

The *tonB* Gene of *Haemophilus parainfluenzae* Demonstrates Strong Sequence Identity with that of *Haemophilus influenzae*

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ABSTRACT

Haemophilus parainfluenzae is a gram-negative bacterium that is a normal habitant and an opportunistic pathogen of the respiratory tract. *H parainfluenzae* has been implicated in several human diseases including infective endocarditis, biliary disease, and exacerbations of chronic obstructive pulmonary disease. The ability of *H parainfluenzae* to acquire iron from its environment is essential to its survival. TonB, a protein located in the periplasm, has been identified as a virulence factor in *Haemophilus influenzae* and is responsible for interacting with receptors in the outer membrane to participate in the scavenging of iron sources. Previous published work involving determination of the *tonB* sequence of multiple clinical isolates of *H influenzae* and *H parainfluenzae* has indicated a low level of sequence identity between the two species and among isolates of the same species. In this current study, we show that *tonB* of *H influenzae* and *H parainfluenzae* clinical strains is high-

ly conserved at the nucleic acid and amino acid levels.

INTRODUCTION

Haemophilus parainfluenzae is a gram-negative coccobacillus frequently found as normal flora in the oropharynx of human hosts.^{1,2} *H parainfluenzae* has a growth requirement of exogenous nicotinamide adenine dinucleotide (NAD), allowing for distinction from *Haemophilus influenzae*, which requires hemin in addition to NAD.^{1,3} Although *H parainfluenzae* is generally regarded as normal flora, it has been implicated in several human diseases including infective endocarditis, biliary disease, and exacerbations of chronic obstructive pulmonary disease.^{1,4} *H parainfluenzae* incorporates lipooligosaccharide onto its surface, which functions as the primary virulence determinant produced by the bacteria. Due to the limited classical virulence factors exhibited by the bacteria, the ability of the organism to persist in the host through metabolic and physiological processes serves an important role in the virulence of *H parainfluenzae*.

Iron is an essential cofactor of enzymes involved in many cellular

Table 1. Bacterial Strains and Plasmids Used in the Study

Strain or plasmid	Description
<i>Escherichia coli</i> DH5 α	Bench strain
NtHi KW20	Genome type strain
NtHi 2019	Clinical isolate
NtHi 3198	Clinical isolate
NtHi egan	Clinical isolate
<i>H influenzae</i> A2	Clinical isolate
<i>H parainfluenzae</i> 4201	Clinical isolate
<i>H parainfluenzae</i> 4190	Clinical isolate
<i>H parainfluenzae</i> 4282	Clinical isolate
<i>H parainfluenzae</i> 1596	Clinical isolate
pHi2019tonB	NtHi 2019 tonB clone
pHi3198tonB	NtHi 3198 tonB clone
pHp4201tonB	H parainfluenzae 4201 tonB clone
pHp4190tonB	H parainfluenzae 4190 tonB clone

processes that are vital to growth of bacteria.^{5,6} Iron chelators and iron-repressible outer membrane proteins are produced by *H parainfluenzae* in response to iron stress.^{7,8} *H parainfluenzae* has been shown to acquire phenolate siderophores but not hydroxamate siderophores, while *H influenzae* lacks the ability to utilize siderophores as an iron source.⁶ Previous studies have shown the failure of *H parainfluenzae* to acquire iron directly from human carrier proteins.^{6,9} These studies would suggest that exogenous iron needed by *H parainfluenzae* would be acquired from the uptake of other organisms' siderophores, a process that is executed by the TonB protein in conjunction with various membrane receptors.

The TonB protein spans the periplasm and is anchored to the cytoplasmic membrane and interacts with receptors in the outer membrane to facilitate the uptake of several nutrients or growth factors, including iron-siderophore complexes.¹⁰⁻¹² In *H influenzae*, TonB has been shown to be responsible for acquisition of heme from several sources in vitro and mutants of tonB revealed decreased ability to cause

bacteremia in an infant rat model.^{10,13} Previous studies have reported on the identification of tonB in clinical isolates of *H parainfluenzae* and *H influenzae* via polymerase chain reaction (PCR) and DNA sequence analysis, and have indicated a weak similarity between tonB sequences of *H parainfluenzae* and *H influenzae*.¹⁴ Our studies seek to address the conservation of tonB in *H influenzae* and *H parainfluenzae*.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

H influenzae and *H parainfluenzae* strains used were clinical isolates, as shown in Table 1. All *Haemophilus* strains were grown in brain heart infusion (BHI) supplemented with 10 $\mu\text{g/mL}$ of NAD and 40 $\mu\text{g/mL}$ of hemin. Cultures were incubated at 37°C in the presence of 5% CO₂. Bacteria bearing plasmids bearing cloned PCR products were grown in Luria broth (LB) with 50 $\mu\text{g/mL}$ kanamycin and incubated at 37°C.

Determination of Factor X and V Requirements

Haemophilus strains listed in Table 1 were streaked for isolation on plated

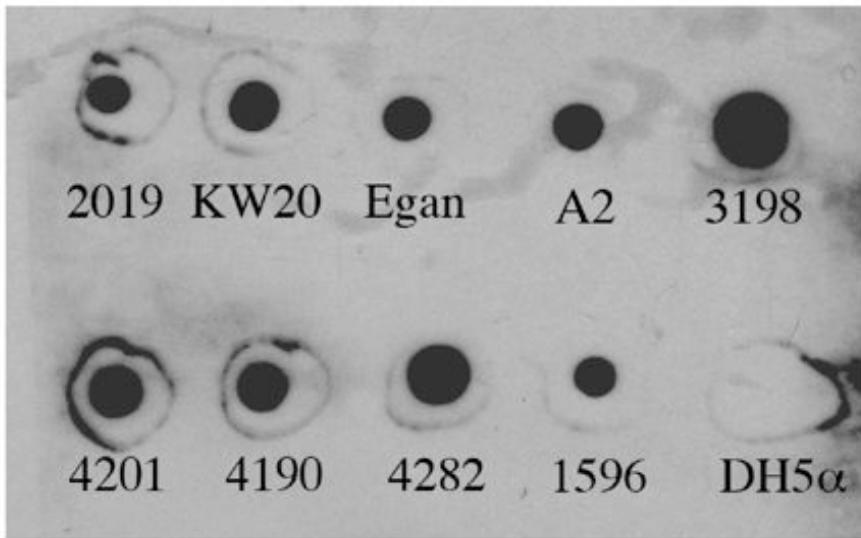
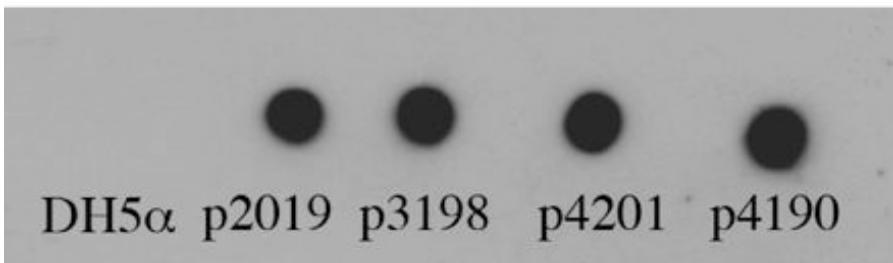
A**B**

Figure 1. (A) DNA dot blot hybridization of genomic DNA isolated from *Haemophilus influenzae* strains NtHi 2019, KW20, Egan, A2; *H parainfluenzae* strains 3198, 4201, 4282, 1596; and *Escherichia coli* DH5 α using a tonB-specific probe. (B) DNA dot blot hybridization of recombinant plasmids bearing cloned tonB PCR products with a tonB-specific probe. Genomic DNA from *E coli* DH5 α is the negative control.

BHI plates containing NAD, hemin, or NAD/hemin. Plates were incubated at previously stated conditions and were checked for growth every 24 hours for 3 days.

DNA Isolation

Genomic DNA was isolated from 100-

mL cultures using a standard phenol extraction procedure.¹⁵ Briefly, a 10-mL overnight culture was pelleted and resuspended in 9.5 mL of TE buffer [10mM Tris-Cl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0]. Sodium dodecyl sulfate (SDS) and proteinase K were added and the suspen-

Table 2. Percent Identities of Nucleotide and Predicted Amino Acid Alignments of Cloned tonB Sequences Amplified from *Haemophilus influenzae* and *H parainfluenzae* Strains

Comparison	Nucleotide % Identity	Amino Acid % Identity
pHi2019tonB & pHi3198tonB	95.1	88
pHi2019tonB & pHp4201tonB	99.4	87
pHi2019tonB & pHp4190tonB	99.1	74
pHi3198tonB & pHp4201tonB	95.2	88
pHi3198tonB & pHp4190tonB	95.0	88
pHp4201tonB & pHp4190tonB	99.8	74

sion was incubated at 37°C for 1 hour. Sodium chloride was added followed by 20-minute incubation at 65°C. An equal volume of phenol/chloroform/isoamyl alcohol was added and the sample was centrifuged to allow for separation of phases. DNA in the aqueous phase was precipitated with the addition of 0.6 volumes of 2-propanol. DNA was dissolved in sterile double distilled water.

Polymerase Chain Reaction

PCR amplifications were carried out in 25 µL reaction mixtures containing 2 mM mixture of deoxyribonucleotide triphosphate (dNTP), 2.0 [Mg²⁺], 1 mM of forward and reverse primers, 1 µg of template DNA, and 1.5 units of Taq polymerase (Promega, Madison, WI). The PCR amplification was performed for 30 cycles and parameters were as follows: 40 seconds at 95°C for denaturation, 45 seconds at 54°C for annealing, and 45 seconds at 72°C for extension. The primer sequences were obtained from a previous study of *H influenzae* and *H parainfluenzae* tonB and were as follows: HitonBF (5') and HitonBR (5'GAAGAG-TAAACTAATTGCACAC-3').¹⁶

TA cloning

tonB-specific sequences were amplified from NtHi 2019, NtHi 3198, *H parainfluenzae* 4201, and *H parainfluenzae* 4190 using primers HitonBF and HitonBR. The PCR products were cloned into pCR 2.1 and plated onto LB-Xgal-kanamycin (Invitrogen,

Carlsbad, CA). Inserts were verified via PCR and nucleotide sequencing.

DNA Sequencing

The cloned inserts were sequenced using Dye Terminator Ready Reaction Mix containing AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA). The samples were analyzed by the ABI Prism 310 Genetic Analyzer (Applied Biosystems).

DNA Dot Blot Hybridization

One µg of genomic DNA from numerous *H parainfluenzae* or *H influenzae* strains was spotted on a nylon membrane and the presence of tonB sequences was determined through DNA-DNA hybridization with a digoxigenin-labeled probe (DIG DNA Labeling and Detection Kit; Roche, Indianapolis, IN) produced from a tonB-specific PCR product obtained using *H parainfluenzae* 4201 genomic DNA as the template. Hybridization was performed at 42°C overnight and high stringency washes of 0.5X standard saline citrate (SSC) were performed at 55°C.

RESULTS

Factor V and X Requirements

All *Haemophilus* strains used in the DNA analysis were tested for their ability to grow in the presence and absence of hemin and NAD. All strains were able to grow on plates containing both hemin and NAD. *H influenzae* strains were unable to grow on plates that did not

	1	11	21	31	41
Hi2019GCCCTTA	TTATGCAAAC	AAAACGTTTCG	CTATTGGGTT	TGCTTATTTTC
Hi3198	TTCGCCCTTA	TTATGCAAAC	AAAACGTTTCG	CTATTGGGTT	TGCTTATTTTC
Hp4190	TTCGCCCTTA	TTATGCAAAC	AAAACGTTTCG	CTATTGGGTT	TGCTTATTTTC
Hp4201	TTCGCCCTTA	TTATGCAAAC	AAAACGTTTCG	CTATTGGGTT	TGCTTATTTTC
	51	61	71	81	91
Hi2019	TTTGATCGCA	CACGGTATTG	TTATAGGATT	TATCTTATGG	AATTGGAATG
Hi3198	TTTGATCGCA	CACGGTATTA	TTATAGGATT	TATCTTATGG	AATTGGAATG
Hp4190	TTTGATCGCA	CACGGTATTG	TTATAGGATT	TATCTTATGG	AATTGGAATG
Hp4201	TTTGATCGCA	CACGGTATTG	TTATAGGATT	TATCTTATGG	AATTGGAATG
	101	111	121	131	141
Hi2019	AGCCAAGTGA	TAGTGCAAAT	AGCGCACAAAG	GCGATATATC	TACAAGTATT
Hi3198	AGCCAAGTGA	TAGTGCAAAT	AGCGCACAAAG	GCGATATATC	TACAAGTATT
Hp4190	AGCCAAGTGA	TAGTGCAAAT	AGCGCACAAAG	GCGATATATC	TACAAGTATT
Hp4201	AGCCAAGTGA	TAGTGCAAAT	AGCGCACAAAG	GCGATATATC	TACAAGTATT
	151	161	171	181	191
Hi2019	TCTATGGAAC	TATTACAGGG	CATGGTGTTG	GAAGAACCTG	CTCCAGAGCC
Hi3198	TCTATGGAGC	TATTACAGGG	CATGGTGTTG	GAAGAACCTG	CTCCAGAGCC
Hp4190	TCTATGGAAC	TATTACAGGG	CATGGTGTTG	GAAGAACCTG	CTCCAGAGCC
Hp4201	TCTATGGAAC	TATTACAGGG	CATGGTGTTG	GAAGAACCTG	CTCCAGAGCC
	201	211	221	231	241
Hi2019	AGAAGATGTA	CAAAAAGAGC	CAGAGCCT---	-----	-----
Hi3198	AGAAGATGTA	CAAAAAGAGC	CAGAGCCTGA	GCCAGAAAAT	GTACAAAAAG
Hp4190	AGAAGATGTA	CAAAAAGAGC	CAGAGCCC---	-----	-----
Hp4201	AGAAGATGTA	CAAAAAGAGC	CAGAGCCC---	-----	-----
	251	261	271	281	291
Hi2019	-----	-GAGCCAGAA	AATGTACAAA	AAGAGCCAGA	ACCAGAAAAA
Hi3198	AGCCAGAGCC	TGAGCCAGAA	AATGTACAAA	AAGAGCCAG	ACCAGAAAAA
Hp4190	-----	-GAGCCAGAA	AATGTACAAA	AAGAGCCAGA	ACCAGAAAAA
Hp4201	-----	-GAGCCAGAA	AATGTACAAA	AAGAGCCAGA	ACCAGAAAAA

Figure 2. Nucleotide alignment of sequences generated from the sequencing of constructed TA clones.

contain both supplements. *H parainfluenzae* strains were able to grow on plates containing NAD only, but not plates containing only hemin.

Survey of Presence of tonB in *H parainfluenzae*

DNA-DNA dot blot analysis was performed on multiple *H parainfluenzae*

strains to determine the presence of tonB in the genome. Hybridization indicated the presence of tonB in the genomes of both of the *H parainfluenzae* strains tested. Figure 1 is a DNA dot blot showing strong hybridization of the *H parainfluenzae* 4201 tonB probe to genomic DNA of all *H influenzae* and *H parainfluenzae* strains.

Sequence Comparison of tonB

TA clones were constructed from several *H influenzae* and *H parainfluenzae* tonB PCR products. The PCR products cloned were generated using primers that anneal to internal sequences of tonB. The clones were sequenced and DNA sequence comparisons were performed using NCBI BLAST, and the analysis revealed an average identity of 97.3% (Table 2). A significant amount of the variation seen was due to the presence of a 33 nucleotide sequence presence within the sequence of HI3198 that was not present in other strains. An alignment of the *H influenzae* and *H parainfluenzae* sequences is shown in Figure 2. The sequences were translated and BioEdit Sequence Alignment Editor (Ibis Therapeutics, Carlsbad, CA) was used to align the resulting amino acid sequences.¹⁷ The translated sequences exhibited an identity of 82.2% with a range of 74% to 88% (Table 2).

DISCUSSION

tonB was observed in numerous *H parainfluenzae* strains via DNA-DNA hybridization and showed high inter- and intra-species similarity. Matar et al indicated an average percent homology of 34% when comparing tonB DNA sequences from numerous *H influenzae* and *H parainfluenzae* clinical and ATCC isolates.¹⁴ Our studies revealed an average percent identity of 97.3% when comparing sequences of constructed TA clones. Furthermore, re-alignment of sequences published by Matar et al using NCBI BLAST resulted in 93% identity.

The function of tonB in *H parainfluenzae* is yet to be determined; however, previous studies have shown the ability of *H parainfluenzae* to acquire siderophores, which is facilitated through tonB in other organisms. In *Escherichia coli*, TonB facilitates uptake of vitamin B₁₂ and iron siderophores,^{11,12}

which could be the case for *H parainfluenzae* due to its ability to acquire enterobactin.⁶ Efforts are currently under way to create a nonfunctional TonB in *H parainfluenzae*. The effects of the mutation will be assessed through analysis of nutrient uptake and growth under low-iron conditions. The ability of *H parainfluenzae* to produce heme indicates a need to determine the role of TonB in the organism. TonB could serve as an alternative pathway to conserve optimal intercellular iron concentrations during times of stress or a pathway to acquire other essential nutrients.

REFERENCES

1. Mitchell J, Hill S. Immune response to *Haemophilus parainfluenzae* in patients with chronic obstructive pulmonary disease. *Clin Diag Lung Dis.* 2000;7:25.
2. NR, Holt JG, eds. *Bergey's Manual of Systematic Bacteriology*, Eighth edition, vol. 1. Baltimore: Williams & Wilkins. 1984:558-569.
3. Wong JC, Patel R, Kendall D, et al. Affinity, conservation, and surface exposure of hemopexin-binding proteins in *Haemophilus influenzae*. *Infect Immun.* 1995;63:2327-2333.
4. Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. *Clin Microbiol Rev.* 2001; 14: 336-363.
5. Litwin CM, Calderwood SB. Role of iron in regulation of virulence genes. *Clin Microbiol Rev.* 1993;6:137-149.
5. Williams P, Morton DJ, Towner KJ, et al. Utilization of enterobactin and other exogenous iron sources by *Haemophilus influenzae*, *H parainfluenzae*, and *H paraphrophilus*. *J Gen Microbiol.* 1990;136:2243-2350.
6. Williams P, Brown MRW. Influence of iron restriction on growth and the expression of outer membrane proteins by *Haemophilus influenzae* and *H parainfluenzae*. *FEMS Microbiol Lett.* 1986;33:153-157.
7. Morton DJ, Williams P. Characterization of the outer-membrane proteins of *Haemophilus parainfluenzae* expressed under iron-sufficient and iron-restricted conditions. *J Gen Microbiol.* 1989;135:445-451.
8. Morton DJ, Williams P. Utilisation of transferin-bound iron by *Haemophilus* species of human and porcine origin. *FEMS Microbiol Lett.* 1989;65:123-128.

9. Jarosik GP, Maciver I, Hansen E. Utilization of transferrin-bound iron by *Haemophilus influenzae* requires an intact *tonB* gene. *Infect Immun.* 1995;63:710-713.
10. Klebba PE, Rutz, JM, Liu J, Murphy CK. Mechanisms of TonB-catalyzed iron transport through the enteric bacterial-cell envelope. *J Bioenerg Biomembr.* 1993;25:603-611.
11. Postle K. TonB protein and energy transduction between membranes. *J Bioenerg Biomembr.* 1993;25:591-601.
12. Jarosik GP, Sanders JD, Cope LD, et al. A functional *tonB* gene is required for both utilization of heme and virulence expression by *Haemophilus influenzae* type B. *Infect Immun.* 1994;62:2470-2477.
13. Matar MG, Kfoury Y, Hadi U. Transcription levels and sequence analysis of *tonB* gene in *Haemophilus parainfluenzae* versus *Haemophilus influenzae* isolates from patients undergoing tonsillectomy and/or adenoidectomy. *J Appl Res.* 2003;3:388-393.
14. Ausebel FM, Brent R, Kingston RE, et al. *Current Protocols in Molecular Biology.* New York: John Wiley & Sons, 1989:2.4.2.
15. Matar GM, Chahwan R, Fuleihan N, et al. PCR-based detection, restriction endonuclease analysis, and transcription of *tonB* in *Haemophilus influenzae* and *Haemophilus parainfluenzae* isolates obtained from children undergoing tonsillectomy and adenoidectomy. *Clin Diagn Lab Immunol.* 2001;8:221-224.
16. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser.* 1999;41:95-98.