	*H. influenzae*
NH212	−	NA	−	NA	−	NA	−	NA	*B. catarrhalis*
NH213	−	NA	−	NA	−	NA	−	NA	*S. pneumoniae*
NH214	−	NA	−	NA	−	NA	−	NA	*S. pyogenes*
NH215	−	NA	−	NA	−	NA	−	NA	*S. pneumoniae*
NH216	−	NA	−	NA	−	NA	−	NA	*B. catarrhalis*
NH217	−	NA	−	NA	−	NA	−	NA	*B. catarrhalis*

[a] +, detectable amplicon; −, absence of an amplicon; NA, not analyzed.
[b] CS, clinical sample. The RT-PCR amplicon was derived from an ear isolate RNA sample.
[c] The PCR amplicon was derived from genomic DNA.

a probe (37). The isolates from specimens lacking *hgpA* products in the RT-PCR mixture failed to yield an amplicon and did not hybridize with the gene-specific probe (data not shown), thus indicating an absence of the gene in the genome. A comparison of the results obtained by RT-PCR and genomic PCR for the genotype of each isolate is shown in Table 3. No amplified products were detected by RT-PCR analysis of the other middle ear aspirates. Culture of these samples variously yielded *Branhamella catarrhalis*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae.*

DISCUSSION

Expression of TbpA, TbpB, and HgpA of *H. influenzae* is repressible by high levels of heme during in vitro culture, while HxuA is apparently expressed in an iron- and heme-rich environment in vitro (6, 12, 30). However, the assessment of the level of protein expression in vivo is problematic due to the small quantities of bacteria. To overcome this barrier, the technique of RT-PCR was applied to the study of clinical samples. The technique relies on the observation that the quantity of product is proportional to the amount of template over the exponential phase of amplification if all other parameters are kept constant (1, 19, 42). The lack of an internal control within the RT-PCR mixture precludes absolute quantitation of the initial template, preventing conclusions regarding the transcriptional regulation of these genes. However, visual comparisons of the quantity of RT-PCR-amplified product of iron- and heme-regulated genes from bacteria cultured in heme-depleted and heme-replete conditions consistently demonstrated an increase in the amount of transcripts following growth in the heme-depleted conditions. The difference in the levels of transcript between the two growth conditions was less dramatic for *hxuA* than for the other genes (Fig. 3), consistent with the results of Cope et al., who demonstrated the expression of the corresponding protein in heme-replete conditions (6). While a precise estimate of the concentrations of template in the RT-PCR mixtures is not possible, it appears that the transcription of the *hin*dIIIM gene was unaffected by the level of heme in the growth media, while the transcription of *tbpA*, *tbpB*, *hgpA*, and *hxuA* was repressible by heme. These data suggest the possibility of a common regulatory mechanism occurring at the level of the transcription of the genes. In many bacterial species genes encoding heme and iron uptake-related proteins are regulated by the ferric uptake regulator (Fur), which, in the presence of iron, binds at specific sites in the gene promoter leading to repression of transcription (26). A *fur* homolog has been identified in the genome of *H. influenzae* Rd (11), and putative Fur binding sites have been identified preceding *tbpB*, *hxuA*, and *hgpA* (6, 14, 20). The functionality of the putative Fur binding sites and the *fur* homolog has not been demonstrated. The regulation of heme and iron uptake proteins in *H. influenzae* remains poorly characterized, and further studies are necessary to address this issue.

There have been few direct studies on the expression of microbial iron acquisition mechanisms in vivo during the infection of the natural host. Siderophores have been isolated directly from the sputum of patients with cystic fibrosis and from peritoneal washes of guinea pigs with experimental *Escherichia coli* infection (15, 17). In addition, iron-regulated outer membrane proteins are expressed by *Pseudomonas aeruginosa* isolated directly from cystic fibrosis sputum without subculture (4). An immunological response to iron-regulated proteins in both naturally occurring and experimental disease has been demonstrated with a wide variety of microbial species (9, 10, 16, 18, 24, 25, 44, 45). The results of the current study demonstrate that there is transcription of *tbpA*, *tbpB*, *hxuA*, and *hgpA* during acute otitis media. The results are consistent with the up-regulation of expression of these proteins displayed by cultures experiencing heme starvation in vitro. Transcripts of *tbpA*, *tbpB*, and *hxuA* were detected in all five clinical samples containing *H. influenzae*. Two samples lacked transcripts of *hgpA*. The strains cultured from these two clinical samples, HI1699 and HI1703, were found to lack a detectable genomic copy of *hgpA* by both Southern and PCR analysis, and these isolates were later identified as two independent isolates collected from the left and right middle ears of the same patient. Although the conditions during otitis media appear to favor transcription, demonstration of in vivo transcription of *tbpA*, *tbpB*, *hxuA*, and *hgpA* does not necessarily indicate the expression of the encoded protein. For example, an additional level of regulation of *hgpA* has been proposed (20). The presence of a series of CCAA repeating units followed by stop codons in two frames within the structural gene *hgpA* suggests that this gene may exhibit slip-strand regulation (29). Thus, protein expression from the mRNA would be dependent on the frame of the gene, leading to either a native protein or a truncated peptide (20). Evidence of posttranscriptional regulation of *tbpA*, *tbpB*, and *hxuA* has not been reported.

H. influenzae utilizes hemoglobin, hemoglobin-haptoglobin, heme-hemopexin, and heme-albumin as heme sources (39). The availability of these heme sources during inflammation in the ear is not known. Transudate in the middle ear contains immunoglobulins and complement (3) and, in a similar manner, may contain serum proteins binding heme or iron. Recently it has been reported that the middle ear effusions of children prone to otitis media have a median lactoferrin concentration of 234 mg/liter, a figure which is significantly higher than that observed for healthy children (38). Regardless of the actual source available, *H. influenzae* may express several mechanisms of acquisition of heme and iron. For example, in vitro heme starvation of *H. influenzae* results in the expression of transferrin-binding and hemoglobin-binding proteins, although neither of these sources is available during growth (12, 30). The source of iron and heme available to *H. influenzae* during otitis media is unknown.

In summary, we have demonstrated the utility of RT-PCR to investigate gene transcription in vivo. Transcription of the *tbpA*, *tbpB*, *hxuA*, and *hgpA* genes of *H. influenzae* is apparently repressible by high levels of heme in vitro. Transcription of

these genes occurs during acute otitis media in children, suggesting that the microenvironment of otitis media starves *H. influenzae* of heme. To accurately assess the degree of up-regulation of these genes, a suitable internal control is required. We are currently constructing recombinant competitors of these genes to facilitate such studies.

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