UNIVERSITY *of* TASMANIA

Potential therapeutic value of normal throat bacteria that produce a novel inhibitory substance for the prevention of respiratory infections.

Brianna A. Atto

BBiomedSc (Hons)

University of Tasmania | School of Health Sciences

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania | March 2022

Declaration of Originality

This thesis entitled “Potential therapeutic value of normal throat bacteria that produce a novel inhibitory substance for the prevention of respiratory infections”, describes original research conducted by the candidate within the School of Health Sciences at the University of Tasmania and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Candidate

Brianna A. Atto

15th November 2021

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Candidate

Brianna A. Atto

15th November 2021

Statement Regarding Published Work Contained in Thesis

The publishers of the manuscripts comprising Chapters 4 to 6 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Due to the inclusion of published material, there is unavoidable repetition of material between chapters in this thesis.

Candidate

Brianna A. Atto

15th November 2021

Statement of Co-Authorship

The following people and institutions contributed to the publication of work undertaken as part of this thesis:

Brianna A. Atto (candidate), University of Tasmania

1. Dr Stephen Tristram (primary supervisor), University of Tasmania
2. Dr Dale Kunde (supervisor), University of Tasmania
3. Dr David A. Gell (supervisor), University of Tasmania
4. Dr Roger Latham (co-author), University of Tasmania

Manuscript 1 | Chapter 4

Atto B, Latham R, Kunde D, Gell DA, Tristram S. *In vitro* Anti-NTHi Activity of Haemophilin-Producing Strains of *Haemophilus haemolyticus*. Pathogens. 2020 Apr;9(4):243.

Author contributions: Lead investigator, B.A.; conceptualisation, B.A., S.T. and D.K.; methodology, B.A., S.T., D.K. and R.L.; software, B.A.; validation, B.A.; formal analysis, B.A.; investigation, B.A.; resources, S.T., D.K. and D.A.G.; data curation, B.A.; writing—original draft preparation, B.A.; writing—review and editing, B.A., S.T., D.A.G. and R.L.; visualisation, B.A.; supervision, S.T., D.K. and D.A.G.; project administration, S.T. and D.K.; funding acquisition, S.T.

Manuscript 2 | Chapter 5

Atto B, Kunde D, Gell DA, Tristram S. Haemophilin-Producing Strains of *Haemophilus haemolyticus* Protect Respiratory Epithelia from NTHi Colonisation and Internalisation. Pathogens. 2021 Jan;10(1):29.

Author contributions: Lead investigator, B.A.; formal analysis, B.A.; investigation, B.A.; resources, S.T., D.K. and D.A.G.; data curation, B.A.; writing—original draft preparation, B.A.; writing—review and editing, B.A.,S.T. and D.A.G.; visualisation, B.A.; supervision, S.T., D.K. and D.A.G.; project administration, S.T. and D.K.; funding acquisition, S.T.

Manuscript 3 | Chapter 6

Atto B, Kunde D, Gell DA, Tristram S. Oropharyngeal Carriage of *hpl*-Containing *Haemophilus haemolyticus* Predicts Lower Prevalence and Density of NTHi Colonisation in Healthy Adults. Pathogens. 2021 May;10(5):577.

Author contributions: Lead investigator, B.A.; conceptualisation, B.A., S.T. and D.K.; methodology, B.A., S.T. and D.K.; validation, B.A.; formal analysis, B.A.; investigation, B.A. and S.T.; resources, S.T. and D.K.; data curation, B.A.; writing—original draft preparation, B.A., S.T. and D.A.G.; writing—review and editing, B.A.,S.T. and D.A.G.; visualisation, B.A.; supervision, S.T., D.K. and D.A.G.; project administration, S.T.; funding acquisition, S.T.

We, the undersigned, endorse the above stated contribution of work undertaken for each of the published (or submitted) peer-reviewed manuscripts contributing to this thesis:

_____ 15th November 2021
Brianna A. Atto
(Candidate)

_____15th November 2021
Dr. Stephen Tristram
(Primary supervisor)

_____10th December 2021
Professor Nuala Byrne
(Head of the School of Health Sciences)

Statement of Candidature Contribution to Thesis

This thesis comprises interlinked research investigations where the candidate Brianna Atto is the lead investigator, however, the following people and institutions also contributed to the published and non-published work contained within the thesis as follows:

Brianna A. Atto (Candidate, School of Health Sciences, University of Tasmania)

Lead investigator responsible for design of each individual research project, laboratory and experimental analysis, data collection, data analysis, data interpretation, and is the lead author on all resultant manuscripts.

Dr. Stephen Tristram (School of Health Sciences, University of Tasmania)

General supervision, assistance with research project design, conceptualisation, experimental techniques, and manuscript revisions.

Dr. Dale Kunde (School of Health Sciences, University of Tasmania)

General supervision, assistance with research project design, and technical assistance in experiments involving molecular techniques.

Dr. David A. Gell (School of Medicine, University of Tasmania)

General supervision, assistance with research project design, technical assistance in experiments involving molecular techniques, and manuscript revisions.

Contributions to Published Chapters:

Chapter 3 Preparation of recombinant holo haemophilin and SDS-PAGE was performed by David A. Gell (University of Tasmania).

We, the undersigned agree with the above stated, "proportion of work undertaken" for each of the published or non-published chapters, which contribute to this thesis:

_____ 15th November 2021
Brianna A. Atto

_____15th November 2021
Stephen Tristram

_____ 15th November 2021
Dale Kunde

_____ 15th November 2021
David A. Gell

_____ 15th November 2021
Roger Latham

Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University (Tasmania) Network (Approval number: H0016835, approved 11 December 2017).

Candidate

Brianna A. Atto

15th November 2021

Publications and Presentations at Conferences During PhD Candidature

A. Published Manuscripts:

Atto B, Eapen MS, Sharma P, Frey U, Ammit AJ, Markos J, Chia C, Larby J, Haug G, Weber HC, Mabeza G. New therapeutic targets for the prevention of infectious acute exacerbations of COPD: role of epithelial adhesion molecules and inflammatory pathways. Clinical Science. 2019 Jul;133(14):1663-703.

Latham RD, Torrado M, **Atto B**, Walshe JL, Wilson R, Guss JM, Mackay JP, Tristram S, Gell DA. A haem-binding protein produced by *Haemophilus haemolyticus* inhibits non-typeable *Haemophilus* influenzae. Molecular microbiology. 2020 Feb;113(2):381-98.

Atto B, Latham R, Kunde D, Gell DA, Tristram S. *In vitro* Anti-NTHi Activity of Haemophilin-Producing Strains of *Haemophilus haemolyticus*. Pathogens. 2020 Apr;9(4):243.

Atto B, Kunde D, Gell DA, Tristram S. Haemophilin-Producing Strains of *Haemophilus haemolyticus* Protect Respiratory Epithelia from NTHi Colonisation and Internalisation. Pathogens. 2021 Jan;10(1):29.

Atto B, Kunde D, Gell DA, Tristram S. Oropharyngeal Carriage of *hpl*-Containing *Haemophilus haemolyticus* Predicts Lower Prevalence and Density of NTHi Colonisation in Healthy Adults. Pathogens. 2021 May;10(5):577.

B. Conference Presentations:

Royal Society of Tasmania | Public Lecture| Sept 2018 | *"Respiratory probiotics: A new way to fight respiratory infections?".*

Australian Society for Microbiology Interstate Conference | Oral Presentation | March 2019 | *"Exploiting the struggle for haem: a potential probiotic candidate for the prevention of respiratory infections?".*

Australian Society for Microbiology Nancy Millis Student and Early Career Awards Night | Oral Presentation | May 2019 | *"Exploiting the struggle for haem: a potential probiotic candidate for the prevention of respiratory infections?".*

Australian Society for Microbiology National Meeting | Poster Presentation | July 2019 | *"Exploiting the struggle for haem: a potential probiotic candidate for the prevention of respiratory infections?".*

Three-minute thesis finals | Oral presentation | Aug 2019 | "*Recruiting bacterial allies in the fight against respiratory tract infections".*

Australian Society for Medical Research (ASMR) Medical Research Week Virtual Symposia | Oral Presentation | Nov 2020 | "Probiotic Potential of Haemophilin-producing Strains of *Haemophilus haemolyticus* in the Respiratory Tract".

Australian Society for Microbiology Nancy Millis Student and Early Career awards night | Oral Presentation | May 2021 | *"Exploiting the struggle for haem: A novel probiotic approach against infections in the respiratory tract".*

Australian Society for Microbiology National Meeting | Poster Presentation | June 2021 | *"Oropharyngeal Carriage of hpl-containing Hh Predicts Lower Prevalence and Density of NTHi Colonisation in Healthy Adults".*

Australian Society for Microbiology National Meeting | Oral Presentation | June 2021 | *"In vitro Probiotic Potential of Haemophilin-producing Strains of Haemophilus haemolyticus".*

World Microbe Forum| Poster presentation | June 2021 | *"Oropharyngeal Carriage of Hpl-containing Hh Predicts Lower Prevalence and Density of NTHi Colonisation in Healthy Adults".*

World Microbe Forum | Oral Presentation| June 2021 | *"Exploiting the Struggle for haem: A Novel Probiotic Approach Against Haemophilus influenzae Infections in the Respiratory Tract".*

C. Funding Support, Awards and Grants:

University of Tasmania | Nov 2017 | **Research Training Program (RTP) Scholarship**

Australian Society for Microbiology | May 2018 | **Student Travel Award**

Clifford Craig Foundation | Jan 2018 | **Research Grant (CCF 170)**

Clifford Craig Foundation | Sept 2019 | **Research Grant (CCF 192)**

Australian Society for Medical Research (ASMR)| Nov 2020 | **Postgraduate Student Award**

Australian Society for Microbiology | May 2021 | **Early Career Scientist Award**

Australian Society for Microbiology | May 2019 | **Nancy Millis Student Travel Award**

Australian Society for Microbiology National Meeting | June 2021 | **Poster award**

American Society of Microbiology | June 2021 | **Student and Postdoctoral Travel Award**

Acknowledgements

Firstly, I wish to express my sincere gratitude and appreciation to all those who supported and helped me throughout the duration of my candidature. I would like to extend a deep and heartfelt thank you to my supervisors Dr. Stephen Tristram, Dr. Dale Kunde and Dr. David Gell, not only for their enthusi-asm in my research, but also for their invaluable guidance, knowledge and patience. Furthermore, I wish to extend my appreciation to Dr. Kiran Ahuja, my Higher Degree Research coordinator, as well as Professor Nuala Byrne, Head of the School of Health Sciences, for giving me the opportunity to study within the School of Health Sciences.

I would like to extend my gratitude to Dr. Erin Price from the University of the Sunshine Coast, Queensland for her technical expertise in the molecular differentiation and quantification of *Haemophilus* spp. I would also like to acknowledge Dr. Lea-Ann Kirkham from the School of Paediatrics and Child Health from the University of Western Australia, Perth, for provision of some of the *Haemophilus* isolates used in these studies.

I wish to also thank and acknowledge my fellow PhD candidates and the technical staff within the School of Health Sciences, for their general support, and company in both the molecular and microbiology laboratories.

I would like to acknowledge the generous support of the Clifford Craig Medical Research Trust (CCMRT) for their funding of the research projects presented in this thesis, as without their generous support this research would not have been possible. Similarly, the Australian Government Research Training Program (RTP) Scholarship has provided me with the necessary financial security to under-take these research projects on a full-time basis.

Finally, I would like to thank my family, and in particular my partner Matthew Leech, not only for their patience, but also for their general interest in my research. You have kept me grounded and moti-vated throughout my candidature, and for that I am truly grateful.

Yours Sincerely,

Brianna A. Atto

General Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is a major opportunistic pathogen that causes a variety of infections in the respiratory tract, including community-acquired pneumonia, acute exacerbations of chronic obstructive pulmonary disease and otitis media. Collectively, these infections and subsequent complications impose a significant global burden of disease. The impact of NTHi-associated disease is further amplified by the rapidly expanding spectrum and prevalence of antibiotic resistance, and the lack of an effective vaccination strategy. Consequently, novel preventative or therapeutic approaches that do not rely on antibiotic susceptibility or stable vaccine targets are becoming more attractive. One such approach may involve exploiting the bacterium's nutritional dependency for host-derived iron-containing haem, the acquisition of which is a major determinant of NTHi survival and pathogenesis within the respiratory tract. Strategies that interfere with the acquisition of this essential nutrient may therefore have a significant impact on the ability of NTHi to cause disease.

The limited availability and nutritional demand for haem-iron promotes a highly competitive environment between NTHi and other upper respiratory tract inhabitants. We therefore hypothesised that an upper respiratory commensal capable of outcompeting NTHi for haem-iron may have utility as a probiotic therapy by generating an environment inhospitable for NTHi growth. Recently, nasopharyngeal isolates of the closely related commensal *Haemophilus haemolyticus* (Hh), were discovered with the capacity to inhibit NTHi growth by secretion of a novel bacteriocin-like substance (herein referred to as haemophilin; Hpl). Hpl was found to possess structural characteristics consistent with that of a haem-binding protein and thus, we proposed a model by which Hpl inhibits NTHi growth by restricting access to haem. In Chapter 3, this hypothesis was tested by generating and comparing the NTHi-inhibitory capacity of an *hpl* knockout to the wild-type strain under varying concentrations of haem or recombinant Hpl. These experiments indicated that Hpl was only inhibitory under haem-limited conditions. The loss of NTHi-inhibitory activity in media recovered from the knockout strain, and the direct correlation between NTHi-inhibitory activity and *hpl* expression across different Hh strains, confirmed that Hpl was the primary mediator of NTHi growth inhibition by Hh. In addition to the loss of NTHi-inhibitory capacity, the knockout also displayed poor growth on haem supplemented media compared to the wild-type strain, indicating a defect in haem utilisation and a role for Hpl in Hh haem acquisition/utilisation. Growth of NTHi and Hh in media supplemented with either Hpl or the equivalent concentration of free haem demonstrated that Hpl-bound haem is available to Hh as a nutritional source of haem, but not to NTHi. To further characterise the biological role and genetic determinants of Hpl production, whole genome sequences of Hh isolates containing the *hpl* open reading frame

(n=24) were generated. Among all isolates and publicly available genomes, the *hpl* gene occurred in a conserved gene cassette containing genes for a putative secretion protein and a putative Hpl/haem receptor. Together with supporting proteomic investigations, these findings provided evidence that Hpl has a role in haem acquisition by Hh, and that NTHi-inhibitory activity occurs through haem starvation. We therefore proposed the possibility that competition from Hpl-producing Hh (Hh-Hpl+) could antagonise NTHi colonisation in the respiratory tract.

The feasibility of this approach was tested in chapter 4 by direct *in vitro* competition assays between NTHi and Hh strains with varying capacities to produce Hpl. Subsequent changes in NTHi growth rate and fitness, in conjunction with *hpl* expression analysis, were employed to assess the NTHi-inhibitory capacity of Hh strains. The growth rate of NTHi was significantly impaired during co-culture with Hh strains containing *hpl* (Hh-*hpl*$^{+}$), but not with strains lacking the *hpl* open reading frame (Hh-*hpl*$^{-}$), including the knockout strain generated in Chapter 3. During an extended co-culture assay, the competitive advantage of Hh-*hpl*$^{+}$ strains was evident within two generations, culminating in a total loss of NTHi fitness over subsequent generations. Hh strains capable of high levels of *hpl* expression, were able to compete with NTHi more effectively, providing a strong link between the NTHi-inhibitory phenotype, *hpl* expression and favourable outcomes during competitive growth with NTHi *in vitro*. These findings demonstrate that Hh-Hpl+ strains possess characteristics desirable in a probiotic, warranting further investigation into the utility of these strains in preventing NTHi interactions with host cells.

In chapter 5, the capacity of Hh-Hpl+ strains to disrupt NTHi association with airway epithelial cells was tested to determine their probiotic utility against the requisite nasopharyngeal colonisation stage of NTHi infection. Cell culture models of nasopharyngeal (D562) and lung (A549) epithelia were pre-treated with Hh strains with different levels of *hpl* expression prior to NTHi challenge. NTHi attachment and invasion was significantly reduced in cell monolayers pre-treated with Hpl or Hh strains with high levels of *hpl* expression. Among all Hh-Hpl+ strains the production of Hpl was found to be stimulated in response to NTHi challenge and nasopharyngeal cell exposure. Pre-treatment with Hh-Hpl+ strains was more effective than the purified Hpl protein alone, such that Hh-Hpl+ cell numbers 10-100-fold lower than that of the NTHi challenge load, were sufficient to significantly inhibit NTHi host-cell interactions. These data suggest that conditions in the nasopharyngeal niche might support high levels of expression of *hpl* in Hh-Hpl+ strains with associated protection against NTHi adhesion and attachment.

Based on the *in vitro* findings presented in Chapters 3-5, we hypothesised that natural pharyngeal carriage of Hh strains with the Hh-*hpl*$^{+}$ genotype would be associated with a lower prevalence and/or density of NTHi colonisation in healthy individuals. Chapter 6 describes an *in vivo* human study

involving the collection of oropharyngeal swabs from 257 healthy adults in Australia between 2018 and 2019. Real-time PCR was used to quantitatively compare the oropharyngeal carriage load of NTHi and Hh populations with the Hh-*hpl*$^{+}$ or Hh-*hpl*$^{-}$ genotype. The likelihood of acquiring/maintaining NTHi colonisation status over a two- to six-month period was also assessed in individuals that carried either Hh-*hpl*$^{-}$ (*n* = 25) or Hh-*hpl*$^{+}$ (*n* = 25). The carriage of Hh-*hpl*$^{+}$ was associated with a significantly lower proportionate density and prevalence of concurrent NTHi carriage. Additionally, colonisation with high densities of Hh-*hpl*$^{+}$ correlated with lower NTHi carriage loads and a lower likelihood of acquiring/maintaining NTHi colonisation status between visits. These findings suggest a potential protective role of Hh-*hpl*$^{+}$ strains against NTHi pharyngeal colonisation *in vivo*.

In summary, the work presented in this thesis provides *in vitro* and *in vivo* evidence which supports the therapeutic potential of Hh-Hpl+ against NTHi by inhibiting growth and host-cell interactions required for pathogenesis. These findings encourage translational studies of a probiotic which can be applied to the upper respiratory tract as a strategy to prevent NTHi infections which is not compromised by the limitations associated with standard antibiotic or vaccination strategies.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

aa	Amino acid
ABC	ATP-binding-cassette
AECOPD	Acute exacerbations of chronic obstructive pulmonary disease
AOM	Acute otitis media
BLNAR	β-lactamase-negative ampicillin-resistant
BLPACR	β-lactamase-positive amoxicillin-clavulanic acid-resistant
BLS	Bacteriocin-like substance
bp	Base pair
CA	Chocolate agar
CF	Cystic fibrosis
COPD	Chronic obstructive pulmonary disease
CSOM	Chronic suppurative otitis media
DFO	Desferrioxamine B
DFP	Deferiprone
DPBS	Dulbecco's phosphate buffered saline
DUF	Domain of unknown function
fur	Ferric uptake regulator gene
FbpA	Ferric-binding protein
Fhu	Ferric hydroxamate uptake
GaPP	Gallium-protoporphyrin IX
gDNA	Genomic DNA
HbpA	Haem-binding lipoprotein
HBSS	Hank's balanced salt solution
HemR	Haemin receptor
Hgb	Haemoglobin binding protein
Hh	*Haemophilus haemolyticus*
Hh-BW1$^{hpl\text{-}KO}$	Haemophilin gene knockout of *Haemophilus haemolyticus* strain BW1
Hh-Hpl+	Haemophilin-producing *Haemophilus haemolyticus*
Hh-Hpl-	Non-haemophilin-producing *Haemophilus haemolyticus*
Hh-*hpl*$^{+}$	*Haemophilus haemolyticus* containing the *hpl* open reading frame
Hh-*hpl*$^{-}$	*Haemophilus haemolyticus* lacking the *hpl* open reading frame
Hhu	Haemoglobin-haptoglobin binding protein

Hib	*Haemophilus influenzae* type b
HMW	High-molecular weight protein
Hpl	Haemophilin
Hup	Haem-utilisation protein
Hxu	Haem/hemopexin utilisation protein
IgA1	Immunoglobulin A1
MOI	Multiplicity of infection
nHpl	Native haemophilin
NTHi	Notypeable *Haemophilus influenzae*
OM	Otitis media
OMP	Outer membrane protein
ORF	Open reading frame
PBP3	Penicillin-binding protein 3
PD	Protein D
PE	Protein E
PP	Periplasmic protein
rHpl	Recombinant haemophilin
Sap	Sensitivity to antimicrobial peptide
SLAM	Surface lipoprotein assembly modifier
SMM	Skim milk media
sTSB	Supplemented tryptone soya broth
STGG	Skim milk, tryptone, glucose and glycerin media
Tbp	Transferrin-binding proteins
TBDHR	Ton-B dependent haemin receptor
WGS	Whole-genome sequence

Chapter 1 | Thesis Summary

1.1. Background

Respiratory infections caused by the opportunistic pathogen nontypeable *Haemophilus influenzae* (NTHi) impose a substantial global disease burden. This impact is further amplified by the rapidly growing spectrum and prevalence of antibiotic resistance, sufficient to warrant widespread vaccination. However, despite numerous attempts, there are currently no effective vaccination strategies available, largely owing to the high intrinsic heterogeneity and phase variable nature of NTHi antigens. In 2017, the World Health Organization recognised the growing burden of NTHi-associated infections by listing it as a "priority pathogen" for which new therapies are urgently needed. Thus, exploration of alternative strategies may be necessary to successfully manage or prevent infections caused by NTHi.

For many commensals and pathogens alike, iron is an essential micronutrient with critical roles in numerous cellular processes. Iron functions as a protein cofactor both as an inorganic ion and through incorporation into the iron-porphyrin complex, haem. For NTHi, this nutritional requirement is primarily fulfilled by acquisition of haem, from which iron can be liberated or incorporated directly into essential haemoproteins. Acquisition of host-derived haem is not only essential to survival but is also a key determinant of NTHi pathogenesis and persistence within host airways. As such, strategies that interrupt acquisition or utilisation of iron-containing haem may have a significant impact on the ability of NTHi to cause disease. The vulnerability of pathogens to iron or haem restriction has prompted the development of a variety of host- and bacterial-based approaches that target the availability or assimilation of haem/iron in the respiratory tract. Current pharmaceutical approaches in development primarily target iron assimilation pathways and present limited efficacy against NTHi, which compensates through acquisition of haem and other iron-containing moieties. However, even interventions that target haem sources are challenged by the complexity and redundancy of acquisition systems employed by NTHi. Thus, interventions that target multiple modes of haem-assimilation have a higher therapeutic potential against NTHi.

Recently, the therapeutic utility of upper respiratory tract commensals has been recognised, particularly those that are closely related to the target pathogen and compete for similar nutrient sources. New strains of the commensal *Haemophilus haemolyticus* (Hh) that share the nasopharyngeal niche, have been discovered that produce a novel bacteriocin-like substance and elicit inhibitory activity against NTHi *in vitro*. The inhibitory activity of this protein was only observed in haem-limited media and shared structural similarity to the haemoglobin-haptoglobin utilisation protein A found in other members of the Pasteurellaceae family. We therefore proposed an inhibitory mechanism whereby this protein prevents NTHi access to haem-iron and subsequently inhibits growth of the bacterium. These strains of Hh may therefore have utility as a probiotic that can outcompete NTHi for haem-iron and generate a haem-restrictive environment inhospitable for NTHi growth. The research presented in this thesis explores the utility of these Hh strains in preventing NTHi growth and host-cell interactions required for pathogenesis, and thus propose their therapeutic potential as a respiratory probiotic.

1.2. Chronology of Works and Thesis Organisation

Chapter 1 (the current chapter) comprises a general introduction of the overall themes, rationale of the thesis along with a note on the chronology of the laboratory works conducted.

Chapter 2 presents a review of the current literature that summarises the background information on the main themes of the thesis. A general overview of *Haemophilus influenzae* as a human pathogen is provided, emphasizing the need for development of alternative therapeutic approaches. NTHi proteins/regulatory elements involved in haem acquisition are discussed in the context of their role in NTHi survival and pathogenesis in the respiratory tract. Finally, this review outlines strategies under development that disrupt bacterial haem-iron acquisition and their potential utility for use against NTHi in the respiratory tract.

Chapter 3 presents experimental work aimed to characterise and determine the inhibitory role of a novel haemophore (now referred to as haemophilin; Hpl) secreted by an isolate of Hh with the unique capacity to inhibit the growth of NTHi *in vitro*. Given that both species share the same ecological niche and an absolute growth requirement for haem, it was hypothesised that the NTHi-inhibitory activity of Hpl-producing strains of Hh involved competition for haem. This chapter provides novel structural, functional and bioinformatic evidence describing Hpl as a previously unrecognised haem uptake mechanism in Hh and that NTHi-inhibitory activity occurs through haem starvation. These findings

propose the possibility that competition from Hpl-producing Hh could antagonise NTHi colonisation in the respiratory tract, warranting further investigations using direct competition models.

Findings from this chapter were published as a component of the following manuscript:

Latham RD, Torrado M, **Atto B**, Walshe JL, Wilson R, Guss JM, Mackay JP, Tristram S, Gell DA. A haem‐binding protein produced by *Haemophilus haemolyticus* inhibits non‐typeable *Haemophilus* influenzae. Molecular microbiology. 2020 Feb;113(2):381-98.

Chapter 4 presents an edited version of the first full manuscript, which aimed to further test the inhibitory capacity of Hpl-producing strains of Hh by direct *in vitro* competition with NTHi, for the purpose of determining their probiotic potential. Hpl-producing strains of Hh demonstrated a strong competitive advantage over NTHi, compared with Hh strains unable to produce the protein. Parallel expression analysis also established a strong link between the NTHi-inhibitory phenotype, *hpl* expression and favourable outcomes during competitive growth with NTHi *in vitro*. These findings demonstrated the feasibility of Hpl-producing Hh as a probiotic candidate, warranting further investigation into the utility of these strains in preventing NTHi interactions with host cells.

An unedited version of this manuscript was published as:

Atto B, Latham R, Kunde D, Gell DA, Tristram S. *In vitro* anti-NTHi activity of haemophilin-producing strains of *Haemophilus haemolyticus*. Pathogens. 2020 Apr;9(4):243.

Chapter 5 presents an edited version of the second manuscript, which aimed to further investigate the *in vitro* NTHi-inhibitory capacity demonstrated in Chapter 4 by translation into cell line culture models that more closely represent the human respiratory epithelium. Results from this chapter show that in this epithelial culture model, Hh strains capable of high-level Hpl production have a strong protective capacity against NTHi adhesion and invasion and exhibit promising characteristics in the context of a probiotic therapy.

An unedited version of this manuscript was published as:

Atto B, Kunde D, Gell DA, Tristram S. Haemophilin-Producing Strains of *Haemophilus haemolyticus* Protect Respiratory Epithelia from NTHi Colonisation and Internalisation. Pathogens. 2021 Jan;10(1):29.

Chapter 6 presents an edited version of the third manuscript which extends testing into an *in vivo* setting under the hypothesis that natural pharyngeal carriage of Hh strains with the *hpl* open reading frame would be associated with a lower prevalence and/or density of NTHi colonisation in healthy adults (based on findings from chapters 3-5). Oropharyngeal swabs were collected from 257 healthy adults in Australia between 2018 and 2019. Real-time PCR was used to quantitatively compare the oropharyngeal carriage load of NTHi and Hh populations with the Hh-*hpl*$^{+}$ or Hh-*hpl*$^{-}$ genotype. Colonisation with high densities of Hh-*hpl*$^{+}$ correlated with lower bacterial loads and a lower likelihood of acquiring/maintaining NTHi colonisation status. Together with supporting *in vitro* studies, these results encourage further investigation into the potential use of Hh-*hpl*$^{+}$ as a respiratory probiotic candidate for the prevention of NTHi infection.

An unedited version of this manuscript was published as:

Atto B, Kunde D, Gell DA, Tristram S. Oropharyngeal Carriage of *hpl*-Containing *Haemophilus haemolyticus* Predicts Lower Prevalence and Density of NTHi Colonisation in Healthy Adults. Pathogens. 2021 May;10(5):577.

Chapter 7 presents a general discussion and concluding remarks regarding the novel findings from the studies undertaken in this thesis. This chapter also addresses the limitations of the studies conducted and proposes directions and considerations for future translational studies.

Finally, as this thesis comprises a number of published manuscripts that have been reformatted to fit the style requirements for a thesis submitted at the University of Tasmania, there are some important points to note. All references were amended to conform with the Vancouver style, and a combined reference list is presented at the end of the thesis in **Chapter 8**. Supplementary data that accompanies the published manuscripts online has been included at the end of each individual manuscript chapter where applicable. There is also some repetition of abbreviations and terms between chapters, such that chapters may be read as stand-alone manuscripts.

Chapter 2 | General Introduction and Review of the Literature

2.1 Introduction

Over the past decade, nontypeable *Haemophilus influenzae* (NTHi) has gained recognition as a major opportunistic pathogen that imparts a substantial global burden of disease, owing to the high rates of morbidity and sequelae associated with NTHi infections. Further amplifying the global impact of NTHi infections is the increasing spectrum and prevalence of antibiotic resistance, leading to higher rates of treatment failure with both first- and second-line antibiotics (1, 2). In recognition of the growing threat of antibiotic resistance, the World Health Organisation (2017) comprised a list of priority pathogens, including NTHi, for which new therapeutic agents are urgently needed (3). All-cause mortality, healthcare and community burden, and prevalence of resistance ranked highly among the criteria used to determine the placement of NTHi on this list (4). The urgency for new therapeutic agents is exacerbated by the lack of an effective vaccination strategy, the development of which is complicated by the highly heterogenous nature of NTHi antigens (5). Consequently, alternative preventative or therapeutic approaches that do not rely on antibiotic susceptibility or stable vaccine targets are becoming more attractive. One such approach may be the exploitation of the bacterium's reliance on sequestering (i.e obtain and internalise) host-derived sources of haem, a nutrient with a key role in NTHi survival and pathogenesis. This review discusses the role and importance of haem in all facets of NTHi pathogenesis and explores the therapeutic potential of strategies that interfere with its acquisition.

2.2 Nontypeable *Haemophilus influenzae*

2.2.1 Bacteriology and Identification

Members of the *Haemophilus* genus are gram-negative coccobacilli belonging to the Pasteurellaceae family, which is typically comprised of upper respiratory tract commensals. The majority of infections associated with this genus are caused by *H.influenzae*; other species such as *H. aegyptius*, *H. parainfluenzae* and *H. ducreyi* are rarely isolated from clinical samples

but have been documented to cause a variety of mild respiratory or genitourinary infections (2, 6). *H. influenzae* can be further subtyped into encapsulated strains, which express different serotypes of capsular polysaccharide (designated types a–f), and nonencapsulated strains, which are designated NTHi. Prior to the widespread implementation of the *Haemophilus influenzae* type b (Hib)-conjugate vaccination programs in the 1990s, Hib was the most common cause of bacterial meningitis in children under the age of five (7-9). With the decline in Hib disease, the prevalence of NTHi in human carriage and disease has dramatically increased and is now the most common phenotype isolated from clinical sites of infection (2).

The growing clinical importance of NTHi has prompted revisions to identification schemes that accurately differentiate it from commensal members of the genus frequently co-isolated from clinical specimens, particularly the non-pathogenic species, *Haemophilus haemolyticus* (Hh) (2). Although occasionally isolated from sterile sites in immunocompromised patients (10), Hh has not been implicated as a causative agent of respiratory tract infections and isolation from clinical sites may be a result of misidentification of pathogenic NTHi (11-17). Unlike other members of the genus, *H. influenzae* and Hh have an absolute growth requirement for both exogenous X-factor (haemin) and V-factor (nicotinamide adenine dinucleotide). Additionally, serological capsular typing and other phenotypic characteristics cannot reliably differentiate between these species, such that identification schemes that rely solely on phenotypic discrimination have a high misidentification rate (27-40%) of nasopharyngeal and sputum isolates (16, 17). Despite the higher differentiative power of newer molecular-based approaches, no single gene target can unequivocally differentiate between the two species (18-21). This is largely owing to the evolutionary continuum and high degree of recombination between the two species, and the high level of genetic heterogeneity exhibited among NTHi strains (18, 22). Discrimination between NTHi and Hh is improved by identification schemes that simultaneously detect multiple gene targets (12). To date, the highest sensitivity and specificity for the detection of NTHi has been demonstrated by a real-time PCR assay which simultaneously detects novel Hh (*hypD*) and *H. influenzae* (*siaT*) targets, multiplexed with the highly conserved *H. influenzae* L-fucose permease gene (*fucP*) assay (20). However, this assay has not yet been adapted for routine diagnostic use.

2.2.2 Upper Respiratory Tract Colonisation

NTHi colonisation of the nasopharynx occurs within the first years of life with 34% of infants being colonised within two months of birth, and 44% by the age of two (23, 24). Prevalence

of normal NTHi carriage varies considerably with geographical region, age and season, and an individual's susceptibility to NTHi colonisation may be influenced by additional factors such as recent viral respiratory infection, antibiotic therapy, smoking, domestic crowding, airway dysbiosis and underlying chronic respiratory diseases (14, 25-28). Comparisons of carriage rates between populations is complicated by the high degree of inter-study variation largely owing to the different culture- and molecular-based methods employed and difficulty distinguishing NTHi from Hh (14, 25). NTHi carriage is overrepresented in children under the age of five, with global reports varying between 50-80% (17, 29-31). In adults, carriage rates are typically lower (20-30%), but rates as low as 3% have also been reported in adults from Kenya and Nepal (32-34). The prevalence of NTHi carriage is high in Asia-pacific regions, the highest rates of which are reported in Australian Indigenous communities (35-41). Despite a predisposition to NTHi-associated infections (42), information surrounding carriage rates in elderly demographics is limited to studies in Germany (2012-2013) and Brazil (2017), reporting rates of 2-3% (43, 44).

NTHi colonisation is a dynamic and diverse process which is characterised by simultaneous carriage of multiple strains and a rapid turnover rate of constituent genotypes (22-24, 36, 44-49). Week-to-week turnover rates of NTHi genotypes as high as 62% have been reported from healthy children attending day-care (50) and in healthy adults (22). In the majority of cases, NTHi strains are typically replaced within three months of acquisition but persistent colonisation of a single strain up to six-seven months has been reported (23, 24, 46, 50). Clonal conservation within the community is also observed in institutions such as orphanages and day-care centres (24, 49) and within households (41). Persistent airway colonisation is also common in individuals with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF), typically with multiple clones of NTHi with different antimicrobial susceptibilities (51-53).

2.3 NTHi is a Major Pathogen of the Respiratory Tract

NTHi is commonly associated with nasopharyngeal colonisation in healthy adults but in susceptible individuals the bacterium can migrate to other anatomical sites and cause a wide spectrum of disease; from respiratory tract infections to severe invasive disease (54). In particular, non-invasive infections with NTHi such as otitis media, sinusitis, conjunctivitis, acute exacerbations of COPD (AECOPD), and non-bacteraemia pneumonia impart a substantial global disease burden (2).

2.3.1 Mucosal Infections of the Upper Respiratory Tract

NTHi is a common cause of upper respiratory tract infections, including sinusitis, paediatric conjunctivitis and most frequently, acute otitis media (AOM) (2). Globally, AOM is the most common childhood disease for which medical assistance is sought, with approximately 83% of children experiencing at least one episode by the age of three (55, 56). According to the most recent global epidemiological estimate from 2005, there are 709 million cases of AOM each year (57) with NTHi accounting for approximately 60% of cases when taking into account recurrent episodes and seasonal variation (58, 59). The incidence of NTHi-associated AOM varies geographically, tending to be higher in Asia-pacific regions, particularly in Australian Indigenous communities who experience 30% higher rates compared to non-Indigenous populations within the same region (60, 61).

AOM occurs when bacteria within the nasopharynx ascend the eustachian tube and gain access to the middle ear space (50), often due to the presence of predisposing factors, such as previous or concurrent viral infection, young age, familial predisposition, or day-care attendance (62). However, the exact mechanisms that influence the behavioural shift of NTHi from coloniser to pathogen are not understood. Once established in the middle ear, NTHi communities form biofilms that protect bacterial cells from antibiotic and immune clearance, often resulting in chronic and recurrent middle ear infections (2). Recurrent episodes occur in 26-54% of cases (55, 63, 64) and greatly increases a child's risk of developing chronic suppurative otitis media (CSOM), a complication characterised by chronic inflammation of the middle ear cavity with recurrent discharge through tympanic perforation (65). Hearing loss and consequential impairment of childhood psychosocial and cognitive development are common complications of CSOM in children (57) and although rare, neurological sequelae account for 21,000 deaths annually (57, 64, 66).

2.3.2 Lower Respiratory Tract Infections

NTHi in the lower respiratory tract is associated with community-acquired pneumonia, persistent bacterial bronchitis in children and chronic infections in patients with CF (2, 65). More commonly, NTHi is the leading bacterial cause of lower airway infections in adults with COPD, a chronic inflammatory lung disease characterised by chronic obstruction of airflow that

results in impaired breathing and/or destruction or the lung parenchyma (67-69). COPD is estimated to affect 328 million people globally and is the third leading cause of mortality worldwide (68). The mucociliary dysfunction and excess mucus production in COPD airways cultivates an environment that favors bacterial colonisation and a predisposition to respiratory infections (65). Infections are common precipitants of acute exacerbations of COPD; acute events characterised by a worsening of respiratory symptoms that results in additional therapy (70-73). During acute exacerbations, the detection rate of NTHi from bronchus washings or brushings is high (87%), compared to stable COPD (33%) (74) and acquisition of new NTHi strains is associated with the onset of an exacerbation (75). Frequent exacerbations drive clinical and functional decline in COPD and are associated with an accelerated loss of lung function, higher mortality, decreased health-related quality of life and significant economic costs (76). The presence of NTHi in the airways of COPD patients is associated with an increased frequency of exacerbations, and even in the absence of an acute exacerbation, worsening symptoms, inflammation causing tissue damage, and compositional changes to the lung microbiome (65, 77, 78). As such, NTHi is an important contributor to morbidity and mortality in COPD (56, 64).

2.3.3 NTHi as a Cause of Invasive Infections

Prior to introduction of the Hib-conjugate vaccine into national immunisation programs, invasive infections associated with the *Haemophilus* genus were almost exclusively with type-b strains (79, 80). Following the sustained reduction in Hib disease, surveillance programs coincidingly reported a steady year-on-year rise in the incidence of invasive *Haemophilus* infections caused by NTHi (81) from 10-17% in 1989 to 84-90% in 2009-2015 (9, 82-84). It has been proposed that the Hib vaccine drove selection for NTHi, causing pathogen strain replacement; however, the mechanism is not understood (85). Invasive NTHi disease primarily affects individuals in both age extremes, or those with impaired immune function or underlying comorbidities (2). The predominant clinical presentation of invasive NTHi disease varies with age; sepsis without a focus in neonates, meningitis in infants and children, and pneumonia in older adults (86, 87). In both age groups, these infections are associated with serious morbidity and high mortality rates (2, 84, 86). The increasing trend of invasive disease caused by NTHi, particularly in neonates, reinforces the need for an effective vaccination program (84).

2.4 Current Management of NTHi Infections Necessitates Alternative Therapeutic Strategies

There are two broad approaches to managing infections: antibiotic therapy to treat the infection and vaccination to prevent them. Initial empirical treatment usually involves antibiotic therapy, the selection of which depends on the nature and location of the infection. Mucosal infections are typically treated with moderate spectrum β-lactam antibiotics (88). In COPD patients, doxycycline and macrolides are typically used for their broad activity against common respiratory pathogens and additional anti-inflammatory properties (89, 90), and fluoroquinolones are reserved for the treatment of complicated exacerbations (91). However, due to a rapidly evolving resistance profile, there is a growing frequency of treatment failure with these agents, particularly in patients frequently exposed to antibiotics, such as those with COPD, CF or children presenting with recurrent OM (77). The growing threat of NTHi antibiotic resistance was recognised by the World Health Organization's 2017 list of priority pathogens for which new therapies are urgently needed (3, 92).

2.4.1 Impact of Antibiotic Resistance on the Management of NTHi Infections

The most common resistance mechanism expressed by NTHi is a TEM-1 β-lactamase, conferring resistance to commonly used moderate-spectrum β-lactam antibiotics, such as ampicillin, but not to amoxicillin-clavulanate or oral cephalosporins (2, 93). The prevalence of this mechanism varies markedly between regions but is typically reported in 28-50% of clinical isolates and has grown substantially over the past decade (93-98). More recently, studies began reporting the emergence of treatment failure with amoxicillin-clavulanate and cephalosporins used to treat NTHi-associated AOM and community-acquired pneumonia (96, 99, 100). These strains did not produce a β-lactamase but instead contained amino acid substitutions in the penicillin-binding protein 3 (PBP3), resulting in lowered affinity for β-lactam antibiotics (101, 102) and are referred to as β-lactamase-negative ampicillin-resistant (BLNAR) strains. Although global isolation of these strains is currently low (12-17%) (97, 98, 103), their prevalence is rapidly increasing, particularly in regions such as Japan and Taiwan where multiclonal selection has promoted BLNAR strains to the predominant ampicillin-resistant phenotype (102, 104, 105). Since 2007, there have also been increasing reports of strains that combine β-lactamase production with the presence of PBP3 mutations (β-lactamase-positive amoxicillin-

clavulanic acid-resistant: BLPACR) which have further reduced susceptibility to β-lactam antibiotics (106).

Although uncommon, additional mechanisms of acquired resistance to macrolides, quinolones and fluoroquinolones have also been reported (107). Strains containing mutations in the 23S rRNA gene and ribosomal binding proteins, or increased expression of inherent efflux mechanisms exhibit resistance to macrolides. Additionally strains with the plasmid-encoded *aac(6')-Ib-cr* gene, or mutations in the DNA gyrase gene *gyrA* or in the topoisomerase IV gene *parC*, exhibit reduced susceptibility to quinolones, or high-level fluoroquinolone-resistance, respectively (108). Multidrug-resistant isolates of NTHi with a combination of the aforementioned resistance mechanisms have been recovered from pathogenic strains in blood, middle ear and sputum, and colonising nasopharyngeal strains (89, 90, 96, 108-111). These strains exhibit high levels of resistance to β-lactams and macrolides and reduced susceptibility to quinolones (96, 108, 112). The emergence of extensively drug resistant isolates have also been reported in Taiwan (112).

The growing prevalence and spectrum of NTHi antibiotic resistance necessitates changes to antibiotic treatment guidelines that carefully balance intended clinical outcomes with the risk of further promoting antibiotic resistance (89, 96, 113-117). Antibiotic therapy may reduce middle ear damage in some patients with AOM (118) but in 60% of cases, the infection spontaneously clears without antibiotic intervention and without complications (58, 118). Similarly, antibiotic management reduces exacerbation frequency in COPD patients (119, 120) but has no overall impact on airway destruction and disease progression (91). These benefits to clinical outcomes are predicated on successful eradication of the bacterium from the respiratory tract following antibiotic treatment. However, NTHi frequently evades antibiotic clearance through a variety of host-cell interactions, such as invasion and biofilm formation, which through incomplete resolution of the initial infection and persistent bacterial colonisation is likely to influence risk of relapse (70, 121, 122). For this reason, antibiotics do not offer long-term protection against reinfection with different strains and relapse is common, particularly in COPD patients (63, 77, 123). Additionally, prolonged and sub-inhibitory antibiotic concentrations in airways may promote antibacterial resistance by providing a conducive environment for the amplification of antibiotic-resistant subpopulations (121). The prevalence of resistance has the potential to increase quickly due to the ability of NTHi to not only transfer resistant genes on mobile genetic elements, but also by chromosomal recombination (107). This genetic competence, coupled with the high rates of morbidity and long-term antibiotic

prescription associated with NTHi infections, collectively exposes a substantial proportion of the population to antimicrobial agents which drives broad-spectrum antibiotic resistance in the community (106, 118).

2.4.2 Absence of an Effective Vaccine

Despite the enormous success of the Hib-conjugate vaccine and numerous attempts, an effective vaccine for NTHi has not yet been developed (92). Hib vaccines have been highly effective in preventing invasive infections caused by capsular type b *H.influenzae*, however, they have no effect on non-capsulated NTHi (124). A similar vaccine targeting non-capsular strains may play a significant role in reducing NTHi-associated disease burden and need for antibiotic treatment (125, 126). A range of conserved NTHi surface proteins, generally with adhesive functions, have been targeted as vaccine antigens (5). However, the only vaccine that has shown any protection against NTHi disease is the ten-valent pneumococcal conjugate vaccine (PHiD-CV; Synflorix™, GSK Vaccines) licensed in 2008 by a number of countries for active immunisation against AOM caused by NTHi (127). This approach used a pneumococcal polysaccharide conjugated to the *H. influenzae*-derived protein D (PD) which demonstrated a modest 35.3% efficacy against NTHi nasopharyngeal carriage and development of AOM (2, 128). However, this efficacy was not consistent and several randomised controlled trials of infants in Finland and Australian Indigenous populations reported no significant impact on NTHi carriage rates or development of AOM (29, 129). Despite high level induction of PD-specific antibodies, this vaccine was also unable to augment pulmonary clearance of NTHi and thus offered no protection against lung infection (130, 131). Descendent higher valency pneumococcal conjugate vaccines offering protection against more serotypes of *S. pneumoniae* currently offer no protection from NTHi (2, 132).

Challenges in developing an effective vaccine arise from the enormous genetic heterogeneity among strains and the high rates of phase-variable expression of many putative vaccine targets (5, 133, 134). Variability in the expression or sequence of potential vaccine antigens may not induce a broadly reactive immune response or provide cross-protection against multiple NTHi strains. The ineffectiveness of single-antigen target approaches highlights the need for alternative vaccine targets, or development of vaccines with multiple targets important for NTHi pathogenesis (135).

2.5 Haem-iron Acquisition is an Important Mediator of NTHi Pathogenesis

Iron is an essential micronutrient for commensal and pathogens alike, with critical roles in many vital cellular processes, both as an inorganic iron and through incorporation into haem (136). Like other haem auxotrophs, NTHi has a preference for the acquisition of haem (a molecule composed of a porphyrin ring complexed with iron) as a means of fulfilling this nutritional requirement, from which iron can be liberated or incorporated into other essential haemoproteins (137). Haem not only serves as a source of iron but is also important for a variety of cellular processes including respiration, signalling, gas sensing, microRNA processing, and cellular differentiation (138).

The pathogenesis of NTHi is largely dictated by interactions with host airway epithelia. Irrespective of the site of infection, NTHi adhesion and colonisation of the host nasopharyngeal epithelium is required to cause infection (70). Although the exact mechanisms are poorly understood, migration from the nasopharynx to privileged anatomical sites is influenced by a combination of bacterial pathogenic mechanisms and defects in host innate immune defences (70). Survival and persistence at the site of infection is mediated by host-cell internalisation, formation of biofilms, or modulation of the immune response that protect the bacterium from immune or antibiotic clearance (139-141). In addition to being an essential growth requirement, iron-containing haem has demonstrated an important role in the ability of NTHi to perform many of these interactions and as such, the ability to sequester (i.e obtain and internalise) host-derived sources of haem is an important determinant of pathogenesis (**Table 2.1**) (142, 143).

2.5.1 Host Attachment and Colonisation

The high concordance between colonising throat strains and pathogenic strains isolated from lower airways implicates the nasopharynx as a potential reservoir for NTHi infections (144). Both the frequency and density of pharyngeal colonisation has been directly linked to increased risk of developing AOM (17, 145) and AECOPD (48, 146). In COPD, the acquisition of a new strain is frequently associated with the onset of an acute exacerbation (146). Populations with the highest NTHi carriage rates, such as the Indigenous communities in Australia, experience the greatest proportion of AOM (40). A study of 100 aboriginal children (aged 1-3 months) found that early nasopharyngeal carriage of NTHi was associated with a 3.70 times (95% CI 1.22-11.23) higher risk of developing AOM, independent of environmental factors

(61). Similarly, nasopharyngeal carriage of NTHi is associated with lower airway infections in children with bronchiectasis (144). Higher bacterial loads in the nasopharynx have been correlated with an increased risk of developing AOM (17, 147, 148) and a clinically significant increase in respiratory symptoms in COPD, even in the absence of a clinical exacerbation (149).

Successful host colonisation is achieved by a suite of outer membrane proteins that mediate attachment to the nasopharyngeal epithelium (150). Upregulation of several proteins involved in host-cell adhesion have been demonstrated in iron deplete media designed to replicate conditions of the respiratory tract (151, 152). This suggests a co-regulatory relationship between haem-iron acquisition and adhesin expression. Haem-iron acquisition in NTHi is coordinated by the ferric uptake regulator *fur*, a master regulator of genes involved in the uptake of iron and iron-containing moieties (153). Subsequent transcriptome and proteomic analysis of *fur* has revealed additional regulatory management of several molecules shown to mediate attachment and colonisation, such as the high-molecular weight proteins (HMW) (142, 151). HMW1 and HMW2 are major NTHi adhesins, present in 75-80% of isolates. These glycopeptides bind to integrin-receptors on epithelial cell surfaces and are vital to the bacterium's ability to adhere to cultured human respiratory epithelial cells and colonise the upper respiratory tract of rhesus macaques *in vivo* (154).

Proteins involved in host-cell colonisation have also demonstrated moonlighting functions directly or indirectly involved in haem or iron acquisition. The outer membrane adhesin, protein E (PE), is ubiquitous in all NTHi clinical isolates and hijacks host vitronectin-integrin binding to promote cell adhesion (155). The requirement of PE for the optimal adherence and persistence within airways has been demonstrated in mice immunised with anti-PE antibodies (156). More recently, PE has been found to form high affinity interactions with haemin and influence the ability of *H. influenzae* to acquire haem *in vitro*. PE-bound haem could also be donated to haem-starved populations during co-culture, suggesting a secondary role of PE as a storage site for haemin, which can be later distributed to nearby starved cells and promote survivability under conditions of fluctuating haem availability (143, 157). The Sap (sensitivity to antimicrobial peptide) inner membrane ATP-binding-cassette (ABC) transport complex also appears to be important in the ability of NTHi to acquire haem-iron and colonise the host (158). Deletion of the Sap structural ATPase protein, SapF, simultaneously inhibits recovery of depleted internal haem-iron stores and reduces the ability of NTHi to colonise the nasopharynx or cause acute infection in a chinchilla model of AOM (159). The periplasmic binding protein, SapA was also found to be essential for haem transport and utilisation by

haem-starved NTHi (158) and adherence to polarised epithelial cells (160). Inactivation of other well-characterised haem-acquisition systems, such as HbpA and HxuCBA have also attenuated the ability of NTHi to adhere to cultured airway epithelia (143). However, it is unclear if these systems have a direct role in adherence, signalling or if this effect was a consequence of bacterial malnutrition. The shared host-cell adhesion and haem-binding functionality of many NTHi proteins suggests that bacterial haem-utilisation plays an important role in its interplay with host epithelial cells and may provide an adaptive advantage for colonisation in environments with low haem-iron availability (161).

2.5.2 Survival and Pathogenesis in the Respiratory Tract

Haem is essential to the function of haemoproteins that are expressed by NTHi; these proteins are involved in energy generation by the electron transport chain, detoxification of host immune effectors and other vital processes (138). NTHi is unable to synthesise haem and it must therefore secure host-derived sources of haem for continued survival in the respiratory tract (162). NTHi strains with a reduced capacity to acquire haem have a substantially shortened lifespan in broth culture and in chinchilla airways (163, 164).

In addition to being an absolute requirement for growth, the ability to acquire haem from a diversity of host sources may contribute to the pathogenic potential of NTHi. This is highlighted by the higher prevalence of multiple haem-acquisition genes in disease-associated isolates from the middle ear and lungs, than in colonising throat strains or their non-pathogenic relative, *H. haemolyticus* (124, 151). A conserved genetic island unique to disease-associated NTHi features an overrepresentation of genes associated with the acquisition and transport of haem (165). Capacity to utilise specific haem or iron sources such as transferrin and haptoglobin-bound haemoglobin has also been associated with *Haemophilus* spp. isolates from invasive infections in animal models (165, 166). Inactivation of multiple genes associated with haem-utilisation was found to attenuate NTHi virulence-determinants and disease severity/duration in animal models of AOM and lung infection (67, 143, 163, 167). Similarly, an isogenic mutant of two haem-acquisition pathways was unable to sustain bacteraemia or produce meningitis in a rat model of invasive disease (168). Collectively, these data indicate that acquisition genes mediate important virulence mechanisms and contribute to longer and more severe infections by regulating iron within the cell (169).

Table 2.1| NTHi proteins and regulatory elements involved in the acquisition of host-derived haem-iron and non-haem iron sources *in vivo,* and evidence for their potential roles in pathogenesis.

ACQUISITION MECHANISMS	HAEM/IRON SOURCES	POTENTIAL ROLE IN PATHOGENESIS
TON-B DEPENDANT TRANSPORTER SYSTEMS		
HxuCBA	**Haem:** free, hpx-, alb- **Hb:** free, hapt-	**Colonisation:** Mediates adherence to cultured airway epithelia [168, 170] **Virulence:** Higher prevalence in middle ear strains [124]; establishment of invasive disease and mortality in rats [168, 170] **Persistence:** Potentiates antibiotic resistance [143]
TbpAB	**Iron:** transferrin	**Virulence:** Conserved in all invasive NTHi isolates [171]
HgpBCD	**Hb:** free, hapt-, mb-	**Virulence:** Higher prevalence in middle ear strains [124]; establishment of invasive disease and mortality in rats [168, 170] **Survival:** Required for bacterial proliferation during NTHi-induced OM in chinchillas [163]
HemR	**Haem:** free	**Virulence:** Polymorphisms associated with OM isolates, compared to commensal isolates [172]
Hup	**Haem:** free, hpx-, alb- **Hb:** free, hapt-	No data available
HhuA	**Hb:** hapt-	**Survival:** Required for growth *in vitro* [164]
FbpA	**Iron:** transferrin	**Colonisation:** Adhesion molecule of *Listeria monocytogenes* [173]; no data available for NTHi
Fhu	Xenosiderophore acquisition	**Survival:** May utilise siderophores made by other bacteria [174]
TON-B INDEPENDENT PROTEINS		
Protein E (OMP)	**Haem:** free	**Colonisation:** Adherence to cultured and mouse lung epithelia [70, 175] **Survival/persistence:** Invasion of cultured and mouse bronchial epithelia [155]; potentiates antibiotic resistance [143]; inter-bacterial donation of haem [70, 175]
Sap (PP)	**Haem:** free	**Colonisation/persistence:** Adhesion, colonisation and biofilm formation in chinchilla middle ear [160, 176] **Virulence:** Establishment of chinchilla OM [160, 176]

IgA1 Protease	Upregulated by *fur* in response to iron-restricted conditions	**Persistence:** Mediates antibiotic resistance; optimal invasion and long-term intracellular survival in human respiratory epithelial cells [176]
HbpA (PP)	**Haem:** free, alb- **Hb:** Hb, hapt-	**Virulence:** Establishing bacteraemia in a mouse model [177] **Persistence:** Mediates glutathione import and antibiotic resistance [143, 178] **Survival:** Important for periplasmic transport of haem through other outer membrane channels [179]
REGULATORY ELEMENTS		
fur	Master regulator of genes/proteins involved in haem-iron utilisation	**Virulence/persistence:** Critical for bacterial virulence and persistence in a mammalian model of OM [142] **Colonisation:** Additional regulatory management of molecules shown to mediate attachment [142]
icc	Stress response to transient haem/iron limitation	**Virulence/persistence:** Mutations result in epigenetic and morphological adaptations that contribute to persistence and disease severity in experimental models of OM [67, 180]
modA	Downstream phase-variable regulation of haem/iron acquisition proteins	**Virulence/persistence:** Phase variation of antibiotic resistance, biofilm formation and immunoevasion; biphasic switching during chinchilla OM [181]

Haemoglobin, **Hb**; haptoglobin-bound, **hapt-**; albumin-bound, **alb-**; haemopexin-bound, **hpx-**; myoglobin-bound, **mb**-; haem/hemopexin utilisation protein, **hxu**; transferrin-binding proteins, **Tbp**; haemoglobin binding protein, **Hgb**; haemin receptor, **HemR**; haem-utilisation protein, **Hup**; haemoglobin-haptoglobin binding protein, **Hhu**; ferric-binding protein, **FbpA**; ferric hydroxamate uptake, **Fhu**; sensitivity to antimicrobial peptide, **Sap**; immunoglobulin A1, **IgA1**; haem-binding lipoprotein, **HbpA**; ferric uptake regulator, ***fur***; outer membrane protein , **OMP**; periplasmic protein, **PP.**

There is also evidence to suggest that haem-iron accessibility may influence adaptive transitions between commensal and pathogenic states. For many opportunistic pathogens, iron starvation not only triggers activation of genes involved in its uptake, but also those involved in virulence determinants (153, 182, 183). In *Pseudomonas aeruginosa,* positive selection for promotor mutations that increase expression of the bacterial uptake system *phu* (pyoverdine siderophore) occurs exclusively in isolates from patients with CF and enhances bacterial growth (184). A similar microevolutionary response to transient haem-iron restriction has been described in NTHi through mutations of *icc*, which result in epigenetic and morphological adaptations that contributed to persistence and disease severity in experimental models of AOM (67, 180). NTHi haem-iron acquisition proteins are also subject to high rates of phase-variable changes in gene expression, which are differentially regulated during experimental OM (181) and in isolates recovered from COPD airways (185-188). This phase-variable shift from the OFF to ON state within the middle ear produces a phenotypic switch in several genes during infection that increases disease severity, cell adhesion and permits bacterial persistence by increasing invasion and robust biofilm formation (181, 189-191). It has been hypothesised that regulatory switching of haem-acquisition genes provides an adaptive response to fluctuations in haem-iron availability/source influenced by the stage or site of infection (192). Additionally, strand slippage may provide a mechanism to avoid the immunological response of the host by expressing proteins that are functionally similar, but antigenically distinct (185, 188).

2.5.3 Persistence in the Respiratory Tract

NTHi persistence within the respiratory tract, not only enables the potential for disease recurrence/relapse, but also induces constant airway inflammation and promotes disease progression in COPD lungs (69, 193, 194). NTHi populations exposed to haem-restricted conditions, such as those in the respiratory tract, appear to demonstrate a phenotypic shift that favours behaviours associated with persistence (67). To persist within the airways, NTHi must subvert or evade clearance by antibiotics or the innate host immune response. This is achieved by a variety of mechanisms that include specific neutralisation of antimicrobial agents, invasion of host epithelial cells and formation of protective biofilms (72, 163, 195, 196).

An important first-line defence by the host against microbial growth is to reduce circulating concentrations of essential nutrients, such as haem and iron, in an effort to limit microbial

survival; a process known as nutritional immunity. Siderocalins also contribute to this anti-microbial defence by sequestration of microbial siderophores, such as those produced by *Klebsiella* and *Neisseria* species to obtain host sources of iron (197). However, the siderophore-independent nature of haem (and hence iron) acquisition by NTHi renders siderocalins ineffective and may also provide an adaptive advantage over competing bacteria in the respiratory tract that rely on siderophore-mediated acquisition of iron for survival (198, 199). The initial immune response against bacterial infection of mucosal surfaces in the respiratory tract also involves recruitment of immune cells and abundant production of antibacterial peptides (AMPs) (200). IgA1 is the principal immunoglobulin subclass produced by respiratory mucosal tissues and plays a major role in host defence by inhibiting microbial adherence, inactivating bacterial toxins, and promoting humoral immunity (200). NTHi counteracts this response by producing an IgA1 protease, an extracellular endopeptidase which specifically cleaves IgA1 (188). IgA1 protease production appears to be exclusive to pathogenic *Haemophilus* species and is upregulated by *fur* in response to iron-restricted conditions (188).

Coordination between bacterial immune evasion and haem homeostasis has been observed for several NTHi proteins involved in the acquisition/utilisation of haem. HxuCBA, SapA, and PE have been shown to confer resistance to AMP LL-37 and a homologue of human β-defensin in a chinchilla model of AOM (143, 159, 162). The consequence of inhibiting AMP resistance was observed in a SapA mutant which had an attenuated ability to survive in both the nasopharynx and the chinchilla middle ear, compared to the parent strain (162, 201). The versatile periplasmic protein, HbpA, is not only required for transport of haem into the periplasm, but also glutathione, a vital peptide that mediates protection against oxidative, xenobiotic, and metal iron stresses (143, 178). The absence of glutathione has been shown to reduce survival of *H. influenzae* Rd in minimal media (202). Thus, NTHi haem-acquisition systems may promote airway persistence and pathogenesis by conferring resistance to the innate mucosal immune response and oxidative stress (143, 203). This may contribute to the predisposition to NTHi infections in CF and COPD patients who demonstrate compromised lower airway AMP activity despite mounting an inflammatory response (200, 204).

NTHi invasion of host epithelial cells may not only provide a means of evading host immune pressures, but also provide an alternative reservoir for nutrient acquisition when bacteria are exposed to nutrient-limiting conditions (142). Haem and iron acquisition systems have demonstrated a role in sensing host environmental cues to mediate interactions with host epithelial cells. Chinchilla middle ears challenged with *sapA* mutants deficient in haem-iron

uptake demonstrated a tendency towards a more persistent phenotype, favouring intracellular survival and a dampened cytokine response compared to the parent strain (160). Similarly, expression of *fur*-regulated IgA1 protease (142) was required for optimal invasion and long-term intracellular survival in bronchial epithelial cells (176). These observations suggest environments restrictive of iron promote NTHi invasion and persistence, however, contradicting evidence reveals a more nuanced relationship between haem/iron availability and NTHi phenotype. One study observed an impaired ability of NTHi mutants lacking *hxuCBA, hbpA, hpe* or *sapA* haem-acquisition systems to invade type II pneumocytes (143). Mutants lacking the conserved iron-regulon *fur*, also exhibited reduced persistence in middle ears of chinchillas (142). Exposure to excess haem-iron was only found to promote a persistent phenotype in experimental models of OM in NTHi strains previously starved of haem-iron, compared to those continuously exposed to haem-replete conditions (67, 180). This response was attributed to microevolutions through mutations in *icc* during exposure to transient haem-iron restriction (205). Recurrent episodes in a separate pre-clinical model of OM also demonstrated continuous microevolution of a haemoglobin-binding gene that resulted in a highly invasive NTHi phenotype that persisted for at least one month following clinical resolution of infection (206). Thus, the intracellular locale may act as a mucosal reservoir for NTHi following resolution of clinical disease that provides additional sources of haem in addition to protection from immune- or antibiotic-mediated killing, thus promoting bacterial persistence within the airways (160, 182).

The ability to form sedentary communities or biofilms in anatomical sites, such as the middle ear, also contribute to the *in vivo* persistence of NTHi (122). Biofilm-resident bacteria exhibit a reduced metabolism and an altered proteome compared to their planktonic counterparts, features that contribute to their reduced susceptibility to immune effectors and commonly used antibiotic treatments (134). This has contributed to the chronic and recurrent nature of infections associated with NTHi, including bronchitis, AECOPD, conjunctivitis, sinusitis, and OM (65, 134, 207, 208). Haem-iron restriction or loss of the utilisation protein SapF results in morphological plasticity and enhanced community development and biofilm architecture (67, 159). These observations suggest a potential role of haem-iron availability in coordinating the complexity of NTHI biofilm architecture *in vivo* and *in vitro.*

2.6 NTHi Haem-iron Acquisition and Utilisation in the Respiratory Tract

Haem plays an important role in a variety of vital cellular processes; thus, all bacteria must satisfy this nutritional requirement through either synthesis or acquisition. NTHi is incapable of endogenous haem biosynthesis as it lacks the enzymatic capacity to synthesize the immediate precursor to haem, protoporphyrin IX (PPIX) (209). However, NTHi does possesses a ferrochelatase, allowing it to catalyse the insertion of iron into PPIX to form haem. As there is no significant source of free PPIX *in vivo*, this growth requirement is fulfilled by acquisition of haem or other iron-containing moieties (209-211).

2.6.1 Haem-iron Availability in the Respiratory Tract

Iron has critical functions in many cellular processes, such as DNA replication and respiration, making it an essential micronutrient for all microbes, as well as their hosts (212). The redox potential of iron mediates both its catalytic utility in biological reactions, and production of cell-damaging free radicals (213). The host must therefore maintain tight regulation of systemic and cellular iron homeostasis to simultaneously meet the body's iron demand, prevent cellular toxicity and withhold nutrients from invading pathogens. This is achieved by finely-tuned sequestration of free iron by iron-binding proteins (such as ferritin, transferrin, lactoferrin) and by capturing haem within haemoproteins (212, 213) (**Figure 2.1**). The majority of total haem-iron in the body is found complexed as haemoglobin within erythrocytes (60-70%), whereas the major store of inorganic iron is ferritin within hepatocytes and macrophages (25%) (67, 124). As such, plasma iron concentrations are maintained at a low level, which decreases substantially during the acute phase response to infection or other inflammatory stimuli (214). The intracellular sequestration and the paucity of free extracellular haem/iron ensures that the availability of this essential nutrient to pathogens is scarce (213).

Haem-iron levels are estimated to be low in the respiratory tract, the majority of which is sourced from inhaled atmospheric iron and iron-containing particulate matter (215). The upper respiratory tract is exposed to ~10-25 µg iron daily, approximately 1/1000th of that encountered by the gastrointestinal tract (215, 216). Little is known about the mechanisms by which the lung acquires iron independently of inhalation, but it is likely to occur in a similar manner to other organs. Iron may be sourced from the pulmonary vasculature in free-, or transferrin/lactoferrin-bound forms, or as a component of haemoprotein-bound

haemoglobin/haem. The high abundance (44.5%) of transferrin present in bronchoalveolar lavage fluid indicates that iron regulation in the lung may be primarily controlled by transferrin (216). Both alveolar macrophages and the bronchial/alveolar epithelia are able to sequester iron by various mechanisms including receptor-mediated uptake of ferric haem-iron (followed by safe storage within ferritin) and endocytosis of haemoglobin-haptoglobin complexes (215, 217).

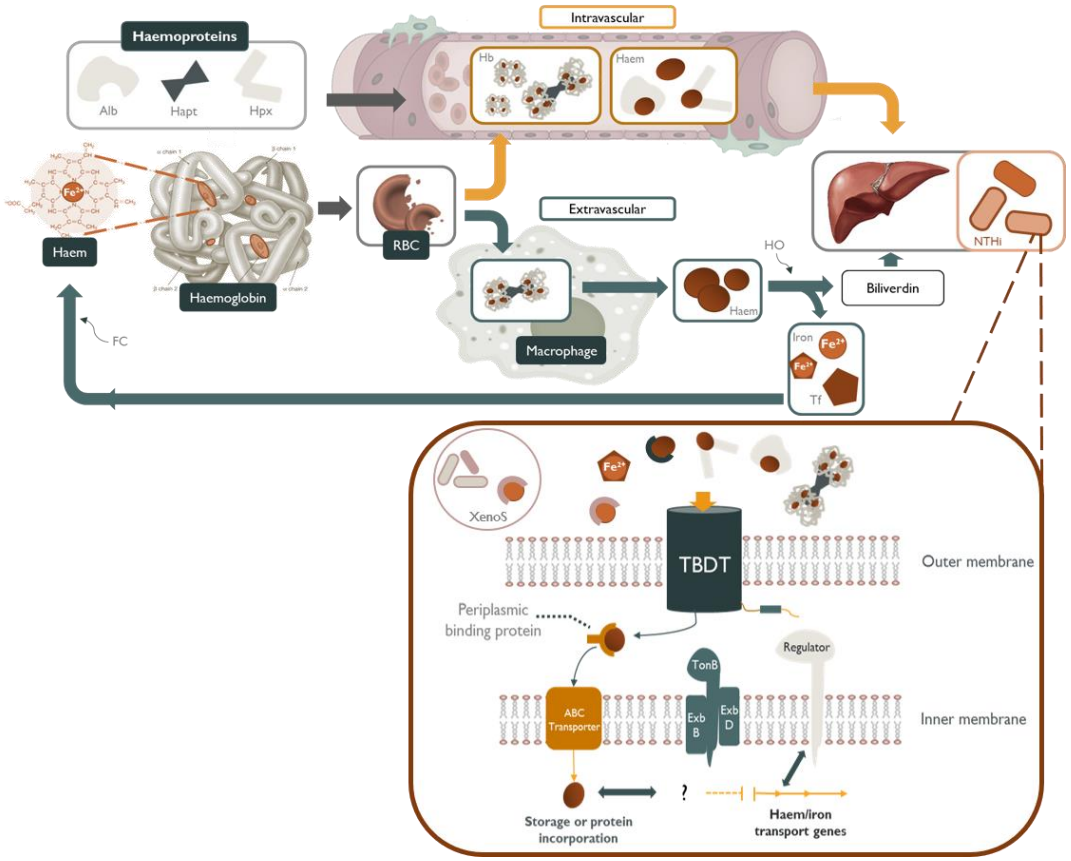


Figure 2.1| NTHi sequestration of host-derived sources of haem and other iron-containing moieties. Haem-iron released into the plasma by erythrocyte (RBC) senescence are quickly scavenged by haemoproteins such as, albumin (Alb) and hemopexin (Hpx). Any haemoglobin (Hb) released into the serum is tightly bound by haptoglobin (Hapt) and subsequently cleared by tissue macrophages (67, 124). Free-haem, free-iron, transferrin-bound (Tf) iron, xenosiderophore-bound (XenoS) and haem-containing haemoproteins are readily available to NTHi as sources of nutritional haem/iron. These sources are transported across the periplasmic space by specific TonB-dependent transporters (TBDT) powered by a cytoplasmic transmembrane protein complex (TonB, ExbB and ExbD), followed by transport across the inner membrane by an ATP-binding cassette transporter (ABC transporter). Haem/iron acquisition in NTHi is regulated in accordance with environmental cues by iron-repressive regulators such as *fur*.

2.6.2 NTHi Acquisition of Host-derived Sources of Haem-iron

The inherently low availability of haem-iron in the respiratory tract, exacerbated by host nutritional immunity, produces an environment deplete of haem-iron. Despite these limiting conditions, NTHi is adept at scavenging a variety of host-derived haem-iron sources from intracellular and extracellular reservoirs, using a highly complex and redundant repertoire of haem acquisition systems (**Table 2.1**) (213, 218, 219). NTHi can uptake haem either directly or by secreted haem-binding proteins, both of which require cell surface receptors that import haem, machinery to shuttle them across the cell wall and membrane, and cytoplasmic components to liberate iron from haem or shuttle intact haem to haem-containing enzymes (149, 213). NTHi is also capable of storing excess haem, creating an intracellular surplus that can be donated to starved bacterial cells in haem-iron deplete conditions (143). Although the mechanisms involved in NTHi inter-bacterial donation and storage are not understood, iron storage in the form of ferritins and bacterioferritins has been described in several pathogenic species; the ability of which mediates growth, protection against redox stress, survival in the host and virulence (213).

Haemoprotein-mediated sequestration from the extracellular environment is the most common mode of haem-iron acquisition used by NTHi. Target-specific secreted or surface-anchored proteins function by taking up free haem or extracting it from hemoproteins in the extracellular medium and delivering it to a TonB-dependent outer membrane receptor (124). Quantitatively haemoglobin and haemoglobin-haptoglobin complexes are likely to be the most significant extracellular sources of haem *in vivo* (168). This is reflected by the sheer number of mechanisms possessed by NTHi for obtaining these molecules and the attenuated pathogenesis of strains unable to utilise them (163). The ability to utilise iron from transferrin or from haemoglobin complexed with haptoglobin is not shared among other members of the *Haemophilus* genus (220) and strains deficient in the ability to acquire haemoglobin-haptoglobin have an attenuated ability to cause systemic infections (168). Individual NTHi strains are capable of producing up to four different haemoglobin/haemoglobin-haptoglobin binding proteins (Hgp) that collectively display affinity for all known human haptoglobin phenotypes (221). The presence of multiple *hgp* genes may allow for selective expression of the Hgp with greatest affinity for the predominant host haemoglobin-haptoglobin phenotype, or alternatively as a strategy to evade the host immune response mounted against a particular Hgp-expressing population (222). There are 2 major alleles of haptoglobin, with each αβ subunit capable of binding one haemoglobin αβ subunit, such that each Hp 1-1 molecule can bind

two haemoglobin αβ subunits, while the polymeric Hp2-1 and Hp 2-2 molecules have the capacity to bind greater numbers of haemoglobin αβ subunits (221). The prevalence of haptoglobin phenotypes expressed varies between populations, with the Hp2-2 phenotype predominating in Indian and Indigenous populations of Australia (222). A growth preference for the Hp 1-1 phenotype has been demonstrated by *H. influenzae in vitro* (221), however the correlation with susceptibility to infection *in vivo* has not been investigated.

Although less common, NTHi (approximately 3% of isolates) also express receptors that directly recognise and extract iron from transferrin and/or lactoferrin (212). In other bacterial species, the most common strategy for obtaining these sources of iron involves the secretion of siderophores, small molecule ferric chelators, which possess an affinity for iron that outcompetes host transferrin (223). Siderophores are synthesised under conditions of low iron availability and are secreted into the extracellular environment where they complex and deliver ferric iron to the cell via surface receptors (223). Unlike the majority of bacteria, NTHi does not possess genes encoding proteins for siderophore synthesis; however, an iron-repressible siderophore utilisation locus was discovered in several NTHi strains that may enable the utilisation of xenosiderophores produced by other microorganisms in the human nasopharynx (224). Such a tactic would expand the pool of iron sources available to NTHi, without the energy burden associated with siderophore synthesis (124).

Once complexed by haem-targeting proteins or xenosiderophores, a set of TonB-dependent transporters (TBDTs) are required to transport the haem/iron into the periplasmic space, following transport across the inner membrane by ATP-binding cassette (ABC) transporters (209). A cytoplasmic transmembrane protein complex composed of three proteins, TonB, ExbB and ExbD, spans the periplasm and interacts with specific TBDTs. This TonB complex transduces the proton motive force of the cytoplasmic membrane to energise transport of substrates through a specific TBDT (225). Once inside the cell, haem-bound iron can be liberated through enzymatic degradation by haem oxygenase (213); the mechanisms governing iron liberation from xenosiderophores has not yet been described. Haem-iron acquisition in NTHi is regulated in accordance with environmental cues by iron-repressive regulators such as *fur*, which is coordinated with storage and efflux to ensure iron homeostasis (213). Tight regulation of haem-iron uptake is critical for balancing the metabolic requirement with the potentially toxic consequences of excess (142).

2.6.3 Dysregulation of Host Haem-iron Homeostasis

High iron availability has been shown to increase the pathogenic potential of many bacteria in tissue and animal infection models by enhancing growth, cellular adhesion and invasion, and epithelial damage (226-228). Thus, disorders that interfere with iron-restricting host responses may predispose individuals to NTHi infections. High susceptibility to infection with a variety of bacterial genera, including *Haemophilus* has been described in several iron overload disorders, such as hereditary haemochromatosis, β-thalassemia, sideroblastic anaemia, transfusion-dependence, or chronic liver disease (229-234). In these conditions, the transferrin-binding capacity is exceeded, resulting in low affinity bonds with iron and excess non-transferrin-bound iron in plasma (213). These conditions provide a source of iron that is easily accessible for microbes. Studies have also demonstrated that iron overload is associated with defective chemotaxis and phagocytosis of neutrophils and macrophages as well as decreased bactericidal activity, contributing to decreased immune function (235-240). Another study demonstrated impaired killing of *Pseudomonas aeruginosa* by iron-overloaded human macrophages due to dysfunction of lysosomal acidification (241). Some opportunistic pathogens, such as *Capnocytophaga canimorsis*, *Yersinia spp., Vibrio vulnificus, Tropheryma whippelii*, and *Legionella pneumophila* are sufficiently impaired in iron acquisition ability so as to be dangerous mainly in hosts with iron loading conditions (234). Thus, a combination of impaired immune function and increased iron availability may contribute to the heightened susceptibility to infection in conditions of iron-overload (240).

High levels of iron and/or ferritin have been detected in the airways of individuals with COPD and CF, and in heavy smokers, suggesting dysregulation of iron homeostasis in these conditions (216, 242). The subcellular location of this excess iron appears to reside intracellularly in alveolar macrophages and to a lesser extent, the epithelial lining fluid of the lung (243). One may therefore postulate a role of excess iron in the characteristically high predisposition to NTHi infections observed in these patient groups (244), however; correlative studies are required to determine if this is the case.

2.7 Disruption of Haem-iron Assimilation as an Effective Therapeutic Strategy Against NTHi Infection

The diminishing effectiveness of current antibiotic treatments and the challenges associated with vaccine development have encouraged exploration of novel therapeutic strategies to prevent and/or treat NTHi infections. The dependence for haem-iron at all stages of NTHi pathogenesis exposes a vulnerability that may provide promising targets for the development of therapies that disrupt its uptake (169, 242). Extracellular iron restriction has previously been effective in preventing infection and dissemination of pneumonia in animal models infected with a variety of gram-negative bacteria, including *H. influenzae* (245, 246). Similarly, disruption of haem or iron acquisition mechanisms significantly affects the ability of NTHi to cause disease in animal models (169). Currently, there are a variety of host- and bacterial-targeted approaches in development which have been used to disrupt haem and/or iron assimilation by a range of pathogens, and which may have utility against NTHi (**Table 2.2**).

2.7.1 Pharmacotherapy

There are currently a variety of pharmaceutical agents in development that target microbial haem-iron acquisition with proposed therapeutic utility against NTHi infections. These agents can be broadly categorised by their intended approach; either those that inhibit haem-iron availability or bacterial acquisition pathways, or toxic haem/iron-mimicking compounds that gain entry to bacterial cells through existing uptake systems (213).

One of the earliest antimicrobials used for treatment of tuberculosis, para-aminosalicylic acid, inhibits synthesis of the bacterial siderophore, mycobactin (247). Subsequently, a large number of siderophore biosynthesis inhibitors have been developed that have the capacity to prevent bacterial growth under iron-limiting conditions (248). Flucytosine, a synthetic fluorinated pyrimidine used to treat fungal respiratory tract infections, has also been shown to inhibit expression of the pyoverdine siderophore-biosynthesis gene in *Pseudomonas aeruginosa,* and subsequently suppress pathogenicity in a mouse model of pulmonary infection (249, 250). Similarly, disruption of the function of the pyoverdine protein also mitigated *P. aeruginosa* pathogenesis in a nematode host (251). Although NTHi possess the capacity to utilise siderophores, genes associated with siderophore biosynthesis have not been identified (199). Therefore, the utility of these compounds in restricting NTHi access to iron is limited

and may only disrupt a minor source of iron by proxy of decreased xenosiderophore production by local microbial communities. Additionally, local microbiota may have the potential to develop adaptive resistance through production of structurally modified siderophores (199, 252, 253).

An alternative approach is the use of xenosiderophores covalently linked to antibiotics, known as sideromycins, that encourage antibiotic uptake through existing iron uptake systems. Sideromycins have demonstrated bactericidal activity against β-lactamase producing Enterobacteriales, and other antibiotic resistant strains of *P. aeruginosa, Stenotrophomonas maltophilia* and *Acinetobacter baumannii*, including producers of the class B metallo-β-lactamases and class C serine-β-lactamases (254-257). Sideromycins have also been used *in vivo* to successfully treat infections with *P. aeruginosa* and prevent systemic infection with *S. pneumoniae* and *Y. enterocolitica* in mouse models (258, 259). However, translation into human use has historically faced diminished clinical utility due to compound instability *in vivo,* emergence of adaptive resistance during exposure, or side-effects accompanying treatment (260, 261). As a result, only the novel catechol siderophore-conjugated cephalosporin antibiotic, Cefiderocol has progressed beyond the first phase of human safety trials, owing to a unique combination of structural features derived from cefepime and ceftazidime that overcomes that stability problems associated with earlier iterations (256).

Cefiderocol has recently been approved as a last-line treatment for complicated urinary tract infections and pneumonia caused by antibiotic resistant gram-negative bacteria, such as carbapenem-resistant *P. aeruginosa, A. baumannii, and K. pneumoniae* (262, 263). However, clinical data has reported a higher all-cause mortality in patients treated with Cefiderocol for hospital-acquired pneumonia, and thus treatment regimens require close monitoring to avoid toxic side-effects, particularly in elderly patients (264). The uncertain safety profile in elderly patients and lack of data in children lends poorly to routine treatment of NTHi infections which primarily affects these age groups. Additionally, NTHi is adept at scavenging iron from a variety of sources *in vivo* and siderophore-mediated iron acquisition is a minor contributor to total iron acquisition in NTHi (174). This characteristic have been implicated in the intrinsic resistance of NTHi to siderophore-targeting compounds observed *in vivo.* In a nasal colonisation model, production of lipocalin, an acute-phase inhibitor of siderophore-mediated iron uptake, was upregulated but did not affect the ability of *H. influenzae* to acquire host-derived sources of haem-iron (198, 213, 265). Additionally, NTHi cannot utilise catechol-containing siderophores (220, 266) and so is unlikely to possess the necessary machinery to

uptake Cefiderocol. Thus, it is unlikely that the activity of catechol sideromycins against NTHi will be greater than that of the antibiotic alone. Additionally, production of multiple β-lactamases have been shown to contribute to the emergence of Cefiderocol non-susceptibility in several gram-negative isolates (267). Thus, sideromycins may be vulnerable to the same antibiotic resistance mechanisms faced by traditional antibiotic therapies.

An alternative approach that mitigates the potential for adaptive resistance involves targeting multiple iron sources through chelating agents (231). Chelation therapy prevents accumulation of excess iron and reduces its availability to invading pathogens (268). This approach has been effective in protecting mice against *K. pneumoniae* pneumonia and dissemination (245). However, as is the case with xenosiderophores, iron-containing chelating molecules such as desferrioxamine B (DFO), used in the treatment of iron-loading disorders, can be utilised as a source of iron by bacteria that harbour the necessary receptor, including NTHi (220, 269, 270). Chelation therapy with DFO may therefore increase iron availability and increase the risk of infection. More severe infections, and higher liver and kidney bacterial burdens have been demonstrated in DFO-treated mice following intravenous challenge with *S.aureus* (270). A positive correlation between DFO use and higher rates of infection with *S. aureus* and other opportunistic bacteria in patients suffering from thalassemia-associated iron overload has also been reported (229, 271). Research has therefore moved towards synthetic iron chelators which can be taken orally, such as Deferiprone (DFP) which does not promote bacterial growth (272). DFP has been found to not only reduce the growth of some strains of common gram-negative nosocomial pathogens, but also reduce the minimum inhibitory concentrations of antibiotics (273). However, the activity of these compounds against NTHi have not been tested and their effectiveness may be subverted by acquisition of haem-iron.

The majority of NTHi iron requirement is fulfilled by acquisition of haem, therefore; compounds that restrict haem-assimilation may have higher therapeutic value than those solely targeting iron acquisition. Haem assimilation was first targeted with the analogue gallium-protoporphyrin IX (GaPP), a bactericidal metalloporphyrin that uses existing haem uptake machinery to gain entry to the bacterial cell (274). Incorporation of Ga(III) in place of iron disrupts the iron-dependent redox process as Ga(III) cannot be reduced to Ga(II) under physiological conditions, and thus cannot be liberated from porphyrins by haem oxygenase (275).

Table 2.2 | Strategies that target bacterial haem-iron assimilation and their potential application for the prevention or treatment of NTHi infections.

STRATEGIES	EXAMPLE	ANTIMICROBIAL ACTIVITY	UTILITY AGAINST NTHI?	CHALLENGES/LIMITATIONS
PHARMACEUTICAL APPROACHES				
INHIBITORS OF SIDEROPHORE BIOSYNTHESIS/ FUNCTION	**Flucytosine:** nonribosomal peptide synthetase (NRPS) enzymes inhibitor	Suppresses *P. aeruginosa* pathogenicity in a mouse model of pulmonary infection [249]		
	Baulamycins A and B: NRPS independent siderophore synthetase enzymes	*In vitro* antibacterial activity against *S. aureus* (staphyloferrin B) and *B. anthracis* (petrobactin) [276]	Siderophore-inhibiting molecules do not disrupt the ability of *H. influenzae* to acquire haem-iron *in vivo* [198]	**Efficacy:** adaptive resistance through production of structurally modified siderophores has been described [199, 252, 253]
	Small molecule inhibitors	Mitigates *P.aeruginosa* pathogenesis in a nematode host [251]		
SIDEROMYCINS	**Cefiderocol (Fetroja):** Siderophore-mimicking compound conjugated to a cephalosporin	Bactericidal activity against β-lactamase producing and multidrug-resistant gram-negative species *in vivo* [262]	Effectiveness has only been demonstrated against NTHi *in vitro* under conditions with artificially restricted haem-iron availability [263]	**Safety profile**: toxic side-effects in the elderly; no safety data for children **Efficacy:** inhibition of siderophore-mediated iron-acquisition may be overcome by acquisition of haem **Acquired resistance:** has been demonstrated in gram-negative bacteria [267]
IRON CHELATORS	**Desferrioxamine B (DFO):** intravenous iron chelator	Some antibacterial and antibiotic potentiating activity observed *in vitro* [277]	NTHi can utilise iron sequestered within DFO as an iron source [220]	**Safety profile**: may increase susceptibility to infection [229, 271] **Efficacy:** can be used as an iron source, which promotes growth of some bacteria [269, 273]
	Deferiprone (DFP): oral iron chelator	Activity against common gram-negative nosocomial pathogens [273]	Activity against NTHi has not been tested.	**Efficacy:** may be subverted by acquisition of haem-iron
HAEM-DEGRADATION INHIBITORS	**Gallium-protoporphyrin IX:** haem analogue	*In vitro* activity against multidrug-resistant gram-negative species, including intracellular and biofilm communities [275, 278-280]	NTHi is highly reliant on exogenous haem-acquisition; activity against NTHi has not been tested	**Application:** no *in vivo* evidence; currently limited to topical applications
	Haem-oxygenase inhibitors	*In vitro* activity against *P.aeruginosa* [281]	Not dependent on haem-source; capable of blocking the oxidation of haem at high concentrations [281]	

IMMUNE-BASED APPROACHES				
VACCINE TARGETS	**Protein E:** Outer-membrane protein	Induces an antibody response capable of blocking haem-acquisition and epithelial adhesion	Elicits potent bactericidal immune response in mice [282] ;a PE-PilA fusion protein protected against NTHi colonisation and biofilm integrity in the mouse nasopharynx [131]	**Efficacy:** no protection against *H. influenzae* in the lungs of COPD patients [283]; high mutability, phase-variability and redundancy of NTHi -acquisition systems may limit efficacy
	Multiple haem/iron epitope targets	Protects mice from infection caused by uropthogenic *E. coli* [284, 285] and against intravenous challenge with *S. aureus* [286]	Interference of multiple haem-utilisation systems attenuates NTHi virulence and disease severity/duration in animal models of OM and lung infection [67, 143, 163, 167]	**Application:** no candidates currently available for NTHi
ANTIBODIES AGAINST HAEM-UTILISATION	**Na-APR-1;** hookworm haemoglobinase	Significantly reduced parasite burden in experimentally-infected canines [213]	No antibacterial activity	**Application:** development of a similar strategy requires full elucidation of mechanisms governing NTHi haem-utilisation
BACTERIOTHERAPY				
GASTROINTESTINAL PROBIOTICS	*E. coli* (Nissle 1917 strain)	Supresses gastroenteritis by outcompeting for the siderophore-mediated iron assimilation of *Salmonella enterica* [199, 287]	Lacks proximity to compete for local haem-iron sources	**Application:** probiotic strain is not a commensal of the upper respiratory tract
ORAL/NASAL PROBIOTICS	α-haemolytic streptococci	Effective in treating [288] and preventing acute pharyngotonsillitis caused by Group A Streptococci, and pneumococcal OM in children [289, 290]	No activity demonstrated against NTHi	**Efficacy:** family-specific antibacterial activity
	Haemophilus haemolyticus (or the rodent equivalent *Muribacter muris*)	Hh inhibited colonisation and transcytosis of NTHi in an *in vitro* cell culture model of human lung epithelium [291]	*M. muris* reduced NTHi colonisation density and infection rate by 45% in a murine OM model [292]	**Application:** effect of Hh therapy on NTHi colonisation has not been tested *in vivo*
BACTERIOCIN-PRODUCING PROBIOTICS	Bacteriocin-producing strains of commensal streptococci and staphylococci	Prevents and treats *S. pyogenes* infections [293], and reduces nasal carriage of *S. aureus* [294]	No activity demonstrated against NTHi	**Efficacy:** family-specific antibacterial activity

	Novel bacteriocin-like substance (BLS) producing *H. haemolyticus*	Potent inhibitory activity against all NTHi isolates using a well diffusion assay [295]	Suspected to prevent NTHi access to haem; commensal of the same family is likely to have highly potent anti-NTHi activity	**Application:** requires further investigation of *in vivo* and *in vitro* activity against NTHi
BACTERIOCIN THERAPY	Mersacidin	Eradicates *S.aureus* from the nasal epithelium of a mouse rhinitis model [296]	No activity against members of the *Haemophilus* species [297]	**Efficacy:** use of a bacteriocinogenic probiotic may offer more consistent protection due to added bacterial interference for pathogen host cell binding sites **Application:** requires further investigation of *in vivo* and *in vitro* activity against NTHi
	Novel bacteriocin-like substance (from *H. haemolyticus*)	Potent inhibitory activity against NTHi isolates using a well diffusion assay [295]	Suspected to prevent NTHi access to haem	

GaPP has demonstrated inhibitory effects against *A. baumanii* and *P. aeruginosa* in model respiratory cell lines, and other multidrug-resistant gram-positive and gram-negative species *in vitro* (278-280). This compound is also effective against biofilm and intracellular communities (275). The activity of GaPP is enhanced when combined with DFP which has been demonstrated by a topical hydrogel with anti-biofilm and antibiotic-potentiating properties against *S. aureus* in an artificial wound model (298, 299). Although this strategy is likely to have activity against NTHi, it is limited to topical applications and cannot be used to prevent future infections. Beyond GaPP, exploration of haem-targeting compounds is scarce. Additional haem-oxygenase inhibitors against *P. aeruginosa* and *Neisseria meningitidis* have only been identified through virtual screening and an *in vitro* growth assay, which were capable of blocking the oxidation of haem at concentrations in excess of that available to pathogens in the respiratory tract (281).

2.7.2 Immunotherapy

Pharmaceutical approaches that restrict NTHi access to haem-iron offer viable approaches for the treatment of infections; however, they do not offer protection from future infections. Immunological-based approaches for the prevention of infection are limited by the inherent mutability of NTHi. Previous attempts to develop a vaccine against NTHi, such as those incorporating antigen candidates PilA or PD, failed to provide satisfactory protection to warrant clinical trials. Recently, vaccine strategies have exploited the haem-dependant pathogenesis of NTHi by incorporating the surface haemoprotein receptor, PE as a vaccine antigen. As previously discussed, PE is an adhesin of NTHi with functions involved in haem binding, storage and inter-bacterial donation (70). PE is expressed by 98% of NTHi strains, the epithelial cell-binding region of which is highly conserved among strains (175). Serum from mice immunised with recombinant truncated PE demonstrated a strong bactericidal effect against NTHi (282). Incorporation of PE in a fusion protein with the PilA enhanced immunogenicity and protected against NTHi colonisation and disrupted biofilm integrity in the mouse nasopharynx (131). More recently, the PE-PilA fusion protein, combined with Protein D, has completed phase 2 clinical trials, demonstrating an acceptable reactogenicity and safety profile in adults with moderate/severe COPD (300). However, the isolation of *H. influenzae* from sputum samples did not differ between the vaccine and the placebo group (283).

The effectiveness of PE approaches may be undermined by the same genetic heterogeneity and phase-variable expression of other potential NTHi surface antigens (5, 133, 134). A high

degree of polymorphisms within the gene encoding an NTHi haemoglobin-binding protein has been reported, which alters the protein affinity for iron capture/usage (70). This is exacerbated by the high level of redundancy and multi-functionality of NTHi proteins, particularly those involved in the acquisition of haem-iron (218). Therefore, an antibody response capable of blocking a variety of epitopes may cause sufficient malnutrition to inhibit survival and host-pathogen interactions (301). This approach has been used against uropathogenic *E. coli* strains; mucosal immunisation with six outer membrane iron receptors or siderophores protected against urinary tract infection in mice (284, 285). Antibodies targeting the IsdA and IsdB haem-acquisition systems of *S. aureus* protected mice against intravenous challenge (286). However, a human trial using the vaccine candidate IsdB alone, did not reduce the rate of serious post-operative *S. aureus* infections, perhaps demonstrating the advantage of multivalent approaches when targeting pathogen iron acquisition systems (302, 303). In addition to targeting iron acquisition proteins, some efficacy has been achieved with vaccines targeting iron homeostasis in pathogens. The Na-APR-1 protease from human hookworm, *Necator americanus*, is essential for enzymatic activity to support blood feeding. Vaccination with a mutated form of Na-APR-1 significantly reduced parasite burden in experimentally-infected canines (213).

A similar strategy that targets multiple haem-iron acquisition systems, or their regulation may offer similar protection against NTHi infection. Interference of multiple NTHi haem-utilisation systems has been shown to successfully downregulate virulence determinants and disease severity/duration in animal models of AOM and lung infection (67, 143, 163, 167). Similarly, mutants lacking the conserved haem-regulon, *fur*, also exhibit reduced persistence in middle ears of chinchillas (142).

2.7.3 Bacteriotherapy

The inherent issues associated with pharmacological- and immunological-based approaches has necessitated the exploration of alternative routes of therapeutic development against NTHi infections. The vital role of haem-iron in the survival of NTHi and other bacteria in the upper respiratory tract raises the stakes for evolutionary conflicts to arise in the struggle for this limiting nutrient (212). Thus, a commensal bacterium that can outcompete NTHi for haem-iron may have potential as a probiotic therapy by making the environment inhospitable for NTHi growth (304). A probiotic is defined as a live microorganism that, when administered in adequate amounts, confers a health benefit to the host (305). Probiotics that outcompete

pathogens for iron have demonstrated high levels of protection against infection in the gastrointestinal tract. The Nissle 1917 strain of *E. coli* has been applied as a probiotic treatment that supresses gastroenteritis by outcompeting for the siderophore-mediated iron assimilation of *Salmonella enterica* serovar Typhimurium (199, 287). This inter-bacterial relationship exposes the protective potential of beneficial microbes to combat pathogens through iron sequestration.

Probiotic administration has almost exclusively targeted gastrointestinal tract health and their utility in preventing respiratory tract infections has only recently been recognised. Although, there are reports of immune-mediated protection against upper respiratory tract infections following orally administered probiotics, the evidence is weak and inconsistent between studies owing to unclear study methods, blinding, sample sizes and varying probiotic formulations (306-309). Probiotics administered directly to the upper respiratory tract have closer proximity to pathobionts and may therefore interfere with colonisation and development of disease by competing for host cell binding sites and nutrients (310). Bacteriotherapy has demonstrated effectiveness in preventing a range of respiratory infections. Nasal and oral α-haemolytic streptococcal probiotic sprays are effective in treating (288) and preventing episodes of acute pharyngotonsillitis caused by Group A β-haemolytic streptococci, and pneumococcal AOM in children (289, 290). Prevention of experimental meningococcal meningitis in mice using intranasal delivery of the closely related commensal *Neisseria lactamica* (311), and pneumococcal pneumonia in mice using intranasal delivery of *Streptococcus mitis* (312) has also been demonstrated. In all cases, the antagonising commensal bacterium belongs to the same family and occupies the same niche as the pathogenic species, suggesting these characteristics predict, or are required for microbial interference. For this reason, the closely related commensal species Hh that shares not only the nasopharyngeal niche, but also the growth requirement for exogenous haem-iron acquisition, has been proposed as an ideal probiotic candidate for the prevention of NTHi infection (291). In an *in vitro* cell culture model of human lung epithelium, pre-treatment with Hh significantly inhibited colonisation and transcytosis of NTHi to cell monolayers (291). Intranasal administration of *Muribacter muris*, the rodent equivalent of Hh, reduced NTHi colonisation density and infection rate by 45% in a murine AOM model (292).

Closely-related commensals may also elicit inhibitory activity through production of antibacterial substances known as bacteriocins, strain-specific toxins that target bacterial neighbours competing for the same ecological niche, or sequester nutrients to gain a growth advantage

(313). Nisin, a broad-spectrum bacteriocin isolated from lactic acid bacteria, has *in vitro* activity against several multi-resistant gram-positive pathogens, with additional anti-biofilm and antibiotic-potentiating properties (Hols, Ledesma-García et al. 2019). In the context of the upper respiratory tract, bacteriocin-producing strains of commensal streptococci have been commercialised as probiotics to prevent and treat *S. pyogenes* infections (293), and bacteriocin-producing strains of *Staphylococcus lugdunensis* have been associated with reduced nasal carriage of *S. aureus* (294). Although some bacteriocins demonstrate broad-spectrum activity against a variety of bacteria, those produced by commensals within the same family have a higher potency. Bacteriocin-like substances produced by *S. salivarius* demonstrate inhibitory activity against some NTHi strains (314). However, a protein thought to be a novel bacteriocin-like substance (BLS) recently isolated from a nasopharyngeal *H. haemolyticus* isolate elicited potent inhibitory activity against all 100 tested NTHi and 20 Hib clinical isolates using a well diffusion assay (295). The inhibitory activity of this BLS was only observed in haem-limited media and shared structural similarities to the haemoglobin-haptoglobin utilisation protein A found in other Pasteurellaceae members such as *Kingella denitrificans* and *Neisseria gonohorreae* (315). The authors therefore proposed an inhibitory mechanism whereby this substance prevents NTHi access to haem and subsequently, inhibits growth of the bacterium. However, further studies are needed to determine the inhibitory potential of these BLS-producing strains of Hh to determine their utility as a probiotic candidate against NTHi infection. Provided significant NTHi-inhibitory activity can be demonstrated during direct competition with NTHi, Hh possesses favourable characteristics suited to probiotic applications. Although the distinction of Hh as a true commensal has been obscured by misidentification of isolates from infection sites (11-16), recent data has shown that the bacterium does not cause AOM or lower airway infections (16, 17). The normal nasopharyngeal habitat also provides Hh with all the necessary characteristics to thrive in the niche amongst other microbial inhabitants (304).

In addition to a bacteriocinogenic probiotic, the NTHi-inhibitory capacity of Hh cell-free supernatants (295), also indicates utility of the novel BLS as a pharmabiotic and/or novel alternative to existing antibiotics (316). Intranasal application of the bacteriocin mersacidin, produced by *Bacillus* sp., successfully eradicated *S.aureus* from the nasal epithelium of a mouse rhinitis model (296). Although mersacidin has no activity against members of the *Haemophilus* species (297), similar application of the Hh BLS may have a similar effect against NTHi nasopharyngeal colonisation and infection. This raises the question of the therapeutic utility of bacteriocins versus treatment with their bacteriocinogenic counterparts. The sustained

production and high local concentrations of bacteriocins generated by adherent probiotic strains, may offer more consistent and longer-term protection than bolus applications of the protein alone. The presence of competitive probiotics in the nasopharynx may also provide additional inhibition of pathogen colonisation through interference for host cell binding sites. However, use of live bacteria requires appropriate safety evaluations to ensure they do not pose a health threat, particularly in immunocompromised individuals (317). Conversely, bacteriocins may offer more immediate treatment of an existing infection and their diverse and modular nature may be used to bioengineer chimeric bifid molecules with broad activity against gram-negative and gram-positive bacteria (318, 319). Alternatively, an adjuvant-style therapy combining a probiotic strain and the bacteriocin may take advantage of the benefits from both applications. Regardless, the approach of using a bacteriocin, or a bacteriocin-producing probiotic delivers several benefits over standard antibiotic approaches which make them an asset to dampen the emergence of resistance. The narrow spectrum of activity does not damage host tissue and does not provoke collateral effects on the whole microbiota or promote enrichment of resistant clones/strains (320). A two-peptide bacteriocin produced by *Bacillus thuringiensis* was shown to be as effective as conventional antibiotics (e.g. metronidazole and vancomycin) in an *ex vivo* model of *Clostridium difficile* infection but did not result in major alterations of gastrointestinal populations, a contributing factor in recurrent *C. difficile* infection (321). The structural stability of bacteriocins may also mitigate low remnant and sublethal concentrations that favour development of bacterial resistance (318, 319).

2.8 Conclusion

The growing prevalence of resistance to first- and second-line antibiotics, in the absence of an effective preventative strategy necessitates the need for alternative therapies that can reduce the enormous disease burden associated with NTHi infections. Strategies that target haem-iron assimilation of NTHi have a potentially high impact on the ability of NTHi to survive and cause disease within host airways. Unlike pharmacological- and immunological-based approaches, bacteriotherapy may provide an effective strategy that both treats, and prevents NTHi infections, which is not compromised by antigenic heterogeneity or bacterial resistance mechanisms.

Chapter 3 | Haemophilin Mediates Exploitative Competition Between Haemophilus Species

The findings discussed in this chapter form part of an original research article entitled "A haem-binding protein produced by *Haemophilus haemolyticus* inhibits non-typeable *Haemophilus* influenzae" by R. Latham, M. Torrado, **B. Atto**, J. L. Walshe, R. Wilson, J.M Guss, J.P. Mackay, S.Tristram and D.A. Gell which was published as a collaborative effort in Molecular Microbiology in November 2019 (doi: 10.1111/mmi.14426). The functional and bioinformatic evidence discussed in this chapter comprises my contribution to the above paper, which together with proteomic data collected by collaborators, establishes the mechanism by which haemophilin mediates haem-uptake and NTHi-inhibitory activity of Hh.

3.1 Abstract

Pathogenesis of the opportunistic respiratory pathogen nontypeable *Haemophilus influenzae* (NTHi) is largely dependent on adequate sequestration of iron-containing haem from the host (as previously discussed in Chapter 2.5.). Thus, strategies that interfere with the acquisition of this essential nutrient may have a significant impact on the ability of NTHi to cause disease. Recently, strains of the closely related commensal species *Haemophilus haemolyticus* (Hh) were isolated with the capacity to produce a novel haemophore (herein referred to as haemophilin; Hpl) and inhibit the growth of NTHi. Given that this inhibitory activity was only observed under haem-limited conditions, we hypothesised that growth inhibition occurs by limiting NTHi access to haem. To test this hypothesis, an *hpl* knockout was generated and subject to growth in media supplemented with varying concentrations of haem or recombinant Hpl. The loss of NTHi-inhibitory activity in media recovered from the knockout strain and the correlation between NTHi-inhibitory activity and *hpl* expression, confirmed that Hpl was the primary mediator of the observed *in vitro* NTHi-inhibitory activity. The knockout also displayed poor growth under haem-restrictive conditions compared to the wild-type strain suggesting a defect in haem utilisation. The role of Hpl in Hh haem acquisition was supported by bioinformatic analysis which revealed that *hpl* occurs in a conserved gene cassette that includes genes for proteins predicted to be involved in haem import. Finally, NTHi demonstrated no growth in media supplemented with Hpl, but robust growth when provided with the equivalent amount of free haem, indicating that haem bound to Hpl is not available to NTHi. Together with supporting structural investigations, these findings provided evidence for Hpl involvement in haem acquisition by Hh, and that NTHi-inhibitory activity occurs through haem starvation. Further investigations using direct competition models are warranted to determine if competition from Hpl-producing Hh could antagonise NTHi colonisation in the respiratory tract and thus have therapeutic utility as a respiratory probiotic.

3.2 Introduction

NTHi is a common constituent of the nasopharyngeal microbiome in healthy adults (322). However, in infant and elderly populations in particular, this bacterium is also a major opportunistic pathogen, primarily causing acute otitis media (AOM) and exacerbations of chronic obstructive pulmonary disease (COPD) (2). Despite significant efforts, there are currently no effective vaccination strategies to prevent infections, owing to the intrinsic heterogeneity and phase-variable expression of NTHi surface antigens (181, 323). Additionally, treatment of existing NTHi infections is becoming increasingly complicated by the rapidly expanding spectrum and prevalence of antibiotic resistance, resulting in

treatment failure with first- and second-line antibiotics (1). Thus, exploration of alternative approaches is warranted to overcome the growing disease burden of NTHi.

The ability of NTHi to survive and cause infection in the respiratory tract is heavily dependent on its ability to sequester adequate host-derived sources of haem-iron (142, 143). The essential role of haem-iron in the survival of NTHi and other bacteria in the upper respiratory tract raises the stakes for evolutionary conflicts to arise in the struggle for this limiting nutrient (212). Thus, a commensal bacterium that can outcompete NTHi for haem-iron may have utility as a probiotic therapy by generating an environment inhospitable for NTHi growth (304). Probiotics that outcompete pathogens for iron have demonstrated high levels of protection against infection in the gastrointestinal tract. The Nissle 1917 strain of *E. coli* has been applied as a probiotic treatment that supresses gastroenteritis by outcompeting for the siderophore-mediated iron assimilation of *Salmonella enterica* (199, 287). This inter-bacterial relationship highlights the protective potential of beneficial microbes that combat pathogens through competitive sequestration of iron.

The closely related commensal species *Haemophilus haemolyticus* (Hh), which shares the same nasopharyngeal niche and nutritional requirements, has been proposed as an ideal probiotic candidate for the prevention of NTHi infection. In an *in vitro* cell culture model of human lung epithelium, pretreatment with Hh significantly inhibited colonisation and transcytosis of NTHi to cell monolayers (291). Intranasal administration of *Muribacter muris*, the rodent equivalent of Hh, reduced NTHi colonisation density and infection rate by 45% in a murine AOM model (292). A novel haemophore was recently isolated from a nasopharyngeal Hh isolate (Hh-BW1) that elicited inhibitory activity against a collection of 100 NTHi and 20 Hib clinical isolates during a well diffusion assay (295). The inhibitory activity of this novel protein, now referred to as haemophilin (Hpl), was only observed in haem-limited media. Additionally, an X-ray crystal structure of recombinant Hpl showed that each Hpl monomer contained a single haem molecule bound with a stereochemistry consistent with a specific haem binding function (315). We therefore hypothesised that Hpl may be involved in Hh haem acquisition, and that inhibition occurs by limiting NTHi access to haem. To test this hypothesis, an *hpl* knockout was generated and subject to growth in media supplemented with varying concentrations of haem or recombinant Hpl. Whole genome sequences of Hh isolates containing the *hpl* open reading frame (n=24) were also analysed to gain insight into the biological role and genetic determinants of Hpl production.

3.3 Materials and Methods

3.3.1. Bacterial strains and culture conditions

Hh strains were acquired from the respiratory tracts of patients from five Australian hospitals and were identified by 16S rRNA sequencing and qPCR for species-specific markers as previously described (13). Hh strain BW1 (Hh-BW1) has previously demonstrated the ability to produce high levels of Hpl (295).

NTHi and Hh isolates were propagated from liquid nitrogen frozen glycerol stock, followed by two overnight passages on chocolate agar (CA) containing 2% (v/v) Vitox® (Oxoid Ltd) at 37 °C with 5–10% CO_2 prior to experimentation. Isolates were stored long-term at –80°C in 10% w/v sterile skim milk media (SMM).

3.3.2 Preparation of haematin solutions and recombinant Hpl

Solutions (1–5 mg mL^{-1}) of ferriprotoporphyrin IX were prepared by dissolution of porcine haematin (ferriprotoporphyrin IX hydroxide, Sigma-Aldrich) solid in 0.1 M sodium hydroxide. Recombinant haemophilin protein (in holo form) purified from *Escherichia coli* was provided by Dr. David Gell (University of Tasmania, Hobart, TAS, Australia); the preparation of which has previously been described (315).

3.3.3 Generating an *hpl*-knockout

To investigate the role of Hpl in the haem-utilisation and NTHi-inhibitory activity of Hpl-producing Hh strains, an *hpl* gene knockout mutant of Hh-BW1 (Hh-BW1$^{hpl\text{-}KO}$) was generated by insertional inactivation with a kanamycin resistance cassette. A partially assembled whole-genome sequence of Hh strain 11P18 (Seq ID: LCTK01000015, contig 00016) was used to acquire sequence flanking the *hpl* ORF for PCR primer design. These PCR primers, NIS-KO-F (GCTAGACGTGCTGATGTT) and NIS-KO-R (TGTTGTTCTTGTCGTTGTTG) were then used to generate a 1691 bp fragment using genomic DNA from strain BW1 as template. This 1691 bp fragment containing the *hpl* ORF (bp 700–1518) and a unique BspTI site was cloned in to pGEM-T (Promega) according to the manufacturer's instructions. A 1132 bp kanamycin resistance cassette, generated by PCR with BspTI tagged primers Kana-Bsp-F (5'-GCGCCTTAAGTAAACCTGAACCAAA-3') and Kana-Bsp-R(5'-GCGCCTTAAGGTCGTCAGTCATAAA-

3') using pLS88 (Genbank L23118) as the template, was then sub-cloned into the BspTI site using standard methods. The *hpl* ORF containing the kanamycin cassette was then PCR amplified using NIS-KO-F and NIS-KO-R primers and transformed into Hh-BW1 using the MIV method (324). Transformants were selected by growth on CA supplemented with 50 mg L^{-1} kanamycin. The presence of the insert was confirmed by gel electrophoresis and Sanger sequencing of the *hpl* region (AGRF, Brisbane). A whole-genome sequence of Hh-BW1$^{hpl\text{-KO}}$ was generated (and compared to the Hh-BW1 wild-type genome) using the MiSeq (Illumina) platform to exclude insertion of the kanamycin cassette elsewhere in the genome (see section 3.3.8. for assembly and mapping details). Sequence verified transformants were tested for the NTHi inhibitory phenotype by ammonium sulfate extraction and estimation of Hpl bioactivity (as described in section 3.3.4).

3.3.4 Ammonium sulfate extraction of native Hpl

Supplemented tryptone soya broth (sTSB; Oxoid) was prepared following manufacturer specifications and autoclaved at 121°C for 30 min. Once cooled to 50°C, TSB was supplemented with 1% (v/v) Vitox® (Oxoid) and porcine haematin at 0.0-15.0 mg L^{-1}. Hpl production by Hh (determined by NTHi-inhibitory zones from cell-free supernatants) was higher in media with low concentrations of haem (Supplementary Figure S3.1). The optimal concentration of haematin supplementation (5.0 mg L^{-1}) was determined as the minimum concentration required to support Hh growth over a 24-h period.

Cultures of Hh-BW1 were grown on CA for 12–16 h, suspended in pre-warmed (37 °C) sTSB to a density of 0.05 (OD_{600}), then incubated with 200 RPM agitation at 37 °C for 24 h. Culture broths were clarified by centrifugation at 7000 × g for 30 min. Hpl was enriched by ammonium sulfate precipitation at 4°C, with the 50–70% saturation cut collected and redissolved in a volume of PBS equal to 1/20th of the initial culture broth volume, then dialysed using a 3500-Da molecular weight cut off dialysis membrane (Thermofisher) for 24 h at 4°C against 50 mM Tris-HCl, pH 7.5.

3.3.5 Estimation of native Hpl bioactivity

A well diffusion assay adapted from Schillinger, *et al*. 1989 (325) and modified by Latham, *et al*., 2017 (295) was optimised to detect bioactivity of native Hpl extracts. Solid test media consisted of 18.5 g L^{-1} Heart Infusion Agar (Oxoid) solidified with 7.5 g L^{-1} Bacteriological Agar

(Oxoid), autoclaved at 121°C for 30 min, cooled to 50°C, then supplemented with 1% (v/v) Vitox® (Oxoid) and 2.5 mg L^{-1} of porcine haematin (Oxoid). Molten agar (25 mL) was dispensed into a 90-mm Petri dish. Indicator strains (NTHi strain NCTC 4560 and NCTC 11315) were grown for 6–12 h on CA then suspended in Dulbecco's phosphate buffered saline (DPBS, Gibco) to a density of 1 x 10^6 CFU mL^{-1}, a 100 µL aliquot of which was evenly spread over the solid test media using a hockey stick**.** Circular holes (5-mm diameter) were cut in the agar using a sterile cork-borer. Test solutions of native Hpl extracted from Hh culture supernatants (as described in section 3.3.4) were pipetted (25 µL) into wells. Plates were left open in a biological safety cabinet for one 1 h and then then incubated for 18–24 h at 35°C in 5% CO_2. Inhibitory activity was measured by the annular radius of cleared zones. To compare the Hpl-producing capacity among Hh strains, the concentration of Hpl recovered from conditioned media was corrected for final bacterial density (OD_{600}).

3.3.6 Propagation of Hh and NTHi in growth-limiting haem conditions

Bacterial suspensions of 1.0 OD_{600} were made in TSB from 8–10 h growth on CA and diluted 1:10 in 5 mL pre-warmed sTSB containing 2% (v/v) Vitox® (Oxoid), and either 15 or 0 µg mL^{-1} porcine haematin (Sigma-Aldrich) to generate haem-replete and haem-starved populations, respectively. Broths were incubated for 14 h at 37 °C aerobically without shaking, centrifuged at 3000g for 10 min at 37 °C and resuspended in pre-warmed TSB to an OD_{600} of 0.5 prior to use in growth experiments.

Haem-starved and haem-replete preparations of Hh-BW1 and Hh-BW1$^{hpl\text{-KO}}$ were diluted 1:10 in 5 mL pre-warmed sTSB containing 2% (v/v) Vitox® and varying concentrations of porcine haematin to determine growth requirements. The effect of recombinant Hpl on growth of NTHi strain ATCC 49247, Hh-BW1 and Hh-BW1$^{hpl\text{-KO}}$ was assessed by diluting haem-starved preparations 1:10 in 2 mL pre-warmed TSB containing 2% (v/v) Vitox®, together with either porcine haematin (Sigma-Aldrich) or an equimolar concentration of Hpl. All broths were incubated aerobically in a benchtop incubator at 37 °C and 220 RPM. Growth was assessed by measuring the optical density (OD_{600}) of 100 µL aliquots in a 96-well plate (Grenier Bio-One) using a plate reader (Infinite 200 PRO, Tecan Life Sciences). Optical density readings were corrected for a pathlength of 1 cm^{-1}. Bacterial viability (determined by colony counts on CA) following pre-incubation in haem-limited conditions did not differ significantly among strains and treatments (Supplementary Figure S3.2).

3.3.7 Expression analysis

Expression of *hpl* was measured using real-time qPCR as described in Chapter 4 (section 4.4.5.).

3.3.8 Whole-genome sequencing and assembly

Whole-genome sequences (WGS) were generated from 24 Hh strains previously identified as containing the *hpl* ORF by PCR and/or which demonstrated NTHi-inhibitory activity *in vitro* (315). An additional three strains lacking the *hpl* ORF (ATCC 33390, NF1 and BW1$^{hpl\text{-KO}}$) were also sequenced for comparative analysis. Hh gDNA was prepared using the DNeasy Blood & Tissue kit (Qiagen) following the standard proteinase K extraction protocol, purified using the QIAquick PCR Purification kit (Qiagen) and eluted in EB buffer (10mM Tris-Cl, pH 8.5). The quality and concentration of gDNA preparations was assessed photospectrometrically (NanoDrop, Thermo Fisher Scientific) and fluorometrically (Qubit DNA BR assay), respectively.

Whole genome sequencing was performed by SCI Ramaciotti Centre using the MiSeq platform with an output of 2 x 150 bp (average coverage of ~85 x). Low-quality reads (<20 N) were filtered out using FastQC (Galaxy version 0.72). Remaining reads were assembled using the SPAdes genome assembler (Galaxy Version 3.12.0) using default settings. Contigs >1000 bp were then mapped and aligned to the Hh ATCC 33390 reference genome using progressiveMauve (Galaxy version 2015_02_13.0); this genome alignment was used to identify conserved regions unique among Hh strains containing the *hpl* ORF (326). A multiple sequence alignment (ClustalW) of the *hpl* gene cluster was performed using MEGA (version X) (327) and visualised using the BLAST Ring Image Generator (BRIG; version 0.95) (328). BPROM (Softberry, 2016) was used to predict potential promoter regions upstream of the *hpl* gene cluster. Sequence similarity to the Hh-BW1 *hpl* gene cluster among publicly available Hh genomes (Genbank, n=67) was conducted by discontiguous MegaBlast. Protein homologues and conserved domains within the *hpl* gene cluster were detected using a hidden Markov model through the Pfam database.

3.4. Results

3.4.1. Haemophilin mediates NTHi-inhibitory activity

Previous work by Latham *et al.* (2017) discovered an Hh isolate (Hh-BW1) capable of inhibiting NTHi growth on agar surfaces by secreting high levels of a novel haemophore, now named Hpl (295). To show that Hpl was the sole mediator of NTHi-inhibitory activity, an *hpl* gene knockout mutant of Hh-BW1 (Hh-BW1$^{hpl\text{-KO}}$) was generated. Inactivation of the *hpl* gene in this strain was confirmed by detection of the 1132 bp kanamycin resistance cassette at position 629 (bp) of the *hpl* gene; this cassette was not detected elsewhere in the Hh-BW1$^{hpl\text{-KO}}$ genome. Presence of a larger *hpl* PCR product consistent with insertion of the kanamycin cassette was detected in Hh-BW1$^{hpl\text{-KO}}$ (Figure 3.1A). The ~30 kDa protein (estimated Hpl size) was also absent in chromatographic separations and silver staining from growth media of the Hh-BW1$^{hpl\text{-KO}}$ but not the wild-type, despite a similar protein content overall (315). Colonial growth on agar and broth media recovered from cultures of Hh-BW1$^{hpl\text{-KO}}$ displayed no inhibitory activity against NTHi, compared to those of Hh-BW1, which displayed inhibitory activity as evidenced by a clear zone free of visible NTHi growth (Figure 3.1B,C, compare left with right).

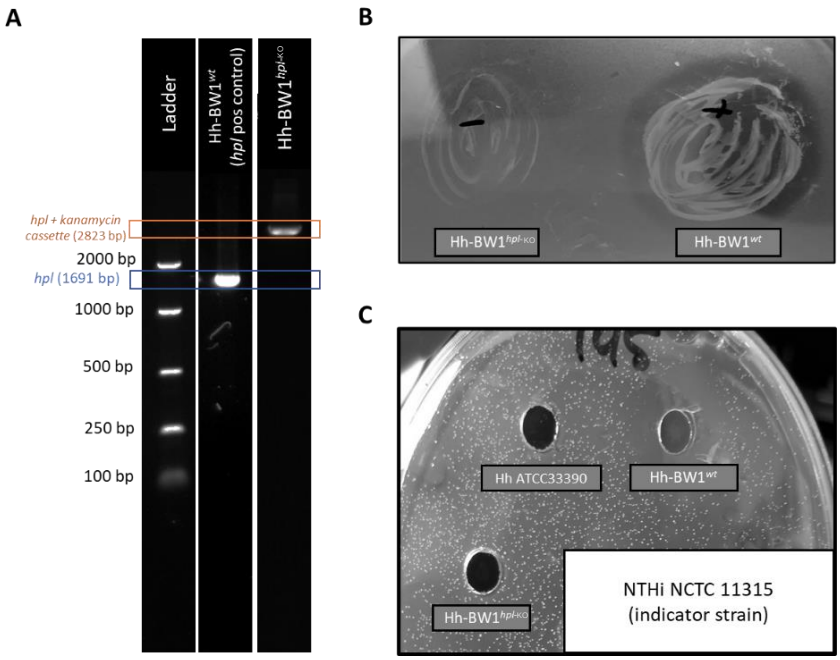


Figure 3.1 | NTHi-inhibitory activity is attenuated in an *hpl* knockout. Insertion of the kanamycin cassette (1132 bp) within the *hpl* ORF (1691 bp) was confirmed by gel electrophoresis showing a larger (>2000 bp) PCR product in the knockout strain (Hh-BW1$^{hpl\text{-KO}}$), compared to the Hh-BW1 wild-type control. Bacterial inoculum (**B**) and media recovered from cultures of Hh-BW1$^{hpl\text{-KO}}$ (**C**) displayed no inhibitory activity against NTHi.

3.4.2. An *hpl* knockout has defects in haem utilisation

Given the haem auxotrophic nature of Hh and the haemophore-like structure of Hpl (315, 329), we hypothesised that Hpl might function to supply nutritional haem to Hh. To investigate this, we compared the growth of Hh-BW1$^{hpl\text{-KO}}$ to the wild-type Hh-BW1 under different haem supplementation regimes. Hh-BW1 propagated in haem-replete sTSB media, continued to grow for some period after haem supplementation was withdrawn (Figure 3.2A), presumably due to accumulation of haem or porphyrin stores from a variety of haem sources under prior haem-replete conditions, consistent with previous reports (330, 331). After a 14-h conditioning period in haem-deficient TSB media, Hh-BW1 showed a strong dependence on haem supplementation for growth (Figure 3.2B). In contrast, Hh-BW1$^{hpl\text{-KO}}$ showed poor growth after conditioning in haem-replete TSB (Figure 3.2C), and after haem starvation (Figure 3.2D). Together, these results suggest that Hh-BW1$^{hpl\text{-KO}}$ has a reduced capacity to utilise free haem.

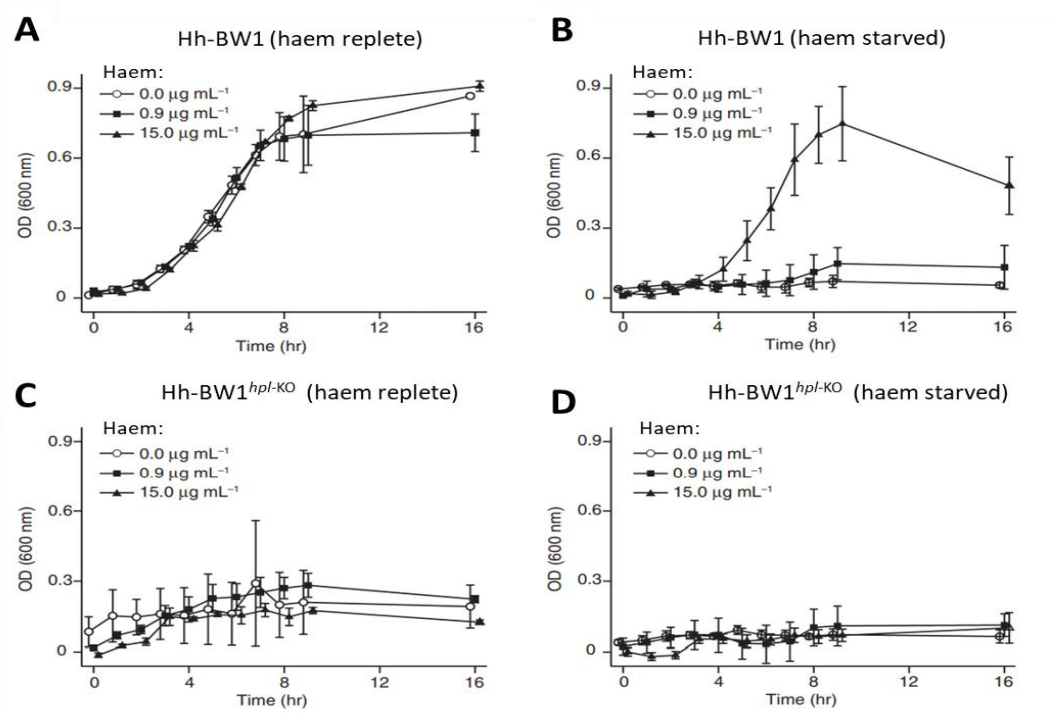


Figure 3.2 | **An *hpl* knockout (Hh-BW1$^{hpl\text{-KO}}$) has defects in haem utilisation.** Haem-replete and haem-starved populations of Hh-BW1 (**A**, **B**) or Hh-BW1$^{hpl\text{-KO}}$ (**C**, **D**) were inoculated into medium containing varying concentrations of haem as indicated. Error bars represent ±1 SD (n=3). The number of viable cells at time t = 0 was equal for all conditions (Supplementary Figure 3.3). Optical density readings were corrected for a solution path length of 1.0 cm^{-1}.

3.4.3. Hpl-bound haem is accessible to Hh but not to NTHi

To directly test if Hpl can deliver haem to Hh, cultures were grown in media supplemented with haem or recombinant holo Hpl (containing bound haem) at matching final molar concentrations. Haem-starved Hh-BW1 inoculated into TSB media containing growth-limiting (0.6 μg mL^{-1}; Figure 3.3A, open circles) or growth-permissive (5.6 μg mL^{-1}; Figure 3.3A, closed circles) concentrations of haem showed the expected dependence on haem in the media for growth. Haem-starved Hh-BW1 inoculated into TSB media supplemented with recombinant holo Hpl (equivalent to 5.6 μg mL^{-1} haem; Figure 3.3A, open squares) produced the same growth as in the medium with the matched, growth-permissive, concentration of free haem, indicating that Hpl-bound haem is readily available to Hh-BW1. Further, when supplemented with Hpl protein, the growth Hh-BW1$^{hpl\text{-KO}}$ (Figure 3.3B, open squares) displayed a level of growth similar to the parent Hh-BW1 strain, despite the fact that Hh-BW1$^{hpl\text{-KO}}$ grew poorly with free haem (Figure 3.3B, closed circles).

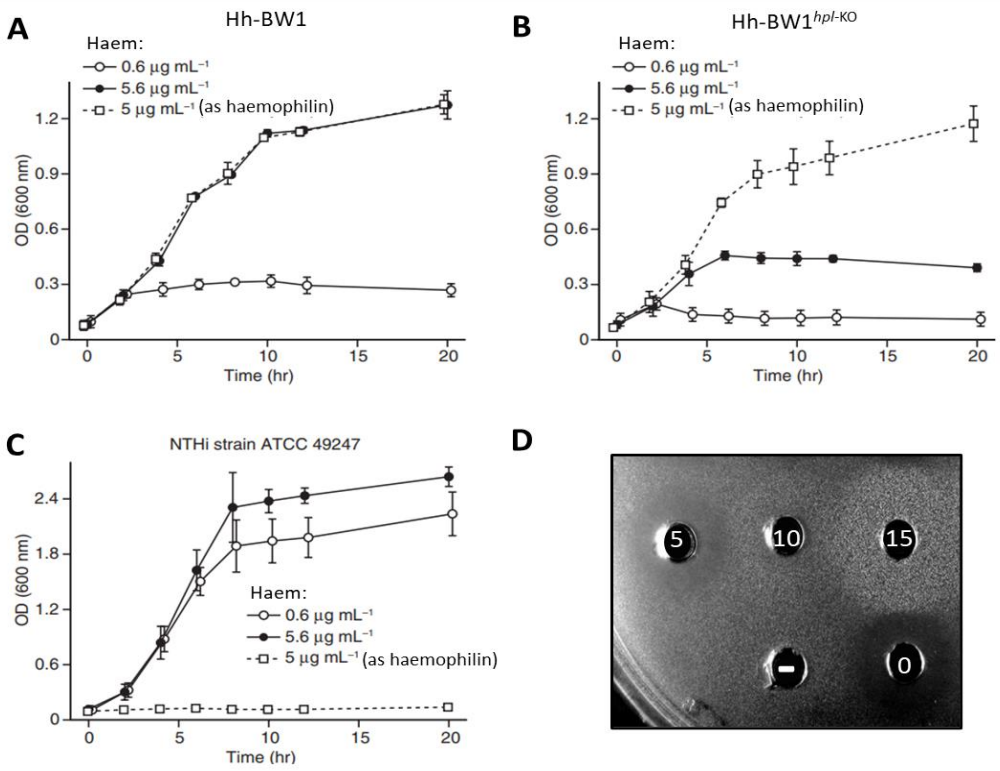


Figure 3.3 | Hpl-bound haem is readily available to Hh-BW1 and Hh-BW1$^{hpl\text{-KO}}$, but unavailable to NTHi. Cultures of Hh-BW1 (**A**), Hh-BW1$^{hpl\text{-KO}}$ (**B**), or NTHi strain ATCC 49247 (**C**) were grown in TSB supplemented with haem at 0.6 μg mL^{-1} (open circles), or 5.6 μg mL^{-1} (filled circles), or with recombinant holo Hpl at 8 μM (corresponding to a haem concentration of 5.6 μg mL^{-1}; open squares). Error bars are

mean ± SD (n=3). An agar well diffusion assay (**D**) showing neutralisation of NTHi-inhibitory activity in wells containing native Hpl extracts mixed with high concentrations of haem (values shown, μg mL^{-1}). Negative control (–) contains DPBS. Indicator is NTHi strain NCTC 11315.

This rescue of Hh-BW1$^{hpl\text{-KO}}$ growth supports the conclusion that the growth defect on media containing free haem is due to loss of the *hpl* gene, and that a substantial fraction of free haem uptake in the parent Hh-BW1 strain occurs through Hpl.

In contrast to Hh-BW1, NTHi showed no growth in media supplemented with Hpl (Figure 3.3C, open squares), but robust growth when provided with the equivalent amount of free haem (Fig. 3.3C, closed circles), indicating that haem bound to Hpl is not available to NTHi. Additionally, the NTHi-inhibitory activity was overcome in the agar well diffusion assay with addition of excess haem (Figure 3.3D). Together, these results suggest that Hpl binds haem in a form that can be utilised by Hh-BW1, but is not accessible to NTHi, and that inhibition of NTHi occurs by haem starvation.

3.4.4. NTHi-inhibitory activity correlates with *hpl* mRNA expression

Previously, additional Hh isolates (n=23) were identified as containing the *hpl* ORF; these strains had a range of NTHi-inhibitory activities (Supplementary Table S3.1) (315). Among these strains, sequence similarity to the Hh-BW1 *hpl* ORF ranged from 79-100%. This was largely representative of the variation described among Hh genomes available in Genbank, in which *hpl*-like genes were detected in 57% (38/67) of Hh genome assemblies with 75-100% nucleotide sequence similarity to Hh-BW1 (Supplementary Table S3.2).

Ten isolates possessed ORFs that were 100% identical to Hh-BW1. Of these isolates, only Hh strain RHH122 produced a similar NTHi-inhibitory activity to BW1 after correcting for bacterial density. Isolates NF5, BW15, BW18, BW36, OP1, OP2, OP3 and OP4 produced intermediary concentrations of Hpl, while no detectable Hpl was produced by NF4 (Figure 3.4A). Among all Hh strains, the relative degree of *hpl* mRNA expression strongly correlated with the amount of Hpl recovered from growth media, and strains lacking expression exhibited a non-inhibitory phenotype (Figure 3.4B). Strains with more divergent sequences (96% and below) to Hh-BW1 *hpl* ORF displayed poor *hpl* mRNA expression compared to Hh-BW1. The PCR test was insensitive to alleles with less than 85% similarity to Hh-BW1, therefore expression of these alleles could not be assessed. Of note, strains L56, CF26, L37 and NF1 had a mutation in the initiation codon predicted to result in a frameshift deletion of the first 11 aa residues,

a truncation/deletion of the signal sequence, and subsequent disruption of protein export to the periplasm. However, no functionally important variants were detected in the other non-producing strains, particularly Hh-NF4, which contained an *hpl* ORF identical to Hh-BW1 and yet secreted no detectable Hpl activity into the growth medium. In particular, no mutations in the haem-coordinating histidine, or pocket residues (315) were detected, indicating that haem-binding is likely to be preserved among all strains. Similarly, no variation in predicted promoter regions (BPROM, Softberry, 2016) were detected among strains; however, the exact promoter region has not been determined experimentally.

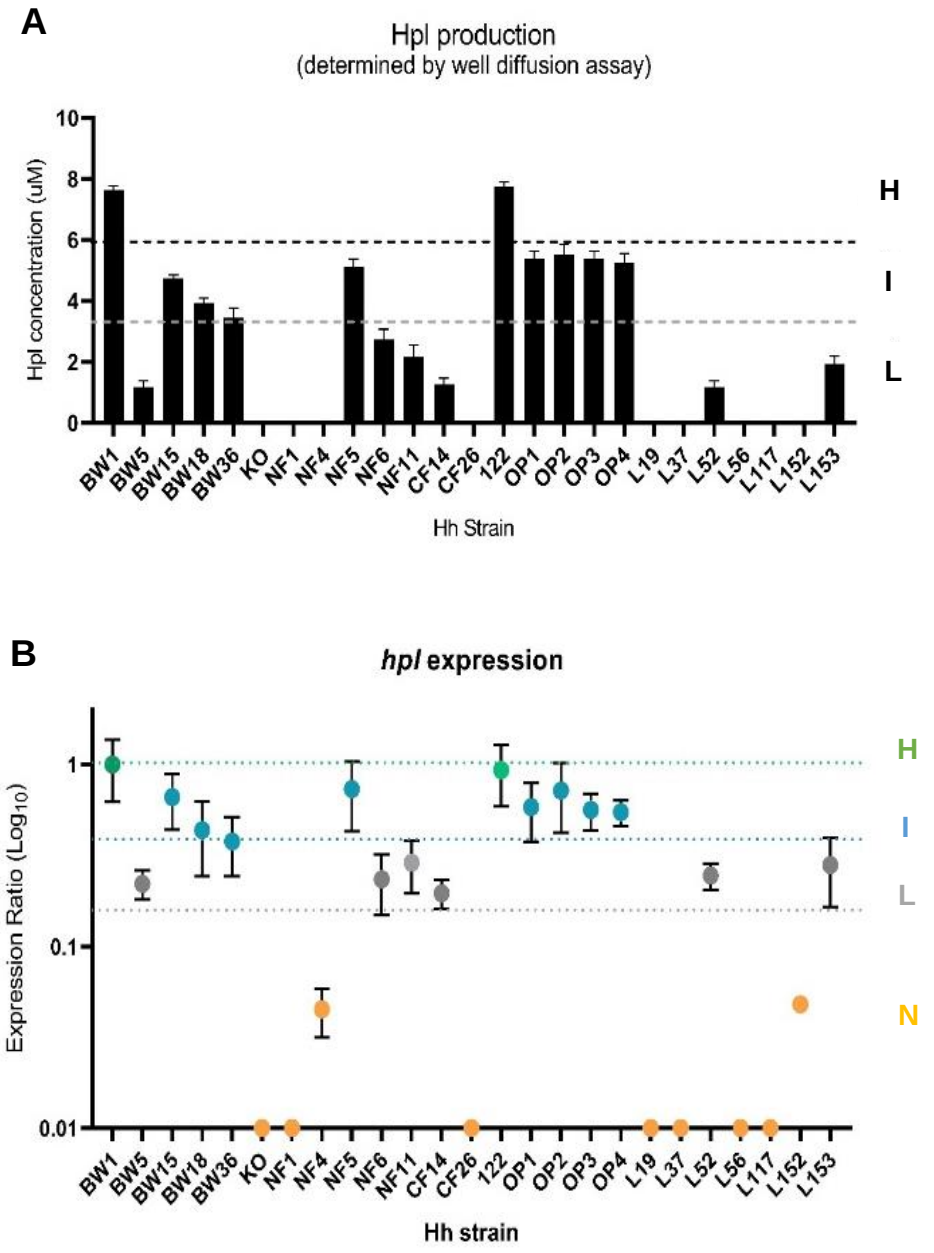


Figure 3.4 | Production of Hpl, and mRNA expression of *hpl* among Hh strains containing variable *hpl* sequences (refer to Supplementary Table S3.1). Hpl concentration (uM) determined by a well diffusion

assay of growth supernatant **(A).** Relative mRNA expression of *hpl* **(B)** determined by rt-qPCR. Hh strains are arbitrarily categorised based on phenotypic capacity for Hpl production; non-producers (N), low-level producers (L), intermediate-level producers (I), high-level producers (H). The Hh-BW1 *hpl* knockout (Hh-BW1$^{hpl\text{-}KO}$) is represented as KO.

3.4.5. The *hpl* gene occurs in a conserved gene cassette that includes genes for a putative secretion protein and a putative Hpl/haem receptor

To determine if nearby genes predicted NTHi-inhibitory phenotype, WGSs of Hh strains containing the *hpl* ORF were compared to those without (reference strain Hh ATCC 33390 and pharyngeal isolate Hh-NF1). In all Hh strains (from the current study) and publicly available genome assemblies (Genbank) containing the *hpl* ORF, a gene predicted to encode a putative Ton-B dependent haemin receptor (TBDHR) was detected immediately upstream, and a surface lipoprotein assembly modifier (SLAM) immediately downstream. These genes were not detected in any strain (Hh ATCC 33390 or Hh-NF1) or genome assemblies lacking *hpl* (Supplementary Figure S3.3), suggesting that they may be important for import of Hpl-bound haem and secretion or transport of Hpl.

Homologues of the Hpl-associated TBDHR in Hh were detected in several species associated with oropharyngeal flora (e.g. *Haemophilus spp, Pasteurella multocida, Neisseria bacilliformis, Aggregatibacter aphrophilus*), with the highest similarity (52.25%) to the HasR outer membrane haem acquisition system receptor from *Pasteurella multocida*. Similarity of the TBDHR among sequenced Hh strains varied between 76-100%, and 78-100% among Genbank genome assemblies (Supplementary Table S3.2). The predicted TBDHR featured a plug domain (porin channel that undergoes conformational change following ligand binding; aa 47-160), and a TonB-dependent receptor domain (that covers the conserved part of the barrel structure, aa 810-1117). A multiple sequence alignment of the TBDHR amino acid sequence revealed a central region (aa 615-822) that was highly conserved in strains capable of producing functional Hpl but not in those unable to produce Hpl (Supplementary Figure S3.4). This region was also missing in all other species containing TBDHR homologues, suggesting that this region may have an important and unique role in the transport of Hpl-associated haem. However, no known conserved domains or motifs were detected in this region.

SLAM homologues have previously been detected in several mucosa-associated genera, including *Neisseria, Moraxella, Aggregatibacter, Eikenella, Kingella* and *Haemophilus* (including *H.haemolyticus*) (332). The predicted SLAM coding sequence was highly conserved among Hh strains in this study (97-100%), and among available Genbank genomes (97-100%; Supplementary Table S3.2). SLAM-related sequences feature a signal peptide (aa 1-30), a periplasmic N-terminal domain (aa 31-203) containing tetratricopeptide repeats, and a predicted membrane-bound 14-stranded barrel domain of unknown function (DUF560; aa 204-488). Identical SLAM sequences were observed among the high- and intermediate-level Hpl producers, while low- or non-producers contained single residue substitutions of unknown significance.

3.5. Discussion

Previous work by Latham *et al.* (2020) discovered an isolate of Hh (Hh-BW1) with a unique capacity to inhibit the growth of NTHi growth *in vitro* by production of a protein with a molecular weight of ~30 kDa (295). Structural and biochemical investigations characterised this secreted inhibitory protein as a novel haemophore which was subsequently named Hpl (315). Given that Hh and NTHi share the same ecological niche and an absolute growth requirement for haem, we proposed that the NTHi-inhibitory activity of Hh strains capable of producing Hpl involved competition for haem.

The absence of NTHi-inhibitory activity by the *hpl* knockout confirmed that this NTHi-inhibitory capacity was mediated by the ability of Hh to produce Hpl. This is supported by parallel investigations showing that purified recombinant Hpl produced by *Escherichia coli* displayed NTHi-inhibitory activity, thus tracing the inhibitory activity to the Hpl polypeptide (315). The attenuated growth of the *hpl* knockout mutant in haem-replete conditions also indicates that Hpl plays a positive role in haem-iron acquisition for Hh. This is supported by the restoration of growth when the knockout is grown in media supplemented with Hpl, demonstrating that Hpl can supply Hh with haem for growth. Like NTHi, Hh is unable to synthesise protoporphyrin (the haem precursor) and relies solely on acquisition of haem from the environment as a source of haem for metabolism and growth (6). The presence of genes involved in the acquisition/utilisation of free- or haemoprotein sources of haem have been described in Hh genomes (124, 333-335). This may explain the capacity of Hh-BW1 and the *hpl* knockout to grow uninhibited when passaged on blood-supplemented media where the predominant haem source is hemoglobin; under these conditions it is possible that outer membrane haem transporters provide alternative and sufficient routes for haem import. Indeed, the Hh-BW1 genome generated by this study shows the presence of *hup* which coordinates acquisition of free- or haptoglobin-bound haemoglobin (211). However, the prevalence and diversity of known genes associated with haem

acquisition in Hh are substantially lower than in NTHi genomes (124). In a screen of 749 NTHi isolates, a high prevalence and diversity of genes involved in haem acquisition/utilisation were detected, including *hxuA* (91%), *hxuB* (98%), *hxuC* (98%), *hemR* (93%) and *hup* (97%) (124). In comparison, the prevalence of these genes in Hh (n=148) is significantly lower (*hxuA,* 9%; *hxuB,* 11%; *hxuC,* 11%; *hemR,* 20%; *hup,* 34%). Notably, very few Hh strains carry the *huxCBA* system, which is considered a virulence factor in *H. influenzae* (170). Thus, Hh has fewer haem uptake pathways than NTHi, which may have created stronger selective pressure for Hh to acquire a haemophore.

Direct acquisition by a secreted haemophore is a novel mode of haem acquisition in the Haemophilus genus. Haemophores are secreted or cell-surface anchored haem scavenger proteins that are found in a subset of bacterial species. In gram-negative organisms, three haemophores have been described in detail: HasA from *Serratia marcesens*, *Pseudomonas* spp., and *Yersinia* spp. (336-339); HmuY from *Porphyromonas gingivalis* (340); and HusA from *P. gingivalis* (341). However, Hpl haem-binding is predicted to be coordinated by a single histidine side chain, which is atypical of haemophores (315). Hpl shares structural similarity with the haemoglobin-haptoglobin utilisation protein (HpuA) haem import system possessed by other Pasteurellaceae; however, the HpuA protein does *not* bind to haem suggesting that proteins with quite different biochemical activities, but nevertheless a shared "higher level" function of haem/iron acquisition, have evolved from a common ancestral gene (342). Given that haemoglobin-haptoglobin is likely the most abundant source of extracellular haem in the respiratory tract, it will be important to establish whether haemoglobin-derived haem is accessible to Hpl; if this were the case it would strongly suggest that Hpl would have a significant role in the haem economy of Hh *in vivo* (168). However, the affinity for different *in vivo* haem-sources available remains to be determined experimentally.

The unique conservation of the predicted TBDHR (homologous with the HasR outer membrane haem acquisition system receptor from *Pasteurella multocida*) and SLAM proteins in strains containing the *hpl* ORF also supports the role of Hpl in Hh haem acquisition. HasAR typically involves type I secretion of the haemophore, HasA, into the extracellular environment which binds and delivers host haem (free-, or haemoglobin-bound) to the HasR haemophore receptor transmembrane channel (343). Less efficient haem-uptake via direct binding of haem by HasR is also possible (343). The highly conserved ~200 aa region within the TBDHR sequence was not only unique among Hpl-producing strains, but also to the Hh species; however, the function of this region is unknown. The SLAM family of proteins are also involved in haem-iron utilisation in several gram-negative species, including Hh. Many SLAM-related sequences are typically located adjacent to, and are essential for surface display of lipoproteins, particularly receptors involved in the transport of iron or haem (332). In *Neisseria meningitidis*,

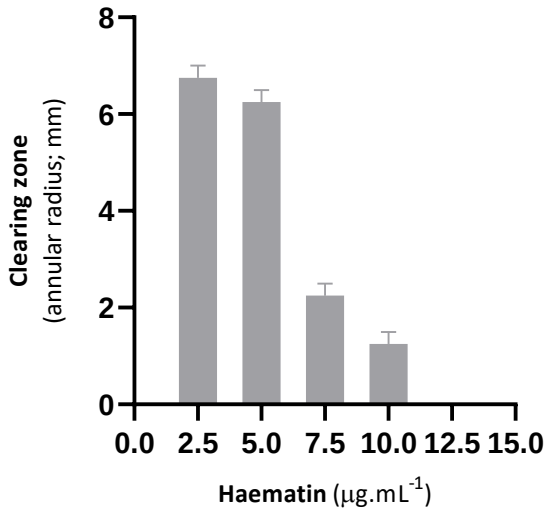
Slam2 is found adjacent to, and is specifically required for the display of HpuA (344). Ton-B dependent receptors, such as HasR, typically form a bipartite receptor which contain an accessory lipoprotein which may be involved in extracting the ligand from the host haem or iron transporter (343). Thus, the predicted SLAM protein may have a role in translocating Hpl to the cell surface, where it functions to capture haem. However, *in vitro* investigations are required to determine if this is the case.

The unusual structure of Hpl may also lend to a model of competitive haem acquisition. The vital role of haem-iron in the survival of NTHi and other bacteria in the upper respiratory tract raises the stakes for evolutionary conflicts to arise in the struggle for this limiting nutrient (212). Competition for iron and haem between bacterial pathogens and their hosts is well accepted, and the ability of the host to impose low concentrations of free iron is one of the most important forms of nutritional immunity (136, 345). The emerging picture is that non-pathogenic probiotic bacteria, as well as pathogenic species, have enhanced iron uptake capabilities that facilitate inhibition of microbial pathogens as well as colonisation of the host (287). Our work suggests that competition for haem between haem auxotrophs, such as Haemophilus *spp.*, might be similarly important. The mechanism by which Hpl-bound haem is unavailable to NTHi remains to be investigated, particularly as NTHi is capable of utilising protein-associated haem when it is non-covalently bound to the protein (209), as is the case with Hpl.

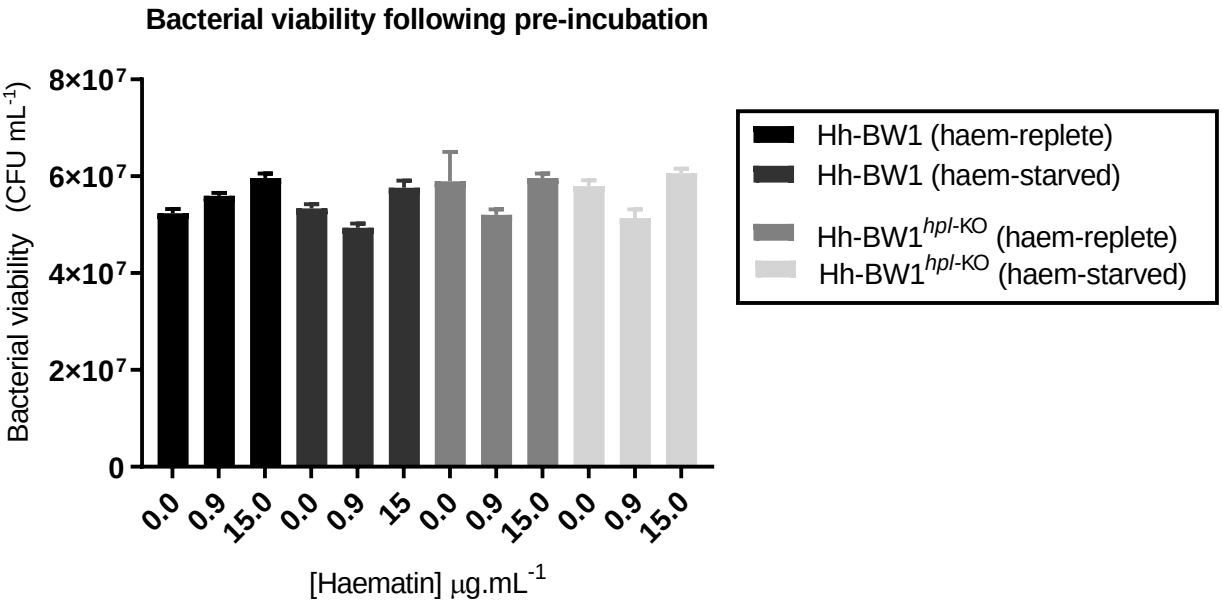
The presence of Hpl-like genes in the majority Hh genomes, suggests that this mode of acquisition may be of considerable importance in the overall haem economy of this species (315). It is therefore unclear why there exists such a high degree of variability in the *hpl* sequence and Hpl-producing (and thus NTHi-inhibitory) capacity among Hh strains. The capacity for intermediate-, and high-level NTHi-inhibitory activity was common only among strains with high *hpl* sequence identity (96-100%), indicating that significant sequence deviations encode a protein with altered function. However, only a subset of strains lacking NTHi-inhibitory activity contained mutations considered to be functionally important. Additionally, sequence variations did not explain the differential mRNA expression/production of Hpl among other Hh strains with identical *hpl* ORFs. The correlation between NTHi-inhibitory activity and mRNA expression suggests differences in transcriptional regulation that may explain the variation in activity between the strains with highly similar *hpl* ORFs (96-100%). Thus, while possession of an intact *hpl* ORF may be required for secretion of a functional protein, further work is required to determine the existence of other regulatory elements which may influence *hpl* expression. However, as the primary aim of this study is to determine the therapeutic potential of Hpl-producing Hh, the regulatory mechanism of *hpl* gene expression is left to future investigation beyond the scope of this thesis.

In summary, this study provides structural, functional and bioinformatic evidence characterising Hpl as a previously unrecognised haem uptake mechanism in Hh that has the potential to block uptake of essential haem by pathogenic NTHi. Since Hh co-colonises the upper respiratory tract and competes for binding sites on epithelial cells (291), Hh strains with high-level expression of particular Hpl alleles, as seen in Hh-BW1, might starve NTHi of haem, thus inhibiting colonisation. Further investigations using direct competition models are warranted to determine if competition from Hpl-producing Hh could antagonise NTHi colonisation in the respiratory tract and thus have therapeutic utility as a respiratory probiotic.

3.6. Supplementary Materials



Supplementary Figure S3.1 | Annular radius (mm) of NTHi clearing zones produced by native Hpl extracts from Hh-BW1 grown in TSB media supplemented with different concentrations of porcine haematin (μg mL^{-1}). Error bars represent ± SEM (n=3).



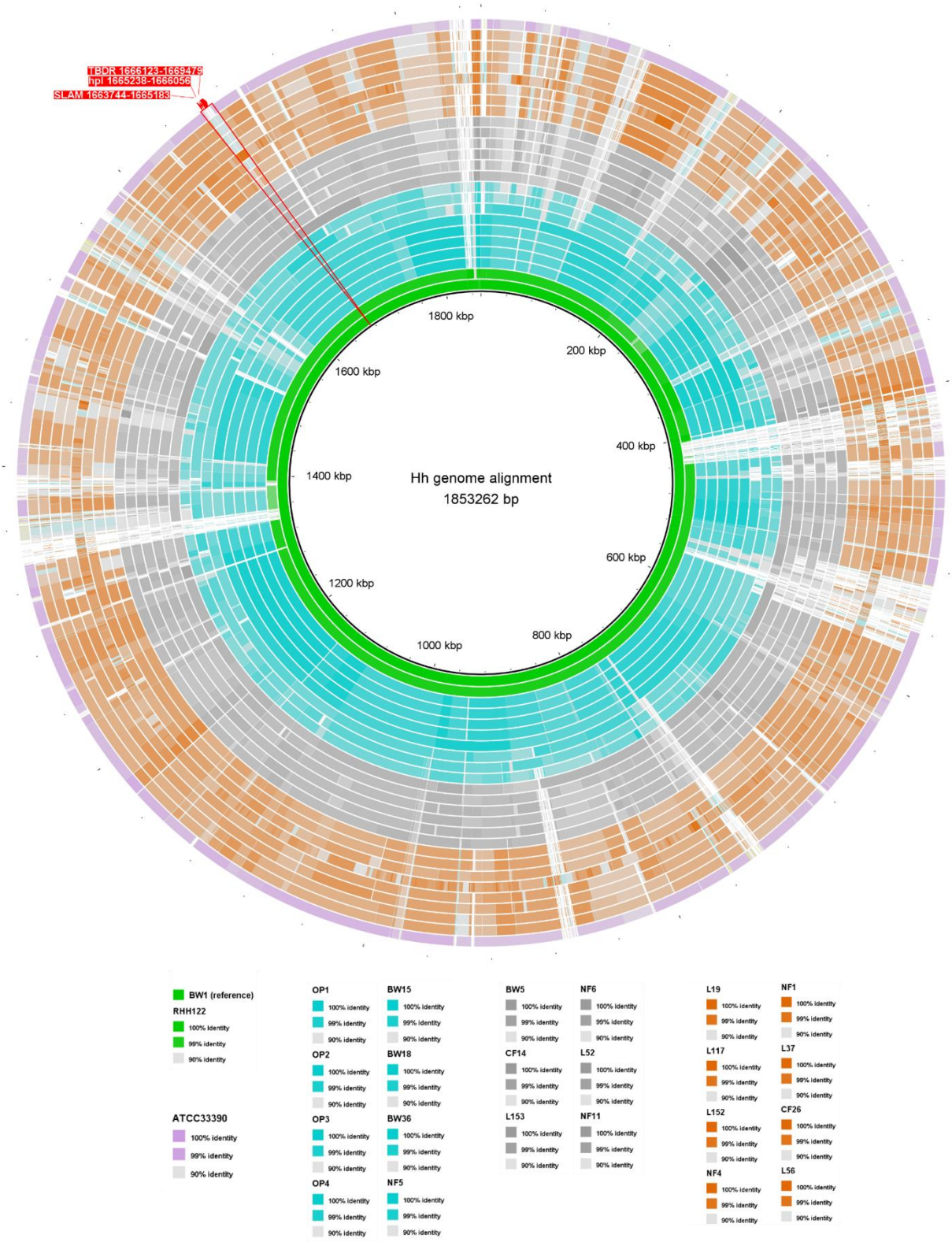
Supplementary Figure S3.2 | Viability of suspensions used for seeding growth experiments (Figure 3.1). Colony counts were performed on all suspensions by plating on chocolate agar to demonstrate that the pre-incubation period did not reduce cell viability and that similar numbers of cells were inoculated into all test conditions. Error bars represent ± SEM (n=3).

Supplementary Table S3.1| Genotypic and phenotypic characteristics of Hh strains containing the *hpl* ORF. Sequences were determined by whole-genome sequencing, performed using the MiSeq (Illumina) platform. ORF similarities are compared to a model Hpl-producing strain (Hh-BW1) that has demonstrated high anti-NTHi activity *in vitro.*

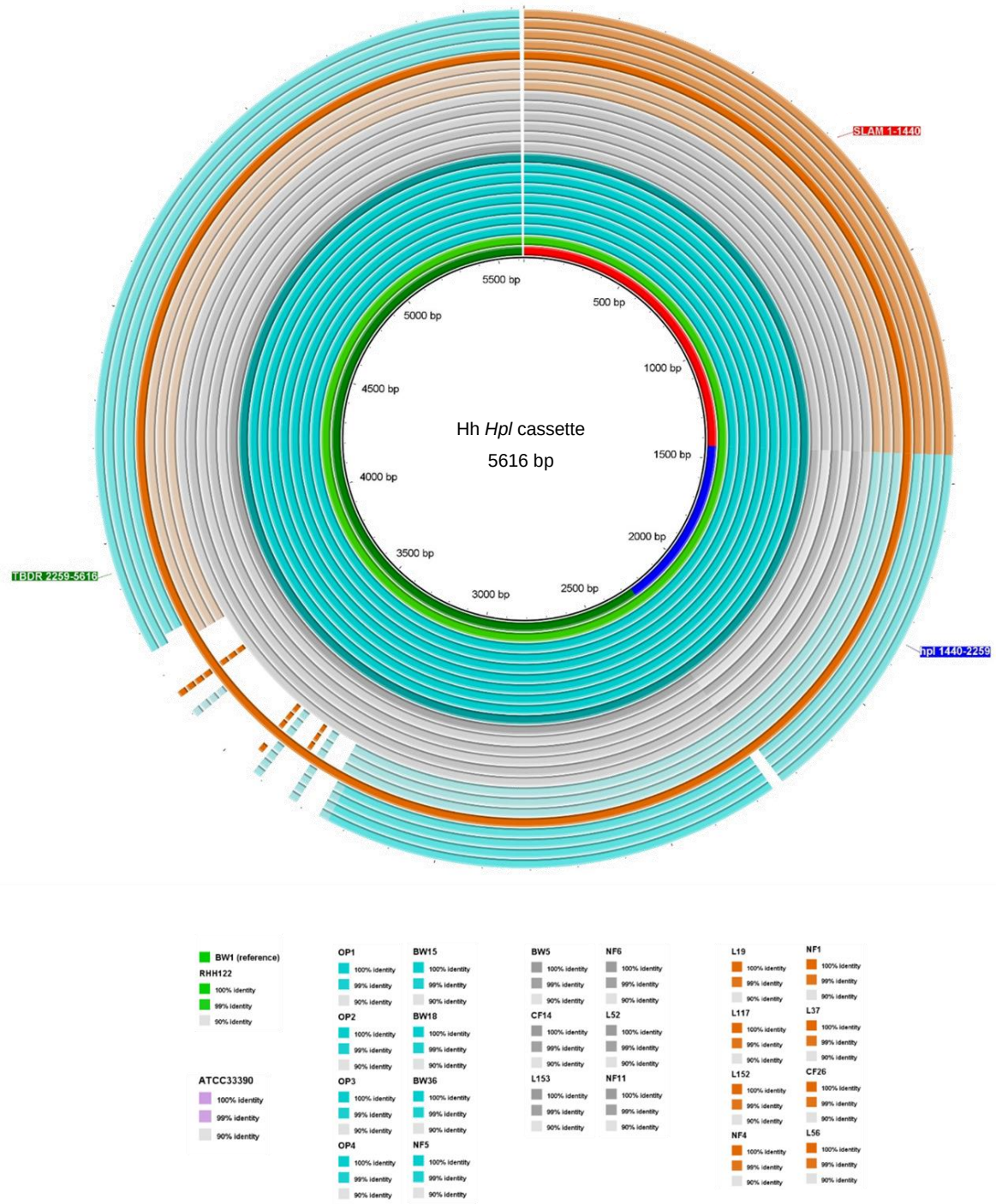
Hh Strain	% Identity of the *hpl* ORF	Hpl production	Hh-*hpl*$^{+}$ by tri-plex PCR
BW1	-	+	+
BW5	99% (807/819)	+	+
BW15	100% (819/819)	+	+
BW18	100% (819/819)	+	+
BW36	100% (819/819)	+	+
CF14	96% (786/819)	+	+
L19	85% (709/833)	-	-
L117	85% (709/833)	-	-
L152	85% (709/833)	-	-
L153	98% (805/819)	+	+
NF4	100% (819/819)	-	+
NF5	100% (819/819)	+	+
NF6	96% (786/819)	+	+
NF1	80% (636/794)	-	-
RHH122	100% (819/819)	+	+
L37	80% (636/794)	-	-
CF26	79% (630/794)	-	-
L52	98% (805/819)	+	+
L56	81% (621/768)	-	-
OP1	100% (819/819)	+	+
OP2	100% (819/819)	+	+
OP3	100% (819/819)	+	+
OP4	100% (819/819)	+	+
NF11	96% (788/819)	+	+

Supplementary Table S3.2 | Sequence similarity of the Hh-BW1 *hpl* gene cassette among 37 (of 67) *H.haemolyticus* (Hh) whole genome assemblies available from Genbank.

	Hpl ORF				TBDR				SLAM			
Hh Genome	Query Cover	E value	Identity (%)	Accession	Query Cover	E value	Identity (%)	Accession	Query Cover	E value	Identity (%)	Accession
M19346	99%	0	79.57	NZ_CP031243.1	84%	0	78.53	NZ_CP031243.1	100%	0	98.33	NZ_CP031243.1
M19345	99%	0	75.9	NZ_CP031240.1	82%	0	78.68	NZ_CP031240.1	100%	0	97.92	NZ_CP031240.1
M28486	100%	0	99.24	NZ_CP031238.1	100%	0	99.91	NZ_CP031238.1	100%	0	98.19	NZ_CP031238.1
M19066	100%	0	100	QQHH01000001.1	100%	0	100	QQHH01000001.1	100%	0	100	QQHH01000001.1
M26157	100%	0	98.39	QQIX01000001.1	100%	0	96.61	QQIX01000001.1	100%	0	97.29	QQIX01000001.1
M11818	100%	0	100	QQHN01000001.1	100%	0	100	QQHN01000001.1	100%	0	100	QQHN01000001.1
M19080	99%	0	79.57	QQIL01000001.1	84%	0	78.53	QQIL01000001.1	100%	0	98.33	QQIL01000001.1
M25342	99%	0	75.25	QQKH01000001.1	82%	0	79.15	QQKH01000001.1	100%	0	97.71	QQKH01000001.1
M19071	90%	0	78.78	QQJF01000005.1	55%	0	100	AFQN01000073.1	100%	0	97.99	QQJF01000005.1
M19155	90%	0	78.7	QQIA01000005.1	84%	0	79.11	QQIA01000005.1	100%	0	97.99	QQIA01000005.1
M19135	90%	0	78.78	QQIS01000005.1	84%	0	78.53	QQIS01000005.1	100%	0	97.78	QQIS01000005.1
65117 B Hi-3	100%	0	96.94	SDPE01000010.1	100%	0	96.46	SDPE01000010.1	100%	0	97.64	SDPE01000010.1
M19122	99%	0	79.57	QQKM01000006.1	84%	0	78.53	QQKM01000006.1	100%	0	98.26	QQKM01000006.1
M19140	99%	0	79.57	QQIR01000006.1	84%	0	78.53	QQIR01000006.1	100%	0	98.19	QQIR01000006.1
M26166	99%	0	79.57	QQLP01000006.1	84%	0	78.85	QQLP01000006.1	100%	0	98.33	QQLP01000006.1
M26173	99%	0	79.57	QQHG01000007.1	84%	0	78.85	QQHG01000007.1	100%	0	98.33	QQHG01000007.1
M19197	99%	0	79.57	QQMK01000011.1	84%	0	78.53	QQMK01000011.1	100%	0	98.33	QQMK01000011.1
M19079	99%	0	78.74	QQGY01000002.1	86%	0	78.73	QQGY01000002.1	100%	0	98.06	QQGY01000002.1
M19201	99%	0	79.57	QQIY01000007.1	84%	0	78.69	QQIY01000007.1	100%	0	98.26	QQIY01000007.1
M26161	99%	0	78.74	QQJW01000005.1	37%	0	84.19	QQJW01000014.1	100%	0	98.06	QQJW01000005.1
CCUG 24149	99%	0	75.63	LZDL01000007.1	82%	0	78.62	LZDL01000007.1	100%	0	97.57	LZDL01000007.1
M19528	99%	0	79.57	QQIN01000007.1	84%	0	78.69	QQIN01000007.1	100%	0	98.33	QQIN01000007.1
M19164	99%	0	79.57	QQFX01000011.1	84%	0	78.58	QQFX01000011.1	100%	0	98.47	QQFX01000011.1
CCUG 15949	100%	0	96.94	SDPH01000014.1	100%	0	96.46	SDPH01000014.1	100%	0	97.64	SDPH01000014.1
M26167	99%	0	78.74	QQKD01000013.1	84%	0	77.97	QQKY01000001.1	100%	0	98.19	QQKY01000001.1
M26176	100%	0	98.06	QQKD01000013.1	86%	0	78.46	QQKD01000013.1	100%	0	98.06	QQKD01000013.1
M19161	99%	0	72.93	QQHM01000014.1	82%	0	78.14	QQHM01000014.1	100%	0	97.99	QQHM01000014.1
11P18	100%	0	99.24	LCTK01000015.1	100%	0	99.97	LCTK01000015.1	100%	0	98.19	LCTK01000015.1
16-549009	99%	0	75.74	RWKG01000004.1	82%	0	78.8	RWKG01000004.1	100%	0	97.78	RWKG01000004.1
CCUG 30218	99%	0	79.44	SDPI01000052.1	55%	0	79.13	SDPI01000012.1	53%	0	98.07	SDPI01000005.1
CCUG 11096	100%	0	98.19	SDPG01000001.1	82%	0	79.13	SDPG01000001.1	100%	0	98.19	SDPG01000001.1
S32F2	100%	0	97.71	JACBKB010000012.1	100%	0	96.43	JACBKB010000012.1	100%	0	97.22	JACBKB010000012.1
27P25	99%	0	75.9	LCTH01000015.1	82%	0	78.19	LCTH01000015.1	100%	0	97.78	LCTH01000015.1
M21127	99%	0	75.74	AFQP01000012.1	82%	0	79.09	AFQP01000012.1	100%	0	97.43	AFQP01000012.1
L3_128_043G1	99%	0	75.74	JAHAHO010000014.1	82%	0	79.09	JAHAHO010000014.1	100%	0	97.29	JAHAHO010000014.1
L3_098_047G1	99%	0	79.44	JAHAHX010000001.1	82%	0	79.13	JAHAHX010000001.1	100%	0	98.19	JAHAHX010000001.1
M21639	100%	0	97.71	AFQR01000004.1	100%	0	96.43	AFQR01000004.1	100%	0	97.22	AFQR01000004.1



Supplementary Figure S3.3 | Multiple sequence alignment of Hh genomes containing the *hpl* ORF used in the current study (constructed using the BLAST Ring Image Generator v0.95). The predicted *hpl* gene cassette is highlighted in red.



Supplementary Figure S3.4 | Multiple sequence alignment of the *hpl* cassette among Hh strains (constructed using the BLAST Ring Image Generator v0.95).

Chapter 4 | Manuscript 1

In vitro **probiotic potential of haemophilin-producing strains of *Haemophilus haemolyticus.***

Brianna Atto [1], Roger Latham [2], Dale Kunde [1], David A Gell [2] and Stephen Tristram [1],

[1] *School of Health Sciences, University of Tasmania, Launceston, Australia*

[2] *School of Medicine, University of Tasmania, Hobart, Australia*

The content of this chapter is reproduced as published in Pathogens MDPI as an original research article (Submitted 27 February 2020; Accepted: 22 March 2020; Published: 25 March 2020), however the text has been reformatted to meet the thesis submission requirements.

4.1. Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is a leading causative organism of opportunistic respiratory tract infections. However, there are currently no effective vaccination strategies, and existing treatments are compromised by antibiotic resistance. We previously characterised *Haemophilus haemolyticus* (Hh) strains capable of producing haemophilin (Hpl), a haem-binding protein that restricts NTHi growth by limiting its access to an essential growth factor, haem. Thus, these strains may have utility as a probiotic therapy against NTHi infection by limiting colonisation, migration and subsequent infection in susceptible individuals. Here, we assess the preliminary feasibility of this approach by direct *in vitro* competition assays between NTHi and Hh strains with varying capacity to produce Hpl. Subsequent changes in NTHi growth rate and fitness, in conjunction with *hpl* expression analysis, were employed to assess the NTHi-inhibitory capacity of Hh strains. Hpl-producing strains of Hh not only outcompeted NTHi during short-term and extended co-culture, but also demonstrated a growth advantage compared with Hh strains unable to produce the protein. Additionally, *hpl* expression levels during competition correlated with the NTHi-inhibitory phenotype. Hpl-producing strains of Hh demonstrate significant probiotic potential against NTHi colonisation in the upper respiratory tract, however, further investigations are warranted to demonstrate a range of other characteristics that would support the eventual development of a probiotic.

4.2. Introduction

The bacterium nontypeable *Haemophilus influenzae* (NTHi) is commonly associated with upper respiratory tract colonisation in healthy adults (22). However, migration to other sites in the respiratory tract frequently occurs in children, the elderly and individuals with underlying respiratory diseases; making NTHi a leading cause of mucosal infections (2). In particular, enormous global morbidity is attributed to otitis media and exacerbations of chronic obstructive pulmonary disease, which are accompanied by long-term health complications and considerable mortality, respectively (72, 133). NTHi has also gained attention as an increasingly important cause of invasive infections (84, 346).

There are currently no effective vaccination strategies for the prevention of NTHi infections, and treatment has been complicated by the rapid development of antibiotic resistance to first- and second-line antibiotics. Resistance is predominantly mediated by β-lactamase production (77); however, the emergence and spread of β-lactamase-negative, ampicillin-resistant strains in many regions of

the world is of substantial concern with treatment failure also being reported in response to macrolides (89, 117, 347) and fluoroquinolones (96, 114, 348).

NTHi infection is preceded by successful colonisation of the upper respiratory tract, and survival in this environment relies on the bacterium's ability to acquire the vital growth factor, haem (124). There is also evidence to suggest haem acquisition genes are important modulators of NTHi virulence factors (67), demonstrated by the increased prevalence in disease-causing strains from the middle ear, compared with colonising throat strains (124). Deletion of multiple genes related to haem-iron scavenging, utilisation and regulation has been shown to significantly reduce NTHi virulence, disease severity and duration in animal models of otitis media (163, 167). Similarly, an isogenic mutant of two haem acquisition pathways was unable to sustain bacteraemia or produce meningitis in a rat model of invasive disease (168). Thus, haem acquisition pathways represent potentially high value targets for the development of novel therapies for the eradication of NTHi from the respiratory tract (169, 242).

NTHi is particularly susceptible to haem restriction as it lacks the necessary enzymes for its synthesis and relies solely on scavenging haem from the host, either in the form of free haem or bound to host carrier molecules (163, 209, 210, 349). Evidence from our laboratory suggests that closely related commensals may present a competitive challenge for haem acquisition in the upper respiratory tract. Previously, we discovered *Haemophilus haemolyticus* (Hh) strains that exhibited inhibitory activity against NTHi (295, 315). Further investigation revealed this inhibition was mediated by the production of a haem-binding protein, haemophilin (Hpl), that restricted NTHi growth by limiting its access to haem (315). Thus, these strains may have utility as a probiotic therapy against NTHi infection by limiting colonisation, migration and subsequent infection in susceptible individuals. Hh strains with anti-NTHi properties have other characteristics that support their potential use as probiotics. Firstly, they share the same upper respiratory niche as NTHi (22) and more importantly, although they have occasionally been reported as pathogens of sterile sites in immunocompromised patients (10), there is convincing evidence that they are not opportunistic pathogens of the respiratory tract (350-352).

Here, we aim to determine the potential of a future probiotic approach by assessing *in vitro* competition between NTHi and Hh strains with varying capacity to produce Hpl.

4.3. Results and Discussion

4.3.1. Validation of a triplex real-time PCR for quantification of NTHi, Hh and detection of *hpl*

The Hpl amplicon was confirmed to be specific and sensitive for the detection of the five previously identified *hpl* sequence variants (315) by *in silico* investigations and by PCR. Specificity of the *hypD* and *siaT* targets was also confirmed by PCR. Complete results of PCR assay validation are detailed in supplementary materials (Supplementary Results S4.6.2). The low limit of quantification values for the *hypD* and *siaT* assays in triplex format were 2×10^{-5} ng and 2×10^{-4} ng, corresponding to 10 and 100 genome equivalents (GE), respectively. The lower limit of detection for the *hpl* assay was 10 GE (Supplementary Figure S4.1). The upper limits of detection/quantification were not explicitly determined as expected DNA levels from sample were unlikely to exceed the maximum 2 ng tested.

Given the high volume of samples generated from growth experiments, a cheap and high-throughput genomic (gDNA) extraction method was required to reliably distinguish and quantify NTHi and Hh in co-culture. Extraction utilising thermal lysis has previously been shown to be an efficient and cost-effective method to harvest bacterial gDNA for quantitative real-time PCR from suspensions of several bacterial species in a range of sample matrices (353-358). Crude DNA extraction methods are also prone to contamination with PCR inhibitors originating from sample matrices (357, 359). There are also reports of intra- and inter-species differences in DNA extractions efficiencies (357, 359, 360). PCR quantification of gDNA extracted by thermal lysis was validated and found to be comparable to quantification by OD_{600} and colony counts (Supplementary Figure S4.4). Complete results of thermal extraction validation, including detection of PCR inhibitors (Supplementary Figure S4.2) and extraction efficiency (Supplementary Figure S4.3) are available in supplementary materials (Supplementary Results S4.6.2).

4.3.2. Baseline NTHi-inhibitory activity of Hh strains containing the *hpl* ORF

We previously identified clinical isolates of Hh with several different *hpl* open reading frame (ORF) sequences and variable inhibitory activities even between strains containing identical

hpl ORF sequences (Supplementary Table S4.1) (315). In order to determine the basis of this phenotypic variation and predict inhibitory potential, selection of Hh strains (Hh-RHH122, Hh-NF4 and Hh-NF5) for investigation was based on identical sequence similarity to the Hh-BW1 *hpl* ORF, previously identified as having the highest NTHi-inhibitory activity (315). Based on results from the well diffusion assay, isolates Hh-BW1, Hh-RHH122 and Hh-NF5 were categorised as having the NTHi-inhibitory phenotype (Hh-Hpl+); no inhibitory activity was detected from Hh-NF4 broth supernatants, categorising it as non-inhibitory (Hh-Hpl-). Hh strains that did not possess the *hpl* ORF (Hh ATCC 33390 and Hh-BW1$^{hpl\text{-}KO}$) were confirmed to be Hh-Hpl-. The degree of inhibitory activity varied between the Hh-Hpl+ isolates and was comparably highest in Hh-BW1 and Hh-RHH122, approximately twice the activity measured for Hh-NF5 (Supplementary Figure S4.5).

4.3.3. Expression of *hpl* correlates with the Hh-Hpl+ phenotype

Given that *hpl* ORF sequence identity was not predictive of NTHi-inhibitory capacity, baseline expression of *hpl* was investigated. The *hypD* target was validated as the housekeeper gene (Supplementary Figure S4.6A), and the optimal growth phase for *hpl* expression analysis was determined (Supplementary Figure S4.6B). Baseline expression of *hpl* was highest in Hh-BW1(Hpl+) and Hh-RHH122(Hpl+), significantly lower in Hh-NF5(Hpl+) (p <0.0001), and completely absent in Hh-NF4(Hpl-) (Figure 4.1). Expression patterns correlate with the NTHi-inhibitory capacity of Hh strains, suggesting a connection between expression of *hpl* and the NTHI-inhibitory phenotype resulting from production of the Hpl protein.

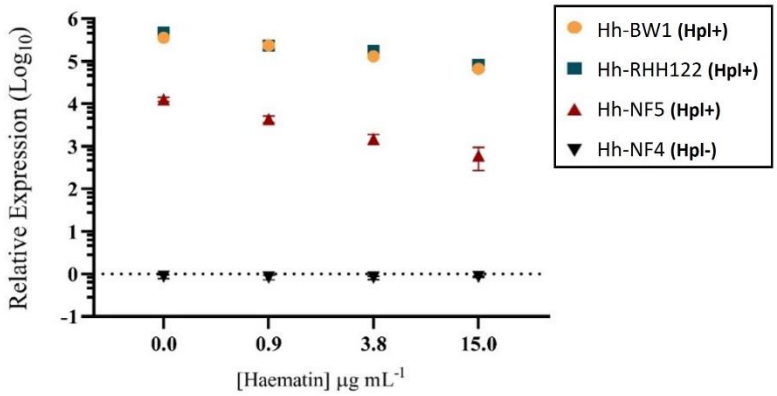


Figure 4.1 | Baseline *hpl* expression. PCR-quantified expression of *hpl* for *Haemophilus haemolyticus* (Hh) strains containing identical *hpl* open reading frames (ORFs) (relative to Hh-BW1$^{hpl\text{-}KO}$). Data points are represented as mean +/- SEM of four biological replicates, performed from duplicate RNA extractions. NTHi-inhibitory phenotype (Hpl+); non-inhibitory phenotype (Hpl)-.

4.3.4. The Hh-Hpl+ phenotype confers a competitive advantage against NTHi

A co-culture assay was used to test the ability of Hh with different levels of Hpl production to compete with NTHi. The growth rate of NTHi was significantly impaired during competition with all Hh-Hpl+ strains, compared with growth without competition ($p < 0.0001$) (Figure 4.2A). This inhibitory effect was more pronounced during competition with strains Hh-BW1 and Hh-RHH122, compared with Hh-NF5, replicating inhibitory patterns observed in the well diffusion assay. The growth rate of NTHi during competition with Hh-Hpl- was not significantly affected (Figure 2B), suggesting that the inhibitory effect observed was a unique characteristic of Hh-Hpl+ strains. Loss of the Hh-Hpl+ phenotype in Hh-BW1$^{hpl\text{-}KO}$, compared with the wild-type Hh-BW1, is evidence for a causative effect of the *hpl* gene on competition. Correlation between competition outcomes and *hpl* gene expression in Hh-BW1(Hpl+), Hh-RH122(Hpl+), Hh-NF5(Hpl+) and Hh-NF4(Hpl-) strains is also consistent with a hypothesis that strains with higher *hpl* expression compete with NTHi more effectively.

For commensals and pathogens living in or invading human tissues, iron-containing haem is often a limiting nutrient, particularly in the respiratory tract where concentrations are considered to be low (216). This is particularly true for haem auxotrophs including NTHi and Hh; for these species', survival in the upper respiratory niche is dependent on their ability to outcompete host proteins and co-existing bacterial populations for haem (163). We previously demonstrated that the NTHi-inhibitory mechanism of Hpl is associated with its ability to bind haem in a form inaccessible to NTHi and that inhibitory activity is lost in conditions where haem concentration exceeds the binding capacity of Hpl (315). While levels of haem/iron are considered to be low in the respiratory tract, there is indirect evidence for increased levels in airways of smokers, chronic obstructive pulmonary disease and cystic fibrosis, which may contribute to increased susceptibility to infection in these individuals (216). Thus, it was important to assess the effectiveness of Hpl with varying concentrations of haem to ensure inhibitory effect in a range of *in vitro* conditions reflecting possible *in vivo* scenarios. The NTHi-inhibitory capacity of Hpl was maintained even in conditions of high haem availability (15 µg mL^{-1}), albeit to a lesser degree than lower haem concentrations (0.0–3.8 µg mL^{-1}) (Figure 4.2A). This suggests that levels of Hpl produced by Hh are sufficient to limit NTHi access to haem in

a dynamic *in vitro* system, even under excess haem conditions unlikely to be encountered *in vivo* (216).

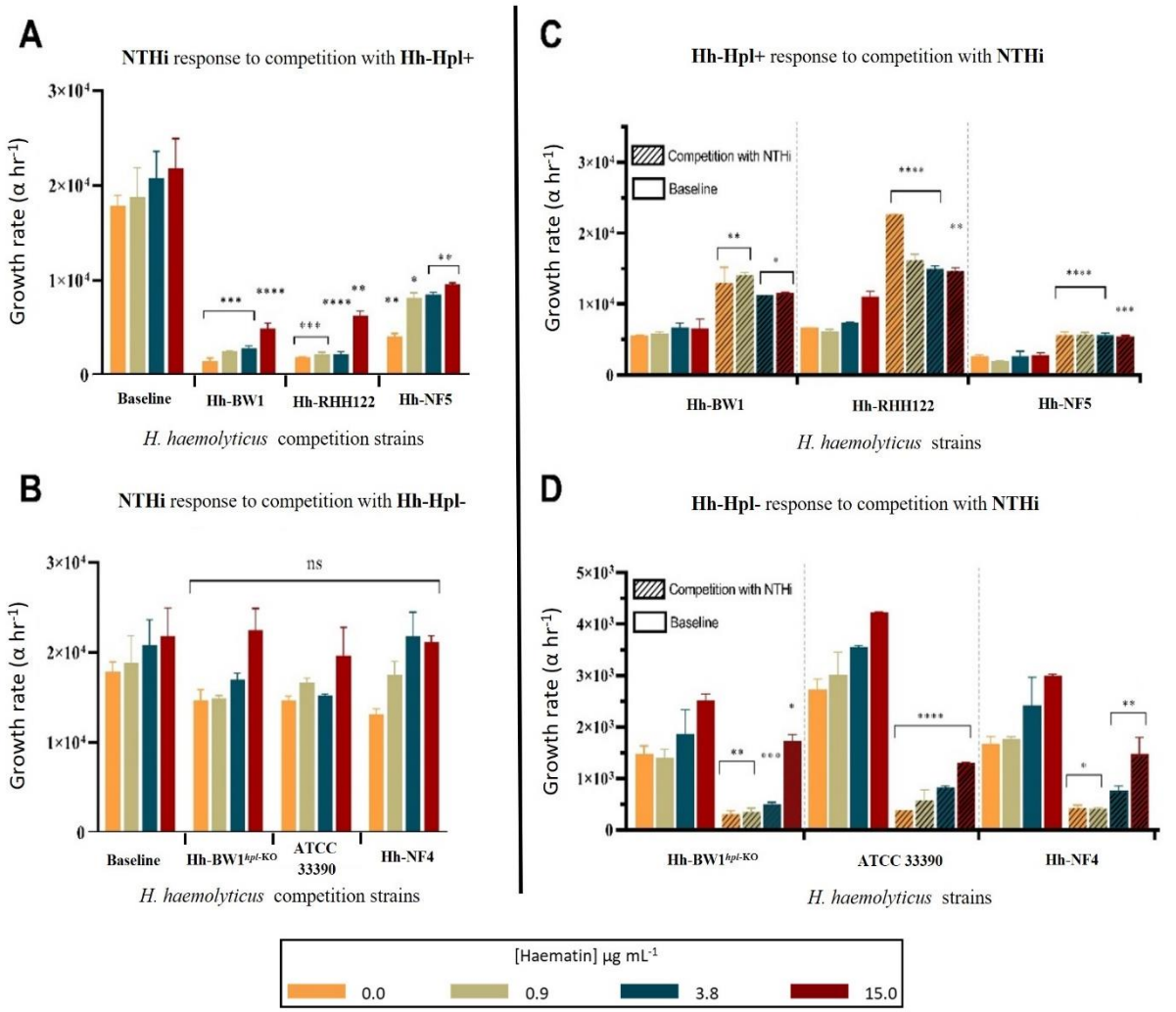


Figure 4.2 | Short-term competition between nontypeable *Haemophilus influenzae* (NTHi) and Hh. Calculated growth rates of NTHi in response to competition with **(A)** Hh-Hpl+ or **(B)** Hh-Hpl-. The growth rate for each **(C)** Hh-Hpl+ and **(D)** Hh-Hpl- strain was also determined. Data points represented as mean +/- SEM of three separate experiments, performed in triplicate; $p < 0.05$*, $p < 0.005$ **, $p < 0.0005$ ***, $p < 0.0001$ ****. The annotated level of statistical significance is relative to the matching baseline reading at each haem concentration.

4.3.5. The Hh-Hpl+ phenotype is associated with a growth advantage

Interestingly, all Hh-Hpl+ strains exhibited a pattern of enhanced growth in response to NTHi competition ($p < 0.0001$) (Figure 4.2C). This effect was maintained across all haem concentrations and was more pronounced for Hh-BW1 and Hh-RHH122. The converse was observed in Hh-Hpl- strains that appeared to experience poorer growth in response to competition with

NTHi (Figure 4.2D). This indicates that NTHi is able to outcompete Hh only in the absence of the Hh-Hpl+ phenotype, which may be a reflection of the highly efficient set of haem-scavenging systems possessed by NTHi.

Given the correlation between competition outcomes and *hpl* gene expression, expression analysis was performed on Hh during competition with NTHi, relative to growth without competition. Upregulation of *hpl* was observed in all Hh-Hpl+ strains in response to competition with NTHi, an effect that was more pronounced in Hh-BW1 and Hh-RHH122 (Figure 4.3). This may explain the enhanced growth rate of Hh-Hpl+ strains in response to NTHi during the short-term competition assays (Figure 4.2C).

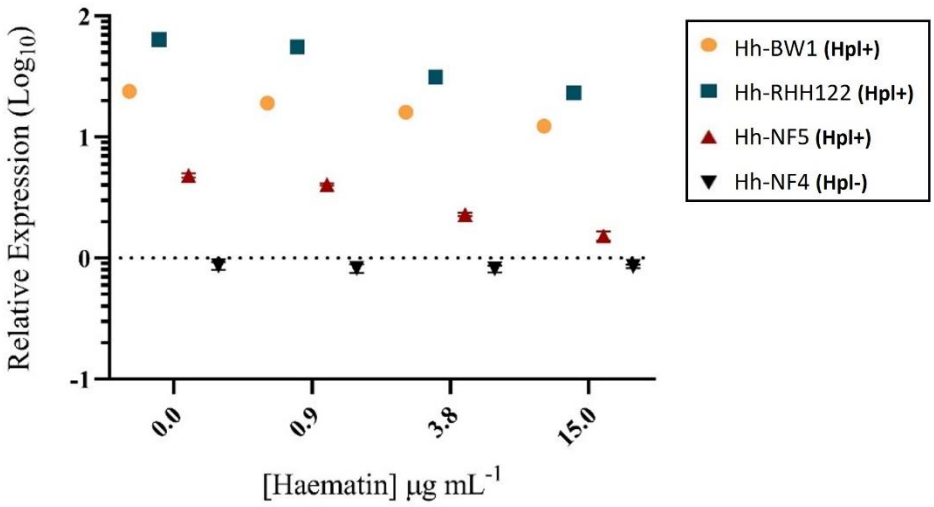


Figure 4.3 | *hpl* expression during competition. PCR-quantified expression of *hpl* during competition with NTHi relative to individual growth. Data points are represented as mean +/- SEM of four biological replicates, performed from duplicate RNA extractions. NTHi-inhibitory phenotype (Hpl+); non-inhibitory phenotype (Hpl)-.

These results show that expression of *hpl* has a significant impact on the NTHi-inhibitory capacity of Hh-Hpl+ strains and eventual therapeutic utility in an *in vivo* setting. Therefore, the huge differential expression of *hpl* amongst Hh-Hpl+ strains must be considered in the future when selecting a potential probiotic candidate for further evaluation. However, our understanding of *hpl* regulation is still rudimentary. Further investigation into potential upstream regulatory components or post-translational modification is needed to elucidate the inter-strain differences in Hpl production and/or biological activity despite complete ORF sequence identity.

4.3.6. NTHi fitness dramatically decreases during extended co-culture with Hh-Hpl+

Short-term competition may highlight the potency of Hpl-mediated inhibition but is not representative of *in vivo* competition dynamics. Thus, a longer-term study was employed to assess the competition between NTHi and Hh-Hpl+ over a period of 6 days (12 generations). The competitive advantage of Hh-Hpl+ strains was evident within the 2nd generation (24 h) for Hh-BW1 and Hh-RHH122 and 4th generation (48 h) for Hh-NF5 (Figure 4.4A). Speculatively, the stunted inhibitory activity exhibited by Hh-NF5 may be due to the lower *hpl* expression levels exhibited by this strain, resulting in slower accumulation of Hpl over the course of the assay. The fitness of NTHi over subsequent generations decreased significantly until complete loss of fitness during the final generations. Competition with Hh-Hpl- strains did not significantly affect the overall fitness of any of the NTHi strains over the 6-day period. However, a small decrease in NTHi fitness was observed after 24 h, followed by complete recovery (Figure 4.4A). This may have arisen from competition for haem prior to the onset of maximum Hpl production.

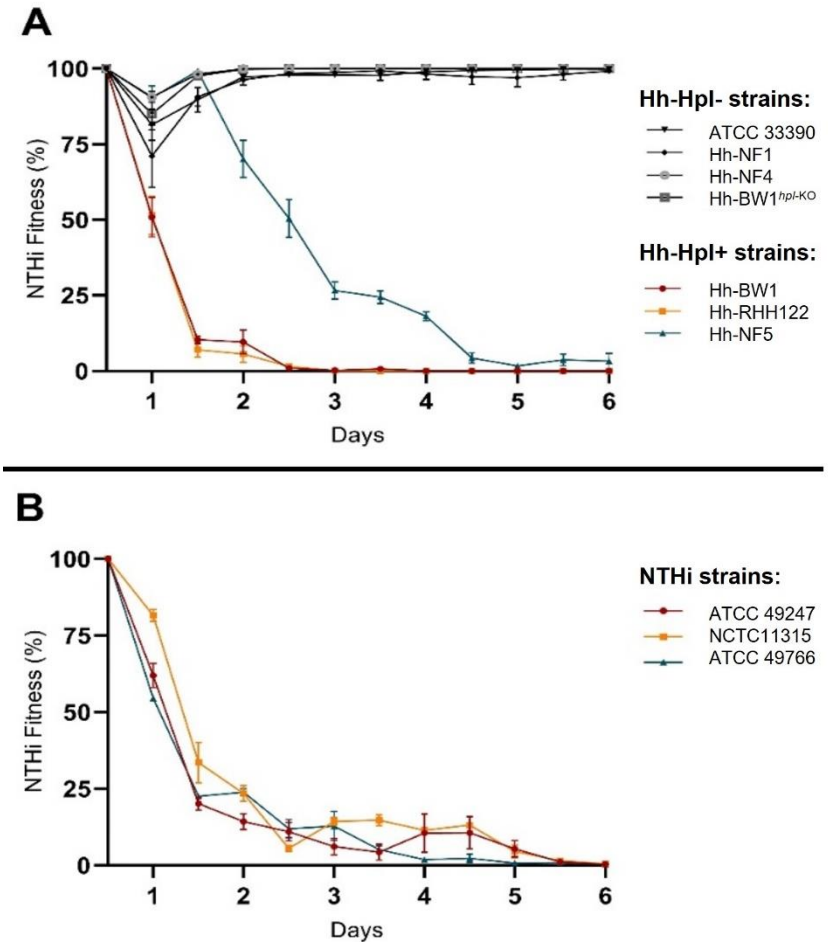


Figure 4.4 | Fitness of NTHi strains during co-culture with Hh. Calculated fitness of NTHi in response to competition with Hh-Hpl+ or Hh-Hpl- relative to growth of the competitor strain. **(A)** Competition between a single NTHi strain and multiple Hh, or **(B)** multiple NTHi against Hh-BW1. Data points represented as mean +/- SEM of three separate experiments, performed in quadruplicate.

To show that loss of fitness of NTHi was not unique to NTHi strain ATCC 49247, additional reference strains NCTC 11315 and ATCC 49766 were tested in competition with Hh-BW1(Hpl+). All three NTHi strains responded in the same manner, culminating in a total loss of NTHi fitness at the end of the 6-day period (Figure 4.4B).

4.4. Materials and Methods

4.4.1. Bacterial growth conditions

Bacterial strains

Hh strains used in this study (BW1, RHH122, NF1, NF4 and NF5) have previously been isolated and screened for the *hpl* open reading frame (ORF) (295, 315). Briefly, Hh strains Hh-BW1, Hh-RH122, Hh-NF4, and Hh-NF5 encode identical Hpl protein sequences but differ in levels of *hpl* expression and Hpl protein production. An *hpl* knockout (Hh-BW1^{hpl-KO}) of the model Hpl-producing strain of Hh (Hh-BW1), constructed using insertional inactivation as previously described (315), and strains Hh-NF1 and Hh ATCC 33390 (PCR negative for the *hpl* ORF) were included as non-inhibitory controls. NTHi strains were ATCC 49247, ATCC 49766 and NCTC 11315.

NTHi and Hh isolates were propagated from liquid nitrogen frozen glycerol stock, followed by two overnight passages on chocolate agar (CA) at 37 °C with 5%–10% CO_2 prior to experimentation. Strains were grown in supplemented Tryptone Soy Broth (sTSB), which consisted of tryptone soy broth (TSB) (Oxoid Ltd., Basingstoke, UK) supplemented with 2% (v/v) Vitox® (Oxoid Ltd.) and 15 µg mL^{-1} of porcine haematin (ferriprotoporphyrin IX hydroxide, Sigma-Aldrich). Exposure to nongrowth conditions was minimised by maintaining suspensions and diluents at 37 °C.

Propagation of haem-replete populations for growth experiments

Strains were propagated under haem-replete conditions prior to use in competition experiments to replenish bacterial haem stores and minimise external stressors that may influence the outcome of competitive growth (158, 159, 315). Bacterial suspensions of ~1.0 OD_{600} were made in TSB from 8–10 h growth on CA and diluted 1:10 in pre-warmed sTSB (5 mL). Broths were incubated for 12 h at 37 °C aerobically with shaking (220 RPM), centrifuged at 4000 × g for 5 min at 37 °C and resuspended in fresh, pre-warmed TSB to an OD_{600} of 1.0 prior to use in growth experiments.

4.4.2. Determination of NTHi-inhibitory activity

A well diffusion assay of broth supernatants was used to categorise Hh strains containing the *hpl* ORF as either inhibitory to NTHi (Hh-Hpl+) or non-inhibitory (Hh-Hpl–), as previously described (295). This assay was also used to establish the relative inhibitory activity of each strain. Testing was conducted on two indicator NTHi strains (ATCC 49427 and clinical isolate NTHi-L15). Media supernatants from strains negative for the *hpl* ORF (Hh ATCC 33390 and Hh-BW1^{hpl-KO}) were included as controls.

4.4.3. Triplex real-time PCR for the quantification of NTHi, Hh and detection of *hpl*

A real-time quantitative triplex PCR assay was designed to quantify NTHi, Hh and detect the *hpl* ORF. The targets used for discrimination of Hh (*hypD*) and NTHi (*siaT*) have previously been described and validated (20). For detection of the *hpl* ORF, primers were designed based on the *hpl* ORF of Hh-BW1 (GenBank MN720274) (315). The FAM, HEX and TET channels were used for simultaneous fluorescence detection of *siaT*, *hypD* and *hpl*, respectively. Primer and probe sequences are detailed in Table 4.1. Primer specificity was confirmed by discontiguous megaBLAST analysis and PCR of a panel of *Haemophilus* spp. and multiple genera representing common upper respiratory flora. PCR assays were extensively optimised and evaluated for detection/quantification limits in triplex format.

Table 4.1 | Summary of primer and LNA probe sequences, and expected amplicon size for the *hypD*, *siaT* and *hpl* targets.

Primers and Probes	Sequence	Amplicon Size (bp)
***hypD* Forward**	5′- GGCAATCAGATGGTTTACAACG	187
***hypD* Reverse**	5′- CAGCTTAAAGYAAGYAGTGAATG	
***hypD* LNA-probe**	/5HEX/CCA+C+AA+C+GA+G+AATTAG/3IABkFQ/	
***siaT* Forward**	5′- AATGCGTGATGCTGGTTATGAC	138
***siaT* Reverse**	5′- AATGCGTGATGCTGGTTATGAC	
***siaT* LNA-probe**	/56-FAM/A+GA+A+GCAGC+A+G+TAATT/3IABkFQ/	
***hpl* Forward**	5′- TATTCCTAATGATCCCGCT	120
***hpl* Reverse**	5′ - TCTTTTTTCGCTACCCCT	
***hpl* LNA-probe**	/5Cy5/AT+CCATTTA+TCGG+CACGTTCT/3IAbRQSp/	

PCRs were performed using the CFX96 TouchTM real-time PCR system (Bio-Rad) in 96-well optical plates. Polymerase activation was performed at 95 °C for 3 min, followed by 40 amplification cycles of denaturation at 95 °C for 15 s, and annealing at 62 °C for 1 min. Each reaction contained 0.25 µM of *hypD*, *siaT* and *hpl* primers, 0.1 µM LNA probes, 1× PrimeTime master mix (Integrated DNA Technologies) and 5 µL gDNA and molecular-grade water, to a total volume of 20 µL. Template gDNA was prepared by a thermal extraction protocol and tested in duplicate. Each run included a positive control for the *hpl* ORF (Hh-BW1), negative control (*H. parainfluenzae* ATCC 7901), no-template control and 10-fold dilutions of a standard containing 2×10^{-8} ng NTHi ATCC 49247 and Hh ATCC 33390 gDNA. Quantification of NTHi and Hh was expressed as genome equivalents (GE) calculated from the standard, as previously described (20). Bacterial quantification from thermally extracted gDNA was validated against conventional bacterial quantification by optical density and colony counts.

Complete details of PCR primer design, assay optimisation and gDNA extraction protocol evaluation are available in supplementary material (Supplementary Methods S4.6.1).

4.4.4. Competition assays

Short-term broth competition

Culture mixes were prepared by adding 100 µL of haem-replete preparations of a single Hh strain (Hh-BW1, Hh-BW1$^{hpI\text{-}KO}$, Hh-RHH122, Hh-NF4, Hh-NF5 or ATCC 33390) together with 100 µL of NTHi ATCC 49247 to 5 mL pre-warmed sTSB containing 0.0, 0.9, 3.8 or 15.0 ug mL^{-1} porcine haematin. Broths containing single strains were also prepared in parallel to determine baseline growth. Broths were incubated aerobically on an incubator shaker at 37 °C (220 RPM) for 16 h. At different time intervals, aliquots of 50 µL were taken for thermal gDNA extraction and subsequent triplex PCR quantification of GE. Aliquots of 500 µL were taken at 8 h for quantification of *hpI* expression. Purity of broth growth was checked by plating on CA after 16 h incubation.

Statistical comparisons were made between strains grown with a competitor and baseline growth by calculating the change in the number of cells per hour (growth rate) using the following formula:

$$ln\frac{N_t}{N_0} = \alpha(t - t_0)$$

where N_t is the number of cells (measured as GE) at time t, N_0 is the number of cells at time zero (t_0), and α is the growth rate where units are determined by the units of t.

Fitness assay

Culture mixes were prepared by adding 100 µL of haem-replete preparations of each of the Hh strains and 100 µL of NTHi (ATCC 49247, ATCC 49766 or NCTC 11315) to 5 mL of pre-warmed sTSB containing 0.0, 0.9, 3.8 or 15.0 µg mL^{-1} porcine haematin. Broths were incubated aerobically on an incubator shaker at 37 °C (220 RPM) for 12 h prior to subculture (200 µL) in fresh sTSB (2 mL) containing the same concentration of haem as the inoculum. The process of 12 hourly incubation followed by subculture into fresh broth was repeated until 6 days had elapsed. After each 12 h incubation, aliquots of 50 µL were taken for boiled gDNA extraction and subsequent triplex PCR quantification of GE. Purity of broth growth was confirmed by plating on CA after each 12 h incubation. Fitness of NTHi at each time point was determined using the following equation (361):

$$w = \frac{ln(\frac{A_t}{A_{t_0}})}{ln(\frac{B_t}{B_{t_0}})}$$

where w is fitness, A and B are the population sizes of the two competitors, subscripts t_0 and t indicate the initial and final time points in the assay. Growth after the first 12 h culture was used as baseline for fitness determination (t_0).

4.4.5. Expression analysis

RNA extraction, purification and quantification

Aliquots taken from broth growth were immediately added to two volumes of RNAprotect Bacteria Reagent (Qiagen) for immediate stabilisation of bacterial RNA. Stabilised aliquots were normalised to an OD_{600} of 0.05 (approximately 5×10^7 cells), pelleted by centrifugation for 10 min at 5000 × g and stored at -20 °C overnight. Bacterial lysates were prepared by resuspending pellets in 100 µl TE buffer (30mM Tris-Cl, 1 mM EDTA, pH 8.0) containing 15 mg mL^{-1} lysozyme and 20 µL proteinase K, vortexed and incubated at room temperature in an incubator shaker (1000 RPM) for 1 h. Following addition of 350 µL buffer RLT, samples were vortexed and centrifuged at 20000 × g for 2 min. The supernatant was purified following the manufacturers protocol for RNeasy Plus Mini Kit, which was semiautomated by the QIAcube (Qiagen). The iScriptTM cDNA Synthesis Kit (Bio-Rad) was used to produce cDNA for subsequent PCR. The validated triplex PCR was used to determine expression of *hpl* ORF in Hh strains, using *hypD* as the housekeeper gene.

Expression validation

Expression analysis was employed to determine baseline expression and suitability of prospective competitive test conditions for *hpl* expression. Given the kinetics of bacterial growth and the haem-binding capacity of Hpl, time and haem availability were targeted as factors that may influence *hpl* expression. The *hypD* target was selected as a potential housekeeper gene and validated for test conditions.

To validate expression analysis, haem-replete preparations of Hh-BW1 and the Hh-BW1$^{hpl-\,KO}$ (100 µL) were added to 5 mL pre-warmed sTSB containing either 0.0 or 15.0 µg ml^{-1} of porcine

haematin. Broths were incubated for 8 h, and aliquots of 500 µL were removed for RNA extraction and purification at 0, 4 and 8 h. Following validation, baseline *hpl* expression was quantified for isolates Hh-BW1, Hh-RHH122, Hh-NF4 and Hh-NF5 relative to Hh-BW1$^{hpl–\ KO}$ from 8 h growth in sTSB without porcine haematin.

4.4.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism V7.04, 2017. Statistical significance was determined by comparison of growth data (growth rate or fitness) between strains grown with a competitor and baseline growth. Data were tested for normality using the Shapiro–Wilk test, followed by a two-way ANOVA with Dunnett's multiple comparison test. Expression ratios and statistical significance were calculated with 2000 iterations by the Relative Expression Software Tool (REST; v 1.0, 2009) (362, 363).

4.5. Conclusions

Previously, we identified an uncharacterised haemophore (designated Hpl) produced by Hh which was able to inhibit NTHi growth by haem starvation (315). Here, we aimed to further test the inhibitory capacity of Hh-Hpl+ strains by direct *in vitro* competition with NTHi, for the purpose of determining their probiotic potential. These results provide a strong link between the NTHi-inhibitory phenotype, *hpl* expression and favorable outcomes during competitive growth with NTHi *in vitro*. Thus, Hh-Hpl+ strains exhibit promising probiotic potential against NTHi colonisation in the upper respiratory tract. Reduction or elimination of NTHi carriage from the upper respiratory tract proposes an effective means of limiting migration and subsequent infection in susceptible individuals. However, significant further investigation is required to determine if the inhibitory capacity demonstrated in this study extends to more complex models of NTHi colonisation and infection, such as cell culture systems and animal models. Further, studies investigating the safety profile of Hh-Hpl+ and their ability to colonise the host are also essential to determine if these strains pose as viable probiotic candidates.

4.6. Supplementary Materials

4.6.1. Supplementary methods

Triplex real-time PCR assay design, optimisation and validation

A triplex PCR assay was designed to detect and quantify NTHi, Hh and the *hpl* ORF. The targets used for discrimination of Hh (*hypD*) and NTHi (*siaT*) have previously been described and validated (364). For detection of the *hpl* ORF, primers were designed based on the *hpl* ORF of Hh-BW1 (GenBank MN720274) (365). Primer specificity was confirmed using discontiguous mega-BLAST analysis performed across 115 complete and 862 draft genome assemblies for *Haemophilus spp.* available from Genbank. A nonredundant nucleotide (nr/nt) collection BLAST search was also conducted to determine amplicon specificity in non-Haemophilus genomes.

Following optimisation in singleplex format, the three assays were merged into triplex format. Annealing temperature was optimised using an 8-step temperature gradient ranging from 53-63 °C. Specificity of amplicons was determined by gel electrophoresis and the optimal temperature was selected based on highest yield of amplicons of the correct size in the absence of non-specific amplification. PCR specificity for NTHi, Hh and *hpl* was determined using 13 Hh strains with varying *hpl* ORF sequence similarity to BW1, as previously defined (365), 13 *H. influenzae* strains and 9 other genera representing common upper respiratory tract flora (Supplementary Table S4.2).

Reaction efficiency of triplex reactions was determined using 10-fold dilutions of control strains over the range of 2 to 2×10^{-8} ng (Hi ATCC 49247, Hh ATCC 33390 and Hh-BW1). Limits of quantification (LoQ) values were determined for *hypD* and *siaT* targets in triplex format based on the following criteria: (i) replicates at a given dilution with a cycles to threshold (Ct) standard deviation (σ) of ≥0.8 were considered to exceed the LoQ; (ii) one or more amplification failures was deemed an LoQ failure. The upper LoQ value was not determined due to the unlikelihood of encountering such high-pathogen gDNA concentrations in clinical specimens. The lower limit of detection was also determined for the *hpl* target and defined from linearity data (Supplementary Figure S4.1).

Boiled DNA extraction protocol

Bacterial suspensions were heated to 99 °C for 10 min in a heat block with shaking (1000 rpm). The tube was vented and vortexed for 10 s followed by a further 10-min incubation at 99 °C. Boiled suspensions were spun for 10, 000 x g for 10 min in a benchtop microcentrifuge. The supernatant was diluted 1:5 in molecular grade water for use in PCR reactions. Given the instability of gDNA extracted by this method, PCR was conducted immediately or stored at 4 °C for no more than 2 h before use.

Validation of boiled gDNA extraction method

A series of 10-fold dilutions ($10^0 – 10^{-10}$) were made made from a heavy suspension (OD_{600} =3.0) of either Hh-BW1 or NTHi ATCC 49427 in TSB. To detect the presence of PCR inhibitors, each dilution was tested in duplicate, and a non-linear regression was generated. A slope between -3.1 and -4.1 (equivalent to amplification efficiency of 75-110%) and a correlation coefficient >0.98 was considered acceptable (366).

To test the accuracy of quantifying both targets simultaneously, each combination of NTHi and Hh dilutions in the range of 10^0-10^{-6} were mixed (50:50) prior to extraction and subsequent PCR. This produced varying proportions of NTHi and Hh to mimic possible scenarios of co-culture.

To compare OD_{600} readings and GE, a growth curve experiment was conducted where 110 µL aliquots were taken from a 5 mL culture in TSB every hour for 8 h and at 18 and 28 h. The OD_{600} of each aliquot was measured in a 96-well microtiter plate (Infinite 200 PRO, Tecan Life Sciences). Of this aliquot, 10 µL was used to produce dilutions for quantitation by colony count on CA and 50 µL was taken for gDNA extraction by boiling.

4.6.2. Supplementary results

Validation of triplex real-time PCR

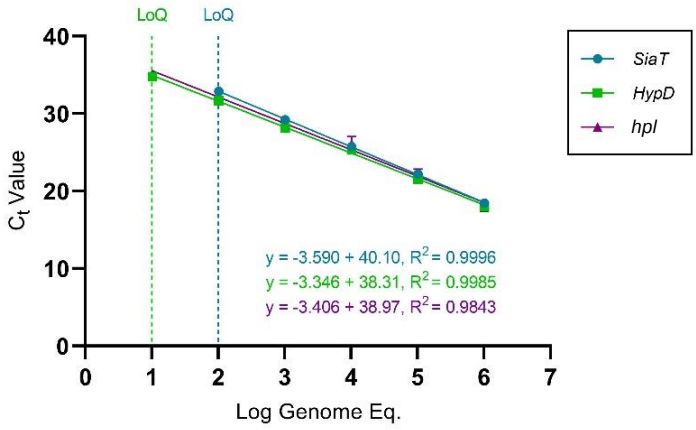
In silico specificity for the *hpl* amplicon revealed 97-100% primer and probe nucleotide sequence identity for 26 of 61 complete or draft Hh genomes available in Genbank. Sequence similarity to the *hpl* amplicon was detected in 20 *H. influenzae* genomes out of 757 complete

and draft genome assemblies. However, all alignments contained a minimum of 4 SNPs in the reverse primer and Taqman probe and did not contain any sequence homology with the forward primer. The *hpl* ORF was also detected in 3 genome assemblies available for *Haemophilus quentini*, which was expected based on previous analysis of the *hpl* ORF (365). Despite close relatedness to Hh, isolation of this strain has only been described in the genitourinary tract so is unlikely to be co-isolated from respiratory specimens (367). Comparison of 11 previously sequenced *hpl* ORFs (365) to these databases yielded the same results, indicating high sensitivity to known *hpl* sequence variants (ranging from 85-100% homology to BW1-Hpl). PCR of these isolates, and additional *H. haemolyticus*, *H. influenzae* and common upper respiratory tract colonisers confirmed specificity for each target.

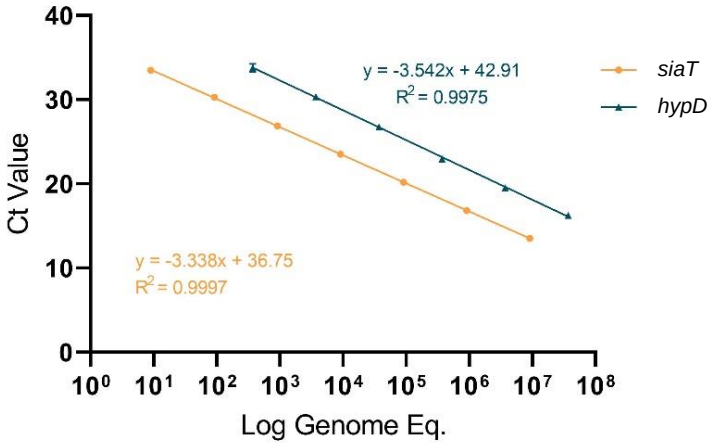
Quantification by PCR of boiled DNA is comparable to optical density

Serial dilutions did not reveal the presence of PCR inhibitors as regression parameters produced amplification efficiency of 99.37% and 91.57% for the *siaT* and *hypD* targets, respectively (Supplementary Figure S4.2). Similarly, amplification efficiencies did not differ between species and their measurement was not affected when present in dramatically different, or similar ratios (Supplementary Figure S4.3). Growth patterns generated by OD_{600} could be replicated by PCR using gDNA extracted by boiling. This was observed visually and by regression statistics directly comparing OD_{600} to GE (Supplementary Figure S4.4).

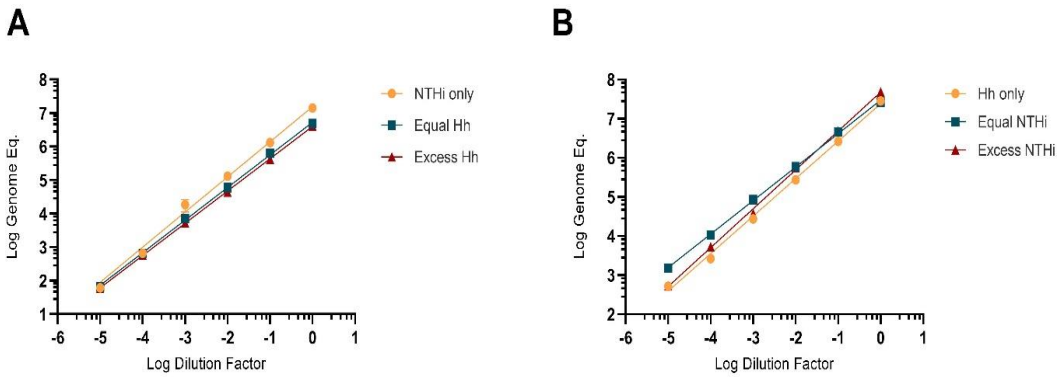
4.6.3. Supplementary Figures and Tables



Supplementary Figure S4.1 |PCR efficiency and measure of LoQ and LoD. Shows PCR C_t values for the *siaT*, *hypD* and *hpl* targets measured from serial dilutions of *H. influenzae*, Hh-*hpl*$^-$ and Hh-*hpl*$^+$ control strain gDNA over the range of 2 to 2 × 10^{-8} ng. Data points are represented as mean +/- SEM of two separate experiments, each conducted in duplicate.



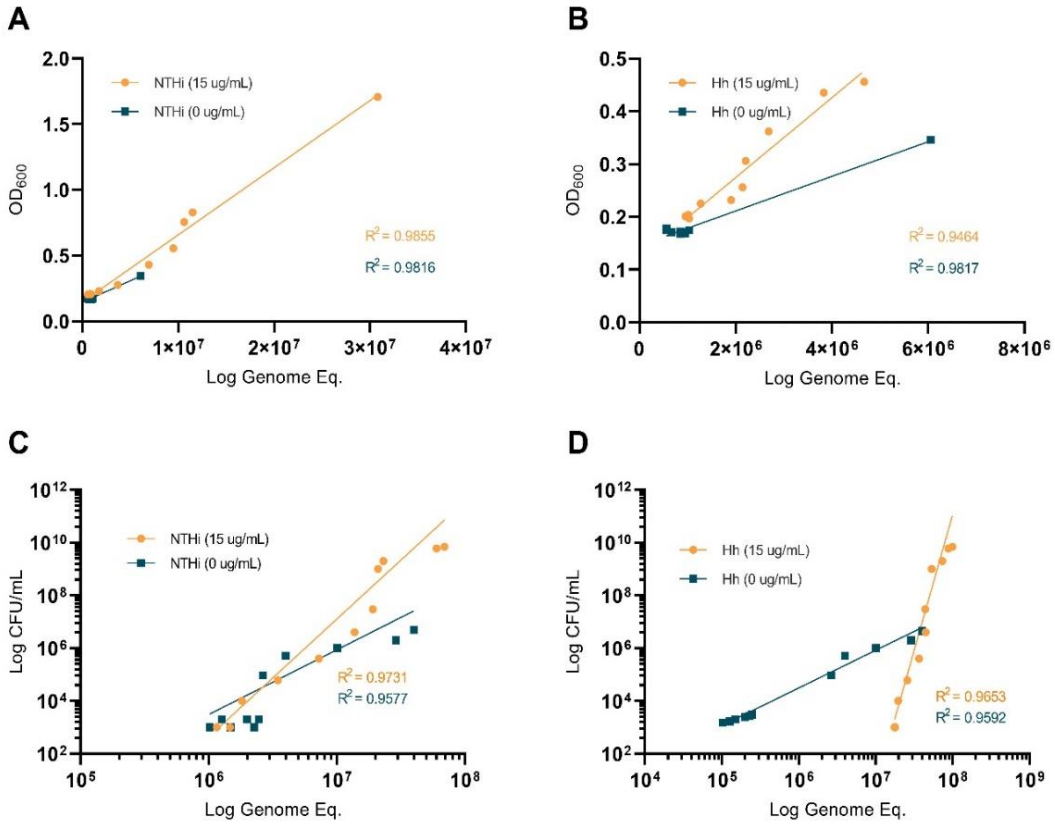
Supplementary Figure S4.2 |Detection of PCR inhibitors. Shows PCR C_t values for the *siaT* and *hypD* targets measured from serial dilutions of DNA extracted by boiling. Data points represented as mean +/- SEM of two separate experiments, each conducted in quadruplicate.



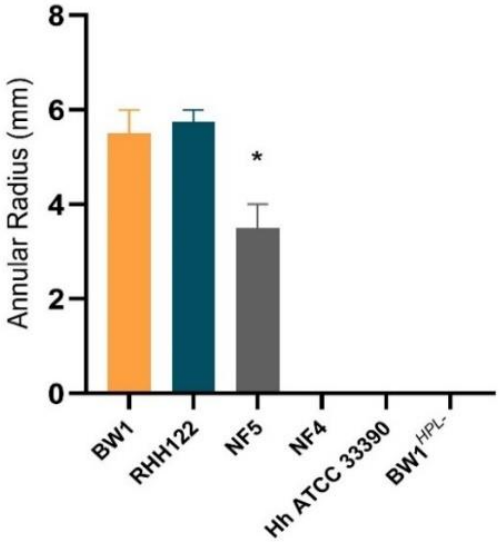
Supplementary Figure S4.3 |Extraction efficiency. Shows calculated genome equivalents from real-time PCR for **(A)** NTHi and **(B)** Hh measured from serial dilutions either alone or spiked with an equal density, or excess density of Hh or NTHi, respectively. Data points represented as mean +/- SEM of two technical replicates. Nonlinear regression model determined there is no significant difference between slopes in any case ($p > 0.05$).

Supplementary Table S4.1 | Bacterial strains used for triplex PCR specificity testing. In the case of Hh, nucleotide sequence similarity to the *hpl* ORF of Hh-BW1 (model Hh-Hpl+) is shown.

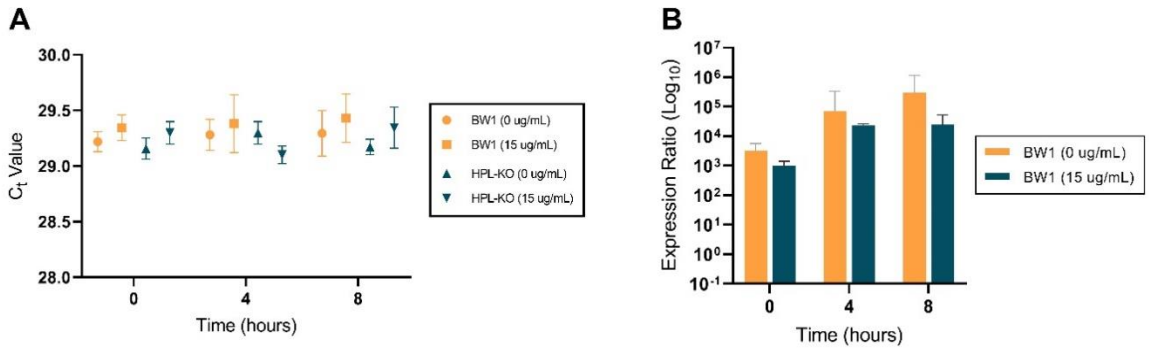
Strains	Species	Similarity to BW1-*hpl* (%)
BW1	*H. haemolyticus*	100
RHH122	*H. haemolyticus*	100
BW15	*H. haemolyticus*	100
BW18	*H. haemolyticus*	100
NF4	*H. haemolyticus*	100
NF5	*H. haemolyticus*	100
BW5	*H. haemolyticus*	99
CF14	*H. haemolyticus*	96
L19	*H. haemolyticus*	85
NF6	*H. haemolyticus*	96
NF11	*H. haemolyticus*	96
ATCC 33390	*H. haemolyticus*	0
NF1	*H. haemolyticus*	0
UTAS252	*H. influenzae*	-
L15	*H. influenzae*	-
CF31	*H. influenzae*	-
CF34	*H. influenzae*	-
CF48	*H. influenzae*	-
Ci2	*H. influenzae*	-
Ci3	*H. influenzae*	-
Ci5	*H. influenzae*	-
ATCC 10211	*H. influenzae*	-
ATCC 43163	*H. influenzae*	-
ATCC 49247	*H. influenzae*	-
NCTC 11315	*H. influenzae*	-
NTCT 4560	*H. influenzae*	-
NCTC 8618	*S. salivarius*	-
UTAS 8	*S. pyogenes*	-
UTAS14	*S. pneumoniae*	-
UTAS 203	*Neisseria sp.*	-
UTAS 387	*S. aureus*	-
ATCC 14990	*S. epidermidis*	-
ATCC 60193	*C. albicans*	-
ATCC 7901	*H. parainfluenzae*	-
UTAS 412	*H. parahaemolyticus*	-



Supplementary Figure S4.4 |Comparison of genome equivalents and OD_{600} and colony counts (CFU mL^{-1}). Growth of NTHi and Hh in TSB over 16 h under haem-replete (15 ug mL^{-1}) or haem-starved (0 ug mL^{-1}) conditions as measured by OD_{600} in 96-well plates, or by calculation of GE from PCR of boiled gDNA. Linear regression models show good correlation between quantification of NTHi, and Hh by OD_{600} (**A, B**) and colony counts (**C, D**). Data points represented as mean +/- SEM of duplicate extractions, measured by PCR in duplicate, over two separate experiments.



Supplementary Figure S4.5 | Baseline NTHi-inhibitory activity of *hpl* PCR positive Hh strains. Zones of inhibition, measured as annular radius (mm) of indicator strains (NTHi ATCC 49427) produced by ammonium precipitate extracts from Hh strains containing the *hpl* ORF (standardised for growth density). Mock extractions were performed on Hh ATCC 33390 and Hh-BW1$^{hpl\text{-}KO}$ (notated BW1^{hpl-}) as negative controls. Data points represented as mean +/- SEM of zones produced across the two indicators strains, from duplicate extractions.



Supplementary Figure S4.6 |Validation of *hypD* (housekeeper gene) and *hpl.* A) Expression of *hypD* by Hh-BW1 and Hh-BW1$^{hpl\text{-}KO}$ (notated as Hpl-KO) during growth under haem-replete and haem-starved conditions. **B)** Hh-BW1 expression of *hpl* during growth under haem-replete and haem-starved conditions. Data points represented as mean +/- SEM of two separate experiments, each with duplicate RNA extractions.

Chapter 5 | Manuscript 2

Haemophilin-producing strains of *Haemophilus haemolyticus* protect respiratory epithelia from NTHi colonisation and internalisation

Brianna Atto [1], Dale Kunde [1], David A Gell [2] and Stephen Tristram [1],

[1] *School of Health Sciences, University of Tasmania, Launceston, Australia*

[2] *School of Medicine, University of Tasmania, Hobart, Australia*

The content of this chapter is reproduced as published in Pathogens MDPI as an original research article (Submitted 3 December 2020; Accepted: 28 December 2020; Published: 1 January 2021), however the text has been reformatted to meet the thesis submission requirements.

5.1. Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is a significant respiratory tract pathogen responsible for infections that collectively pose a substantial health and socioeconomic burden. The clinical course of these infections is largely dictated by NTHi interactions with host respiratory epithelia, and thus, approaches that disrupt colonisation and invasion may have significant therapeutic potential. Survival, successful host–cell interactions, and pathogenesis are reliant on the ability of NTHi to sequester host-derived haem. Previously, we demonstrated the therapeutic potential of exploiting this haem-dependence using a closely related competitor bacterium, *Haemophilus haemolyticus* (Hh). Hh strains capable of producing the novel haem-binding protein haemophilin (Hpl) possessed potent inhibitory activity by restricting NTHi access to haem in a broth co-culture environment. Here, we extend this work to cell culture models that more closely represent the human respiratory epithelium and show that Hh strains with high levels of *hpl* expression protect epithelial cell line monolayers against adhesion and invasion by NTHi. Inhibitory activity was dependent on the level of Hpl production, which was stimulated by NTHi challenge and nasopharyngeal cell exposure. Provided these protective benefits translate to *in vivo* applications, Hpl-producing Hh may have probiotic utility against NTHi infections by inhibiting requisite nasopharyngeal colonisation.

5.2. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is commonly associated with nasopharyngeal colonisation in healthy children and adults but is considered an important opportunistic pathogen in predisposed individuals (22). Common infections include otitis media (OM) in children, pneumonia in the elderly, and exacerbations of underlying lung diseases, such as chronic obstructive pulmonary disease (COPD) (2). Despite the significant health and socioeconomic burdens associated with NTHi infections, there are currently no effective vaccination strategies available, and the treatment of existing infections is compromised by the rapid development of resistance to first- and second-line antibiotics (2, 97, 106, 368-370).

The course of NTHi infection is largely dictated by bacterial interactions with host respiratory epithelial cells. Attachment to respiratory epithelial cells in the nasopharynx is a pre-requisite for colonisation and subsequent bacterial migration to, and infection at other sites in the respiratory tract (155). Although the mechanisms that lead from colonisation to infection are poorly understood, a high

density of NTHi carriage is associated with an increased risk of development of OM (17, 148, 371) and a clinically significant increase in respiratory symptoms in COPD, even in the absence of a clinical exacerbation (372). Following successful attachment, NTHi has demonstrated the ability to invade nasopharyngeal, alveolar, bronchial, and laryngeal cell lines *in vitro* (155, 291, 373-376). Intraepithelial NTHi are protected from bactericidal antibodies and high antibiotic concentrations (155, 377), which are characteristics shown to potentiate infection severity and treatment failure, resulting in bacterial persistence and recurrent infections in COPD airways and the middle ear (169, 194, 373, 378, 379). As such, intracellular NTHi are considered a major determinant of pulmonary morbidity in COPD patients (380) and may contribute to the prolonged and intractable clinical course of acute OM (373).

Successful colonisation and survival within the host relies heavily on the ability of NTHi to acquire and utilise iron-containing haem (124, 138, 373, 381, 382). Although NTHi lacks the necessary enzymes required for *de novo* haem synthesis, it possesses a ferrochelatase that inserts iron into protoporphyrin IX to form haem (383). Thus, NTHi can fulfil haem requirements by acquiring iron in the presence of protoporphyrin or by scavenging haem bound to host haemoproteins. For this reason, NTHi expresses a complex and redundant set of haem acquisition pathways to overcome host nutritional immunity in an environment considered to have an inherently low abundance of haem (215).

As well as being an absolute growth requirement, there is also evidence that haem-scavenging machinery plays a role in pathogenesis by the modulation of virulence factors, antimicrobial resistance, immune evasion, and host–cell interplay (143, 159, 167, 384). Haem-acquisition/utilisation genes have a higher prevalence in NTHi isolated from OM infection, compared to colonising throat strains (124) and are upregulated during infection of the chinchilla middle ear (385). Deletion of these genes reduces the bacterium's capacity to colonise and persist within the nasopharynx, resulting in an infection with attenuated disease severity and duration in animal models of OM and airway infection (67, 143, 159, 168). Similarly, an isogenic mutant of two haem-acquisition pathways was unable to sustain bacteraemia or produce meningitis in a rat model of invasive disease (168). Thus, restricting haem acquisition or utilisation is a potentially high-value target for the development of novel therapies for the prevention of colonisation with, or eradication of NTHi from the respiratory tract (242, 295). However, due to the highly redundant nature of these pathways, approaches that target multiple haem sources or acquisition pathways are likely required to induce sufficient nutritional starvation to elicit a therapeutic response (167).

Previously, we have shown that a closely related bacterium, *Haemophilus haemolyticus* (Hh), competes with NTHi for limited haem resources. Hh strains capable of producing the novel haem-binding protein haemophilin (Hpl) possessed potent inhibitory activity by restricting NTHi access to haem *in vitro* (295, 315, 386). These findings suggest that Hh strains capable of producing high levels of Hpl might disrupt NTHi association with airway epithelial cells and have potential as a probiotic that prevents the requisite nasopharyngeal colonisation stage of infection. Here, we explore this question using Hh strains with different levels of *hpl* expression in cell culture models of nasopharyngeal and lung epithelial cells.

5.3. Results

5.3.1. Cell monolayers pre-treated with Hpl-producing strains of Hh were protected from NTHi attachment and invasion

To investigate interactions of Hh and NTHi with epithelial cells, we used two cultured cell lines, D562 human pharyngeal epithelial cells and A549 human lung epithelial cells. Initial experiments were performed to determine the time course for the adhesion of each Hh or NTHi isolate to epithelial cells. Maximum attachment for all Hh strains was achieved after 4-h incubation (Supplementary Figure S5.1). In comparison, NTHi strains attached in greater numbers and required only 1 h or 2 h to reach maximum attachment to A549 and D562 cells, respectively (Supplementary Figure S5.1). Similar inter- and intra-species variation in adhesion to respiratory cells has been reported previously (291, 373, 375, 376). Hh and NTHi viability in both cell culture media was maintained for up to 8 h (Supplementary Figure S5.3), and A549 and D562 cell lines did not show any substantial changes in viability for up to 8-h post challenge with Hh or NTHi strains compared to media alone (Supplementary Figure S5.4).

Based on these experiments, epithelial cell monolayers were incubated with Hh strains (either Hpl-producing Hh, Hh-Hpl+; or Hh strains that do not produce Hpl, Hh-Hpl-) for 4 h prior to challenge with NTHi strains, and the number of Hh cells in the inoculum was set (Supplementary Table S5.1) to achieve approximately equal attachment of each Hh strain at the 4-h time point (Supplementary Figure S5.5). Following the pre-incubation with Hh, the cell cultures were challenged with a standard dose of NTHi for 1 h (A549 cells) or 2 h (D562 cells) and the number of Hh and NTHi attached to the surface or internalised within the epithelial cells was determined by colony enumeration on appropriate selective media. Cell monolayers pre-

treated with Hh-BW1(Hpl+) or Hh-RHH122(Hpl+) experienced a significant 78.1–99.1% and 98.3–99.5% decrease in attachment of all four NTHi strains tested in the case of A549 (Figure 5.1A, $p < 0.0001$) and D562 (Figure 5.1B, $p < 0.0001$) cells, respectively. Pre-treatment with Hh-NF5(Hpl+) significantly inhibited the attachment of all NTHi strains to D562 cells (82.0–95.6%, $p < 0.001$) but offered limited or no protection in the case of A549 cell monolayers. Pre-treatment with Hh-NF4(Hpl-), Hh-NF1(Hpl-), or Hh-BW1$^{Hpl\text{-}KO}$ did not significantly affect NTHi attachment to either cell line ($p > 0.05$).

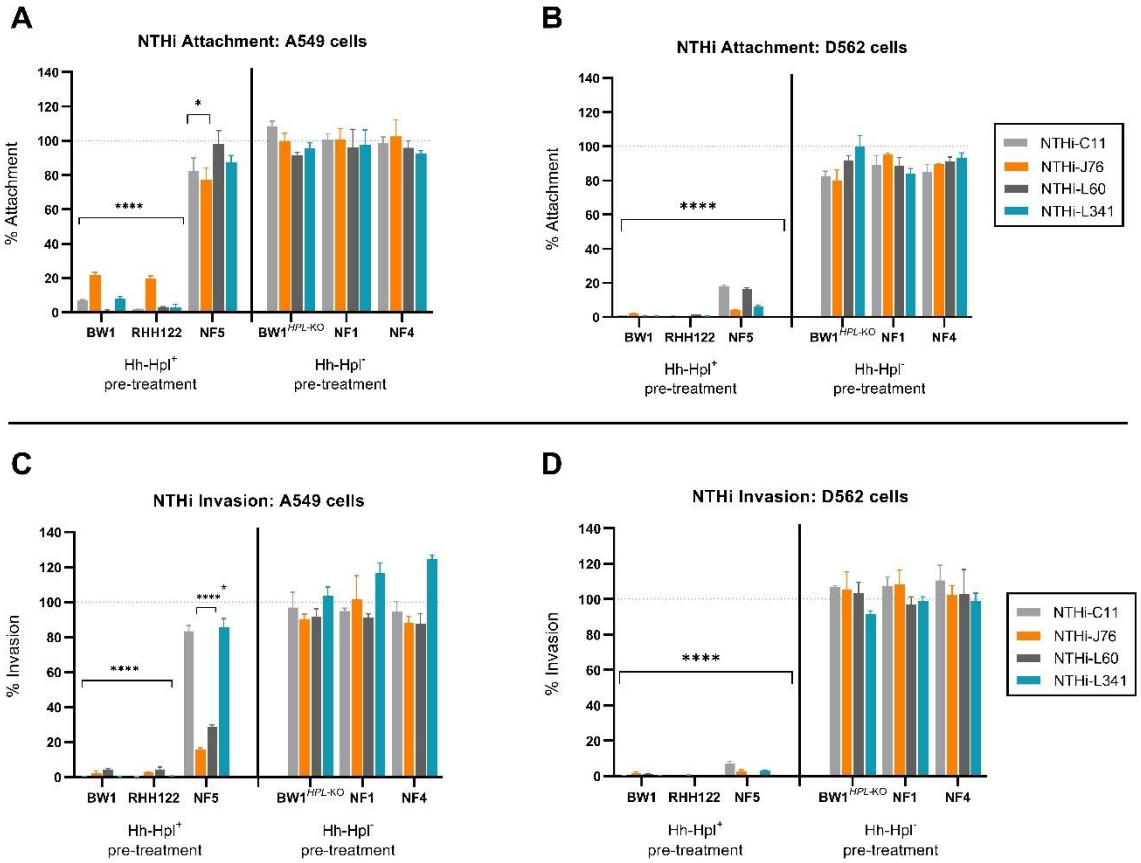


Figure 5.1 | Nontypeable *Haemophilus influenzae* (NTHi) attachment and invasion of A549 and D652 cells post *Haemophilus haemolyticus* (Hh) treatment. The percent attachment of NTHi (compared to media control) to A549 (**A**) and D562 (**B**) cell monolayers post 4-h pre-treatment with Hpl-producing Hh (Hh-Hpl+) or Hh strains that do not produce Hpl (Hh-Hpl-). Percent of internalised NTHi (compared to media control) after exposure to A549 (**C**) and D562 (**D**) cell monolayers post 4-h pre-treatment with Hh-Hpl+ or Hh-Hpl-. Error bars represent the ±SEM of three biological replicates, measured triplicate: *p<0.05, **** $p < 0.0001$. The annotated level of statistical significance is relative to the matching baseline attachment/invasion of each NTHi strain.

In the absence of Hh pre-incubation, strains NTHi-C11 and NTHi-L341 demonstrated strong invasive capacity, whereas other NTHi and Hh strains used in this study were considered non-invasive (Supplementary Figure S5.2), which is defined as the colony-forming units (CFU) of internalised bacteria accounting for less than 1% of the original inoculum (74, 375). After 4 h pre-incubation with Hh-BW1 or Hh-RHH122, the invasive capacity of all NTHi, including highly invasive NTHi-C11 and NTHi-L341 strains, was significantly reduced (Figure 5.1C, D) to levels around that of the non-invasive strains (data not shown). Pre-treatment with Hh-NF5(Hpl+) inhibited NTHi-C11 and NTHi-L341 invasion of one cell line (D562), whereas pre-treatment with Hh-Hpl- strains (Hh-NF4, Hh-NF1, or Hh-BW1$^{Hpl\text{-}KO}$) did not affect NTHi invasion. These experiments demonstrate considerable variation in the ability of different Hh isolates to inhibit the invasion of model epithelium cell lines by NTHi.

5.3.2. Small treatment doses of Hh-BW1 or Hh-RHH122 were sufficient to inhibit NTHi interactions with model epithelium cell lines

To investigate the relative protective potency of Hh-Hpl+ strains with different levels of *hpl* expression, cell monolayers were pre-treated with varying doses of Hh-BW1, Hh-RHH122, Hh-NF5, or Hh-BW1$^{Hpl\text{-}KO}$ (control) prior to NTHi challenge. NTHi-C11 was selected as the competitor strain based on its overall superior attachment (Supplementary Figure S5.1) and invasive capacity (Supplementary Figure S5.2). Pre-treatment with Hh-BW1 or Hh-RHH122 at an Hh:NTHi ratio of 0.1:1 resulted in a 90.0–95.2% reduction in NTHi attachment to A549 cells (Figure 2A, $p < 0.0001$) and a reduction of 90.0 – 90.6% to D562 cells (Figure 5.2B, $p < 0.0001$). At the highest treatment dose (Hh:NTHi ratio 1000:1), pre-treatment with Hh-BW1 and Hh-RHH122 resulted in a 99.0 ± 0.2% inhibition of NTHi attachment compared to pre-treatment with media alone. Pre-treatment with Hh-NF5 required approximately 10-fold higher treatment loads to achieve the same degree of NTHi inhibition as Hh-BW1 or Hh-RHH122 at the low and high Hh:NTHi treatment ratios. A similar pattern of inhibition was seen for NTHi invasion (Figure 5.3A, B). NTHi invasion of cell monolayers was decreased by 92.3–96.3% following pre-incubation with the lowest doses of Hh-BW1 or Hh-RHH122, and no internalised NTHi were found at the highest Hh:NTHi ratio (1000:1). Preincubation with Hh-NF5 gave lower protection against NTHi invasion than Hh-BW1 or Hh-RHH122 at all except the lowest challenge ratio. Except for the highest treatment load (Hh:NTHi ratio 1000:1), no significant reduction in attachment or invasion of NTHi was observed in cell monolayers treated with Hh-BW1$^{Hpl\text{-}KO}$.

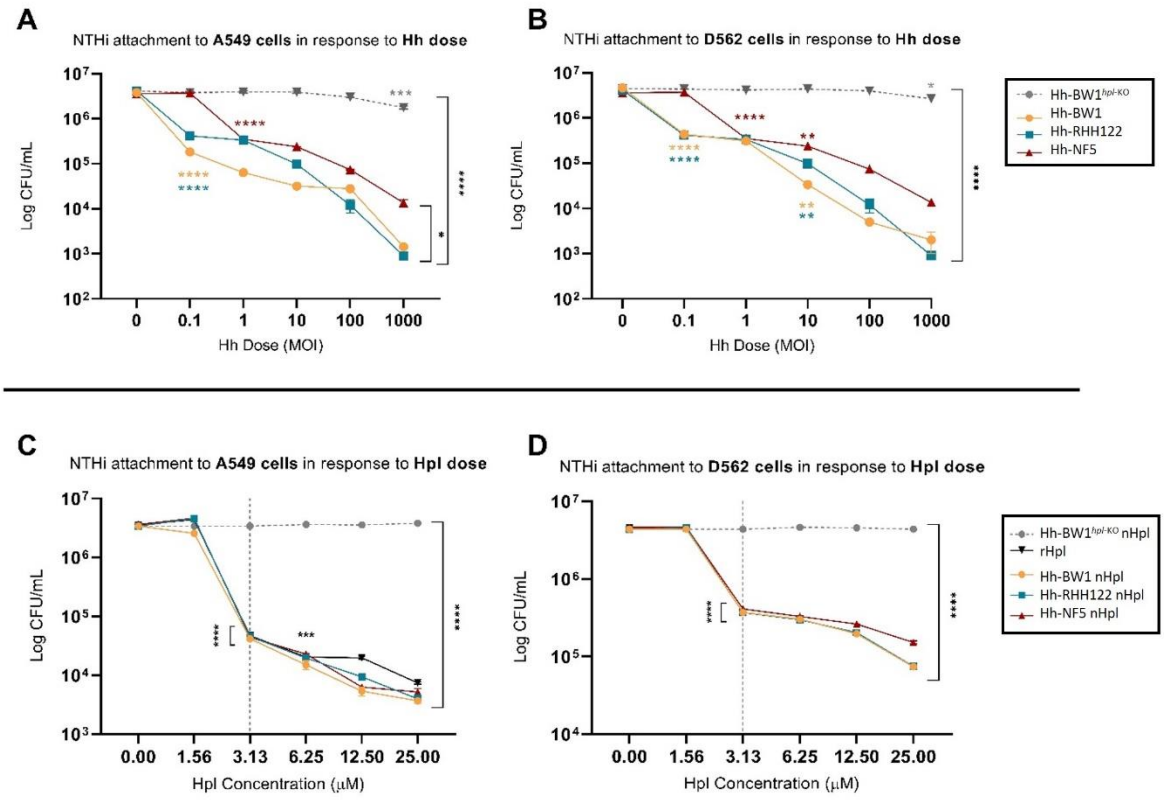
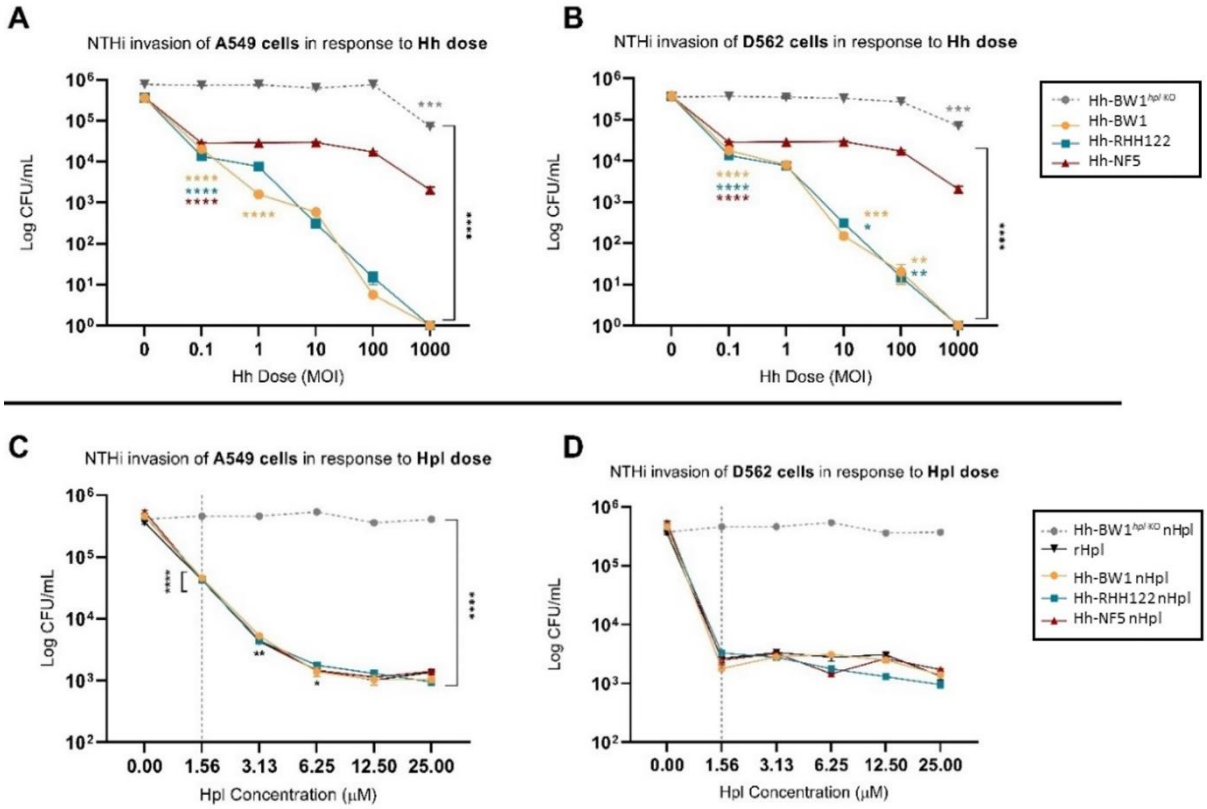


Figure 5.2 | NTHi attachment to A549 and D652 cells treated with varying doses of Hpl-producing Hh or purified Hpl. Colony counts (CFU mL^{-1}) of NTHi attached to A549 (**A**) and D562 (**B**) cell monolayers post 4-h pre-treatment with varying doses of Hpl-producing Hh or 2-fold increasing concentrations of native (nHpl) or recombinant Hpl (rHpl) (**C**,**D**). Error bars represent the ±SEM of three biological replicates. * $p < 0.05$, ** $p < 0.005$ *** $p < 0.001$, **** $p < 0.0001$.

5.3.3. Purified Hpl inhibits NTHi interactions with model epithelial cell lines

In the above experiments, Hh strains previously shown to be high-level *hpl* expressors were more effective at inhibiting NTHi attachment and invasion than other Hh, and the loss of this protective effect in Hh-BW1$^{Hpl\text{-}KO}$ strongly implicates a mechanistic role for Hpl. However, other factors, such as differential expression of cell surface adhesins and physical interactions between bacterial cells could also be important. To investigate a direct role for secreted Hpl, we challenged cell monolayers with NTHi after pre-incubation with recombinant Hpl (rHpl) or cell-free supernatants from Hh cultures. Cell-free supernatants were concentrated by ammonium sulfate precipitation and the concentration of native Hpl (nHpl) was determined by bioassay using purified rHpl as the standard (Supplementary Figure S5.6). NTHi attachment to cell monolayers was significantly reduced following treatment

with purified rHpl and supernatants (nHpl) from Hpl-producing strains (Hh-BW1, Hh-RHH122, and Hh-NF5) but not control extracts from Hh-BW1$^{Hpl\text{-}KO}$ (Figure 5.2C, D). The minimum concentration of Hpl required for a significant reduction in attachment was 3.1 µM ($p < 0.0001$). The NTHi invasion assay was more sensitive to Hpl, only requiring a concentration of 1.6 µM for significant inhibition (Figure 5.3C, D). Higher doses of Hpl reduced NTHi invasive capacity to a level similar to that of the non-invasive strains (data not shown) but, unlike treatment with Hpl-producing Hh bacteria, Hpl protein alone did not completely eradicate NTHi invasion. These experiments suggest that Hpl protein is sufficient to cause a substantial reduction in the adherence and invasion capacity of NTHi, but that additional inhibitory



mechanisms occur in the presence of Hh cells.

Figure 5.3 | NTHi internalisation of A549 and D652 cells treated with different doses of Hpl-producing Hh or Hpl. Colony counts (CFU mL^{-1}) of internalised NTHi in A549 (**A**) and D562 (**B**) cell monolayers post 4-h pre-treatment with varying doses of Hpl-producing Hh or 2-fold increasing concentrations of native (nHpl) or recombinant Hpl (rHpl) (**C**,**D**). Error bars represent the ±SEM of three biological replicates. * $p < 0.05$, ** $p < 0.005$ *** $p < 0.001$, **** $p < 0.0001$.

5.3.4. Expression of *hpl* is stimulated in response to D562 cell culture and NTHi challenge

To investigate whether *hpl* expression is altered following exposure of Hh strains to mammalian cultured cells or NTHi, we performed an analysis of *hpl* mRNA and Hpl protein levels following co-culture experiments. Baseline *hpl* expression in cell culture media was highest in Hh-BW1(Hpl+) and Hh-RHH122(Hpl+), approximately 10-fold lower in Hh-NF5(Hpl+) (Figure 5.4A), and absent in Hh-NF4(Hpl-) and Hh-NF1(Hpl-) (data not shown), which is consistent with findings in bacterial growth medium (315). Hpl mRNA and protein levels did not differ significantly between RPMI and MEM cell culture media (Figure 5.4A, B, $p < 0.05$). Final concentrations of Hpl produced in growth media by Hh-BW1(Hpl+) and Hh-RHH122(Hpl+) at baseline exceeded the minimum concentration required for NTHi growth inhibition, as determined by the agarose well diffusion assay (Figure 5.4B). By the same metric, Hh-NF5(Hpl+) supernatants contained sub-inhibitory concentrations of Hpl. Inhibitory activity was not detected from Hh-NF1(Hpl-), Hh-NF4(Hpl-) (data not shown), or Hh-BW1$^{Hpl\text{-}KO}$ supernatants. Following exposure to D562, but not A549 cell monolayers, *hpl* expression was stimulated in Hh-BW1, Hh-RHH122, and Hh-NF5, compared to expression in media alone ($p < 0.05$, Figure 5.4C). Hh-NF5(Hpl+), which had the lowest baseline level of *hpl* expression, experienced the highest increase in *hpl* mRNA levels upon exposure to D562 cell monolayers (11.59-fold), which is more than double that of the other strains ($p < 0.001$), and this was reflected in the largest increase of Hpl protein concentration to levels that were expected to inhibit NTHi growth (Figure 5.4D). In addition, *hpl* expression (at the RNA and protein levels) was further upregulated after NTHi challenge, compared to cell monolayers alone (Figure 5.4E, F, G, H). The degree of stimulation varied significantly between strains but was generally higher in response to the highly invasive NTHi-C11 than in NTHi-L60 ($p < 0.0001$).

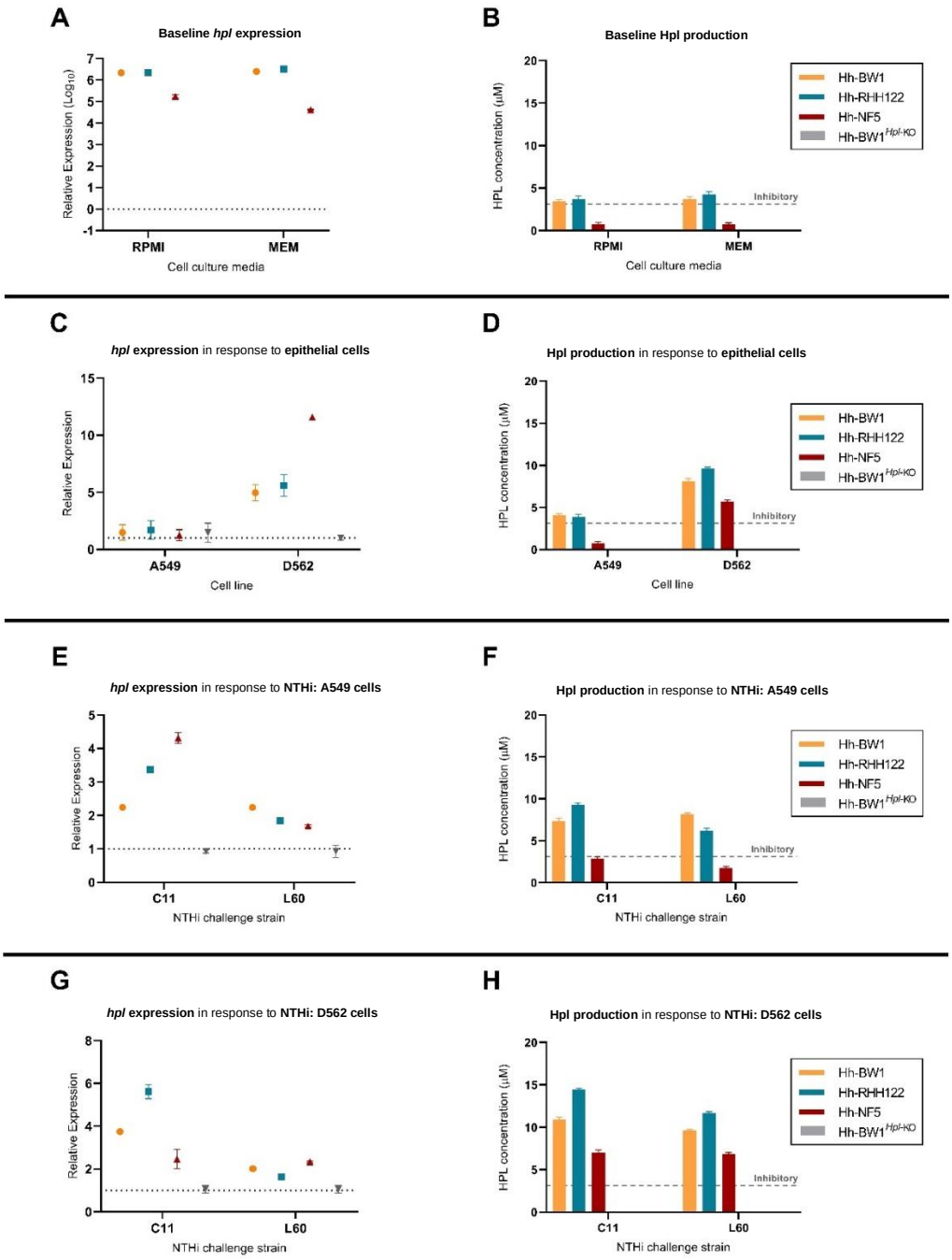


Figure 5.4 | Parallel *hpl* mRNA expression analysis and semi-quantification of Hpl protein production. Baseline expression of *hpl* mRNA and protein production, recovered from 1-h incubation in cell culture media (**A**, **B**) or attached to cell monolayers relative to Hh-BW1$^{Hpl\text{-}KO}$ (**C**, **D**). Expression of *hpl* and nHpl concentration measured from Hh attached to A549 (**E**, **F**) or D562 (**G**, **H**) cell monolayers following NTHi challenge. The minimum concentration of Hpl required to elicit NTHi-inhibitory activity during competition studies is marked on the y-axis by the grey "inhibitory" line. Data points are represented as mean ± SEM of three biological replicates, performed from duplicate mRNA extractions.

5.4. Discussion

We previously demonstrated the NTHi-inhibitory capacity of Hpl-producing Hh strains in a broth co-culture environment and proposed the probiotic utility of these strains against NTHi colonisation of the upper respiratory tract (386). In the current study, we have extended this work to cell line culture models that more closely represent the human respiratory epithelium and show that some Hh strains with high levels of *hpl* expression protect epithelial cell line monolayers against adhesion and invasion by NTHi.

Consistent with literature reports, Hh and NTHi host–cell interactions were highly time-dependant and demonstrated a high degree of inter- and intra-species variability (291, 373-376). In general, NTHi demonstrated a greater capacity to attach to and invade respiratory epithelial cell lines, compared to Hh. The superior attachment of NTHi has previously been reported (291) and may contribute to the bacterium's success as a competitor in the nasopharyngeal niche and as a pathogen (28, 387). Cell interactions were also significantly influenced by the cell line. All NTHi and Hh strains displayed an improved ability to attach and, in the case of invasive NTHi, invade D562 cells, compared to A549 cell monolayers. Differences in susceptibility to NTHi invasion have previously been observed in bronchial epithelial and lung cell lines *in vitro* (375), which may suggest an adaptive preference for nasopharyngeal cells. However, influence of the different media composition or characteristics of the carcinoma-derived cell lines used in this study cannot be ruled out. Hh has previously demonstrated the ability to invade and cause cytotoxicity in respiratory epithelial cells *in vitro*, which are features that are generally associated with pathogenesis and persistence (155, 291). However, none of the Hh strains tested in the current study demonstrated invasive capacity to either D562 or A549 cell lines. Long-term exposure to Hh (24 h) did result in a significant loss of viability in A549 cells, which is an observation that has been made previously but remains unexplained (291). The clinical significance or likely translation to the *in vivo* environment of the demonstrated *in vitro* cytotoxicity is unclear, particularly given the commensal nature of Hh. The lack of cytotoxicity observed in cell lines following treatment with purified Hpl suggests that Hpl is not a cause of cytotoxicity.

In this study, we used five isolates of Hh with differing abilities to inhibit the growth of NTHi in broth co-cultures (386). In these strains, the level of *hpl* expression correlates with the ability of the strain to inhibit adhesion and invasion of model epithelial cell lines by NTHi. Thus, Hh-BW1 and Hh-RHH122 strains with high-level *hpl* expression provided significant protection from NTHi attachment and reduced NTHi invasion to levels observed in non-invasive NTHi strains. Conversely, Hh-NF5, with relatively low *hpl* expression, only offered protection to D562 cells when pre-treated with larger

inoculation doses, and strains that lacked detectable *hpl* expression (Hh-NF1, Hh-NF4) offered no protection to either cell line. In addition, the absence of protection in cell monolayers treated with the insertional deletion mutant, Hh-BW1$^{Hpl\text{-}KO}$, suggests that Hpl plays a causative role in mediating NTHi-inhibitory activity. Purified recombinant Hpl also protected cell monolayers from NTHi adhesion and invasion. Whilst the maximum concentration of purified Hpl used (25 μM) was similar to the maximum level produced by Hh-BW1 and Hh-RH122 in the NTHi cell challenge conditions (≤15 μM), the level of protection conferred by Hh strains was always higher than with Hpl protein alone. One implication is that additional factors, such as the differential expression of surface adhesins and steric interactions between bacterial cells may also contribute to strain-specific differences in NTHi-inhibitory activity. A role, if any, for Hpl in regulating gene expression pathways involved in microbial competition or mammalian cell adhesion is a topic for future investigation. It should also be considered that prolonged production and high local concentrations of Hpl generated by adherent Hh could be more effective than the single bolus application of Hpl protein to the culture. Nevertheless, the experiments presented here identify Hpl as an important effector of the NTHi-inhibitory effect.

We have previously shown that Hpl exhibits NTHi growth-inhibitory activity owing to its capacity to bind haem in a form that is inaccessible to NTHi (386). Although the mechanism by which Hpl acts to protect model epithelial cell lines against invasion by NTHi is not explored in this study, it seems likely that this is also a result of limited haem availability to NTHi, resulting in growth inhibition (315, 386). NTHi lacks the necessary enzymes required for *de novo* haem synthesis, and the deleterious effects of haem starvation on NTHi are demonstrated by NTHi strains lacking either the HxuCBA, PE, SapA-BCDFZ, or HbpA-DppBCDF haem-acquisition systems. Compared to the wild type, these mutants had an attenuated ability to invade A549 airway epithelia or cause airway infection in a mouse model of lung infection, the degree of which was exacerbated when haem availability was restricted (143). The *in vivo* consequence of haem starvation was demonstrated by the decreased persistence in the chinchilla middle ear and nasopharynx by NTHi lacking the SapF-mediated haem-iron acquisition pathway (159).

The Hh-BW1 and Hh-RHH122 strains used in this study were originally identified on the basis of NTHi-inhibitory activity in cell-free supernatants obtained from Hh monocultures, suggesting a constitutive high expression of *hpl* in these strains. Here, we show that *hpl* expression is regulated by environmental conditions, including co-culture with mammalian cells and NTHi. Baseline expression levels were highest in Hh-RHH122 and Hh-BW1 and approximately 10-fold lower in Hh-NF5. These expression levels corresponded to levels of Hpl production that were close to the minimum required concentration for NTHi inhibition, or in the case of Hh-NF5, they were significantly lower. Hpl mRNA and

protein production levels were significantly stimulated during exposure to D562 cells, particularly in the case of Hh-NF5. This response was not replicated in A549 cells. This is particularly significant for Hh-NF5, which without stimulation appears to produce Hpl concentrations that do not exceed the minimum required concentration and translates into its inability to inhibit NTHi during association with A549 cells. The physical or chemical signal that elicits the upregulation of *hpl* remains to be investigated; candidates include iron/haem concentration and surface or secreted products from mammalian cells. Such mechanisms have been described in NTHi, where the type IV pilus, a mediator of adherence, colonisation, and *in vivo* persistence is upregulated in environments of low haem availability and was found to be stimulated by soluble factors released by respiratory epithelial cells (388).

The presence of NTHi was also found to be a significant stimulant of Hpl production, which is a response that varied between NTHi strain and Hh phenotype. Although there is no literature investigating the competitive haem acquisition between NTHi and Hh, there have been reports of siderophore-mediated interspecies competition for iron by other respiratory-associated microorganisms. Many bacterial species have the ability to utilise heterologous siderophores, shifting the cost of production to another organism and simultaneously sequestering iron away from the siderophore producer (389). *Pseudomonas aeruginosa* was shown to upregulate the iron-scavenging siderophore pyoverdine under conditions of low iron availability in response to competition imposed by *Burkholderia cenocepacia* (390) or *Candida albicans* (10).

The strong protective capacity elicited by some Hh strains, despite the superior attachment capacity of NTHi, suggests a potential probiotic activity. Although Hh has occasionally been reported as a pathogen of sterile sites in immunocompromised patients (350), there is convincing evidence that they are not opportunistic pathogens of the respiratory tract (351, 352, 391). In model epithelial cell lines, the significant inhibition of NTHi adherence and invasion was achieved with treatment doses of Hh strains Hh-BW1 and Hh-RHH122 that were 10-fold lower than the NTHi challenge. Based on these *in vitro* data, a translation to clinical significance can be speculated. The presence of healthy carriers of NTHi indicates that a complete eradication of NTHi is not necessary to prevent infection. Furthermore, higher NTHi nasopharyngeal carriage load is correlated with a large increase in susceptibility to OM *in vivo* (371, 392-394) and an increased severity of airway inflammation, exacerbations, and daily symptoms in COPD (362, 372). These *in vivo* observations suggest that even small reductions in NTHi carriage might have a significant impact in reducing infection. The model designed to predict the risk of OM in children based on NTHi nasopharyngeal carriage load (371) can be used to roughly contextualise the potential clinical benefit if the level of protection conferred by Hh-RHH122 and Hh-BW1

to model cell lines, which resulted in a reduction of NTHi attachment from 3.95 – 4.64 x 10^6 CFU to 0.9 – 2.0 x 10^3 CFU, was translated to the *in vivo* situation. Using the *in vivo* model, this would translate into an OM-associated risk from ≈50% down to around ≈10%. Inhibition of NTHi invasion may also enhance the protective capacity and therapeutic utility of *hpl*-expressing strains, as intracellular NTHi within respiratory epithelial cells is associated with persistent airway colonisation and exacerbations of COPD (72, 194) and a prolonged and intractable clinical course of acute OM in children (373). However, our study does not consider the multiple biological haem-sources and complex bacterial communities that may be present in the *in vivo* nasopharyngeal niche, the importance of which requires further investigation.

In conclusion, results from this study show that in the model epithelial cell culture system, Hh strains with a high-level production of the haemophore, hemophilin, have a strong protective capacity against NTHi adhesion and invasion, which are promising characteristics in the context of a probiotic therapy. Further investigation is required to assess factors that may influence the therapeutic potential of a range of *hpl* expressing clinical strains *in vivo*, particularly the influence of other biological haem sources and polymicrobial communities.

5.5. Materials and Methods

5.5.1. Microbial strains

Previously, we showed that production of the Hpl haemophore in five isolates of Hh predicted the ability of these strains to inhibit the growth of NTHi in broth co-cultures (315). Thus, Hh strains containing *hpl* alleles have previously been isolated (315, 386) and screened for *hpl* expression and NTHi-inhibitory capacity during broth co-culture (386). Hh strains Hh-BW1, Hh-RH122, Hh-NF4, and Hh-NF5 encode identical Hpl protein sequences but differ in levels of *hpl* expression. Hh strains Hh-BW1 and Hh-RH122 had the highest expression of *hpl*, as determined by levels of *hpl* mRNA and secreted NTHi-inhibitory activity, and these strains induced a complete loss of growth in multiple NTHi strains in extended co-culture experiments. The Hh-NF4 strain had no detectible expression of *hpl* mRNA or secreted NTHi-inhibitory activity. Hh-NF1 had a truncated *hpl* ORF (non-identical to Hh-BW1) and no secreted NTHi-inhibitory activity. Hh-NF5 had intermediate levels of *hpl* mRNA and secreted Hpl protein and conferred an intermediate loss of growth in co-cultured NTHi. An *hpl* knockout ($BW1^{Hpl–KO}$) of strain Hh-BW1 was constructed using insertional inactivation, as previously described (386). NTHi clinical isolates C11 (sputum), J76 (eye), L60 (throat), and L341 (ear) have previously been collected and categorised

as either “invasive” (C11 and L341) or “non-invasive” (L60 and J76) following infection of respiratory epithelial cells (375).

5.5.2. Microbial growth conditions and propagation of haem-starved populations

NTHi and Hh isolates were propagated from liquid nitrogen frozen glycerol stock, followed by two overnight passages on chocolate agar (CA) containing 2% (v/v) Vitox® (Oxoid Ltd.) at 37 °C with 5–10% CO_2 prior to experimentation. To replicate the predicted haem-restricted environment of the respiratory tract (215) and maximise adherence and invasive capacity (143), haem-starved populations of Hh and NTHi strains were prepared prior to cell association experiments. Bacterial suspensions of ≈0.1 OD_{600} were made in Tryptone Soy Broth (TSB; Oxoid Ltd., Basingstoke, UK) supplemented with 2% (v/v) Vitox® (Oxoid Ltd.) from overnight growth on CA. Broths were incubated for 12 h at 37 °C aerobically with shaking (220 RPM), centrifuged at 4000 × g for 5 min at 37 °C and resuspended in fresh, pre-warmed TSB to an OD_{600} of 1.0 prior to use in growth experiments. Exposure to non-growth conditions was minimised by maintaining suspensions and diluents at 37 °C.

5.5.3. Generation of nalidixic acid-resistant mutants

The differentiation of Haemophilus species was achieved by inducing nalidixic acid resistance in all NTHi isolates. Lawn plates were prepared by spreading 100-200 µL of a 0.5 McF standard suspension of either NTHi-C11, NTHi-J76, NTHi-L60, or NTHi-L341 on CA containing 1 µg mL^{-1} of nalidixic acid and incubated overnight at 37 °C with 5–10% CO_2. Resistant colonies were purified by streaking onto fresh CA containing the same concentration of nalidixic acid as the preceding lawn plate. This process was repeated with resistant mutants by successive passages on CA containing increasing concentrations of nalidixic acid up to 8 µg mL^{-1}.

5.5.4. Epithelial cell culture and maintenance

Cell cultures were maintained in T75 flasks (Thermo Fisher Scientific, Scoresby, VIC, Australia) at 37 °C, 5% CO_2. Immortalised Detroit 562 (D562) human pharyngeal carcinoma epithelial cells (ATCC® CCL-138) were cultured in Minimal Essential Media (MEM) with Earle’s salts and supplemented with 10% foetal bovine serum (Sigma-Aldrich, North Ryde BC, NSW, Australia), 1 mM sodium pyruvate, 2 mM L-glutamine, 1 × non-essential amino acids. Immortalised A549 human lung carcinoma epithelial cells (ATCC® CCL-185) were cultured in RPMI 1640 (Thermo Fisher Scientific, Scoresby, VIC, Australia) supplemented with 10% foetal bovine serum. At confluence, cell

monolayers were disrupted with 1x TrypLE (Gibco) for 15 min at 37 °C, 5% CO_2, washed in Hank's Balanced Salt Solution (HBSS) and resuspended in their respective culture media. Cells were counted with a haemocytometer, and viability was determined using trypan blue staining. Cells were seeded into 24-well plates at 1×10^4 mL^{-1} and grown to confluence prior to bacterial challenge.

5.5.5. Baseline attachment and invasion of respiratory epithelial cell lines

The time required for maximum attachment/internalisation of epithelial cells was determined for individual bacterial strains and cell lines prior to performing more complex competitive cell-association assays. Haem-starved populations of Hh or NTHi strains were diluted in supplemented MEM (D562) or RPMI (A549) cell culture media to a multiplicity of infection (MOI) of 100:1. Cell monolayers were treated (in triplicate) with 1 mL of each Hh or NTHi strain suspended in the appropriate media and incubated for 0.5, 1 ,2, 4, or 24 h at 37 °C, 5% CO_2. Cell monolayers were washed 3 times in HBSS to remove non-adherent populations. Cells were incubated for an additional hour in cell culture media containing 200 µg mL^{-1} gentamicin for the evaluation of intracellular bacteria, or in media alone for cells being evaluated for total bacterial association. Cell monolayers were washed 3 times in HBSS, lysed with 500 µL of 2% saponin v/v in HBSS for 15 min, disassociated from the plate surface by vigorous scraping with a pipette tip, followed by vortexing for 1 min. Collected lysates were serially diluted and 100 µL spread on CA. Colony-forming units (CFU) was determined by counting colonies from plates following 16-24 h incubation. Strains were categorised as "invasive" if the number of internalised bacteria exceeded 1% of the original inoculum (74, 375).

5.5.6. Bacterial and cell viability

Bacterial and epithelial cell survivability was assessed under experimental conditions to ensure that viability was maintained during competition assays. To determine bacterial viability, haem-starved populations of Hh or NTHi strains were diluted in pre-warmed, supplemented MEM or RPMI cell culture media to a density of ≈1 x 10^6 CFU mL^{-1}. Triplicate aliquots of 1 mL were transferred to wells of a 24-well plate and incubated for 24 h at 37 °C, 5% CO_2. Aliquots of 100 µL were retrieved at 0, 1, 2, 4, 8, or 24 h and spread on CA. CFU was determined by counting colonies from plates following 16-24-h incubation.

To determine epithelial cell viability, A549 and D562 cell monolayers were treated (in triplicate) with 1 mL of each Hh or NTHi strain suspended in the appropriate media at a MOI of 1000:1, or

media alone and incubated for 24 h at 37 °C, 5% CO_2. Hh-ATCC 33390 was included as a positive toxic control, as it has previously been shown to produce cytotoxic effects on both A549 and D562 cells *in vitro* (291) . At 0, 4, 8, or 24 h cell monolayers were washed with fresh media, disrupted with 1x TrypLE (Gibco) for 15 min at 37 °C, 5% CO_2, washed in HBSS, and resuspended in their respective culture media. Cells were counted with a haemocytometer, and viability was determined using trypan blue staining.

5.5.7. Preparation and quantification of recombinant and native Hpl

Methods used to prepare recombinant Hpl (rHpl) by purification from *Escherichia coli*, and native Hpl (nHpl) by ammonium sulfate precipitation of Hh culture broths, have previously been described (295, 386). A previously validated well diffusion assay was used for the semi-quantification of nHpl (315). A series of 2-fold dilutions of rHpl solution with a known concentration of 40 µM served as a standard and was tested alongside native extracts. The resultant relationship between zone size and concentration was used to determine the concentration of native extracts. Testing was conducted on two indicator NTHi strains (ATCC 49427 and clinical isolate NTHi-L15). Culture broths from strains negative for the *hpl* ORF (Hh ATCC 33390 and BW1^{Hpl-KO}) were included as negative controls**.**

5.5.8. Competitive cell association and invasion

Considering the high variability in attachment capacity between Hh strains, the initial Hh inoculum densities were normalised to allow for equal cell attachment amongst strains (Supplementary Table S5.1). Additionally, time points used for attachment and invasion measurements were individualised based on earlier baseline cell–interaction dynamics and viability studies. Cell monolayers (n=6) were treated with standardised haem-starved populations of either Hpl-producing or non-producing strains of Hh in the appropriate cell culture media for 4 h, followed by 3 washes in HBSS. Cell monolayers with attached Hh strains were challenged with NTHi-C11, NTHi-J76, NTHi-L60, NTHi-L341, or media alone for an additional 1 h (for A549 cells) or 2 h (D562 cells). A challenge dose of ≈2.5 x 10^7 was maintained among NTHi strains and was based on challenge doses associated with a high risk of OM infection in children (371) and in the induction of OM in mouse models (392, 394). Three replicates were used to prepare lysates for the quantification of adherent or internalised bacteria. Cell monolayers were washed 3 times in HBSS to remove non-adherent populations. Cells were incubated for an additional hour in cell culture media containing 200 ug mL^{-1} gentamicin for evaluation of intracellular bacteria, or in media alone for cells being evaluated for total bacterial association. Cell monolayers were washed 3 times in HBSS,

lysed with 500 µL of 2% saponin v/v in HBSS for 15 min, disassociated from the plate surface by vigorous scraping with a pipette tip, followed by vortexing for 1 min. These lysates were serially diluted and 100 µL spread on CA and CA containing 4 $\mu g mL^{-1}$ nalidixic acid. CFU of NTHi was determined by counting colonies from nalidixic acid CA plates following 16-24-h incubation. CFU of Hh was determined by subtracting colony counts on CA from those counted on paired CA with nalidixic acid. If colony counts of Hh in control wells (without NTHi challenge) varied significantly between Hh strains, the run was discarded. For the remaining three replicates, post-competition suspensions were collected for nHpl quantification by ammonium precipitation and well diffusion, and lysates (prepared as described above) were collected for analysis of *hpl* expression.

Following the same competitive cell association protocol, two additional experiments were performed to assess the effect of varying Hh pre-treatment dose and assess the NTHi-inhibitory capacity of Hpl independent of Hh. Cell monolayers were treated with 10-fold increasing doses of Hpl-producing strains of Hh ranging from $5 \times 10^4 – 5 \times 10^8$ CFU mL^{-1} (equivalent to a Hh MOI of 0.1:1–1000:1) or 2-fold increasing doses (025 µM) of nHpl extracted from broth culture of Hh-BW1, Hh-BW1$^{Hpl\text{-}KO}$ (negative control), or rHpl preparations. Following treatments, cell monolayers were challenged with NTHi-C11, which previously demonstrated consistently high attachment and invasive capacity.

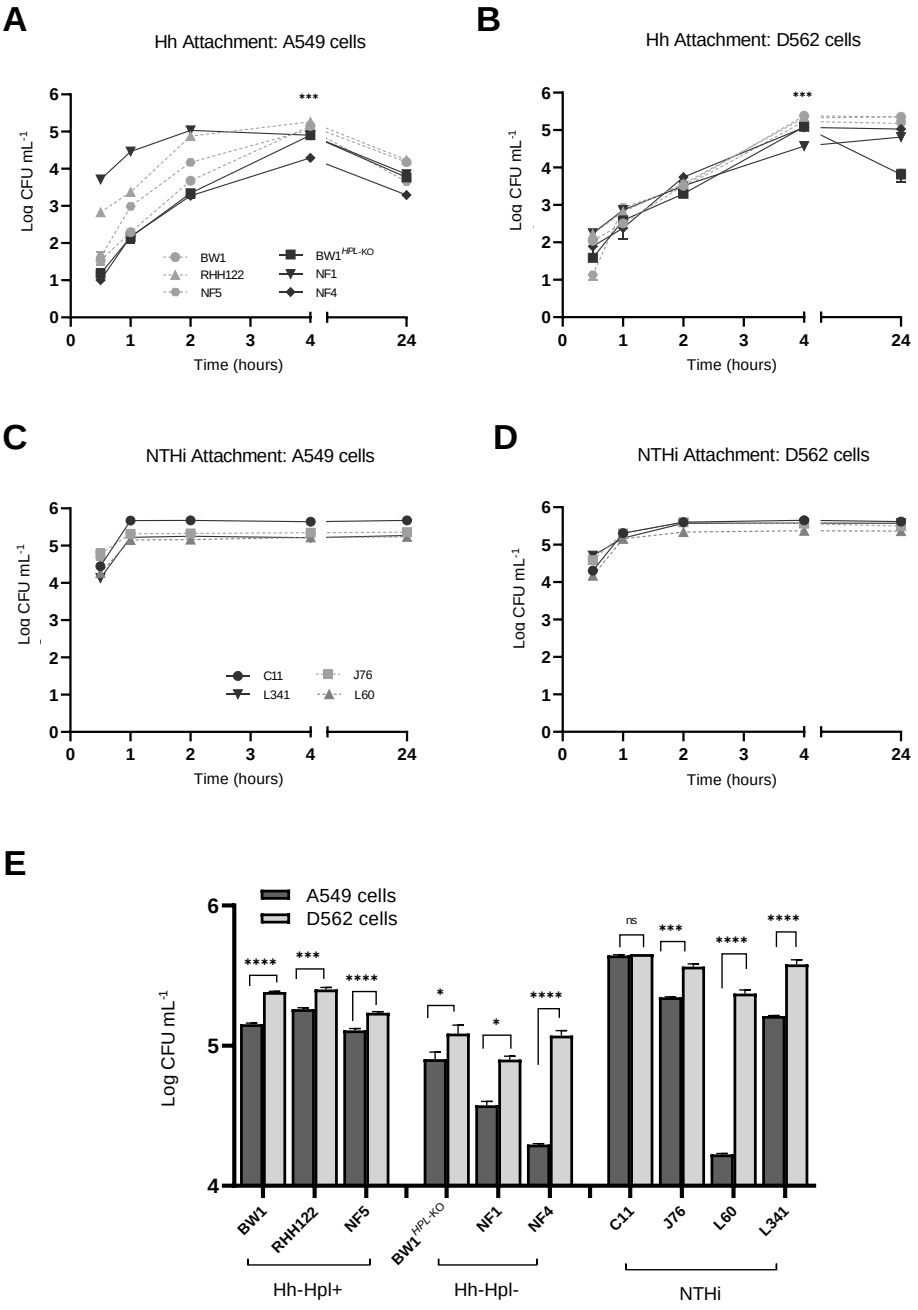
5.5.9. Expression analysis

Lysates recovered from competitive colonisation and invasion assays were immediately added to two volumes of RNAprotect Bacteria Reagent (Qiagen, Chadstone, VIC, Australia) for stabilisation of bacterial mRNA. Baseline *hpl* expression was determined from Hh suspensions incubated for 1 h in MEM or RPMI cell culture media without eukaryote cells or NTHi challenge. Extraction of mRNA and real-time PCR quantification of *hpl* expression was determined, as previously described (386).

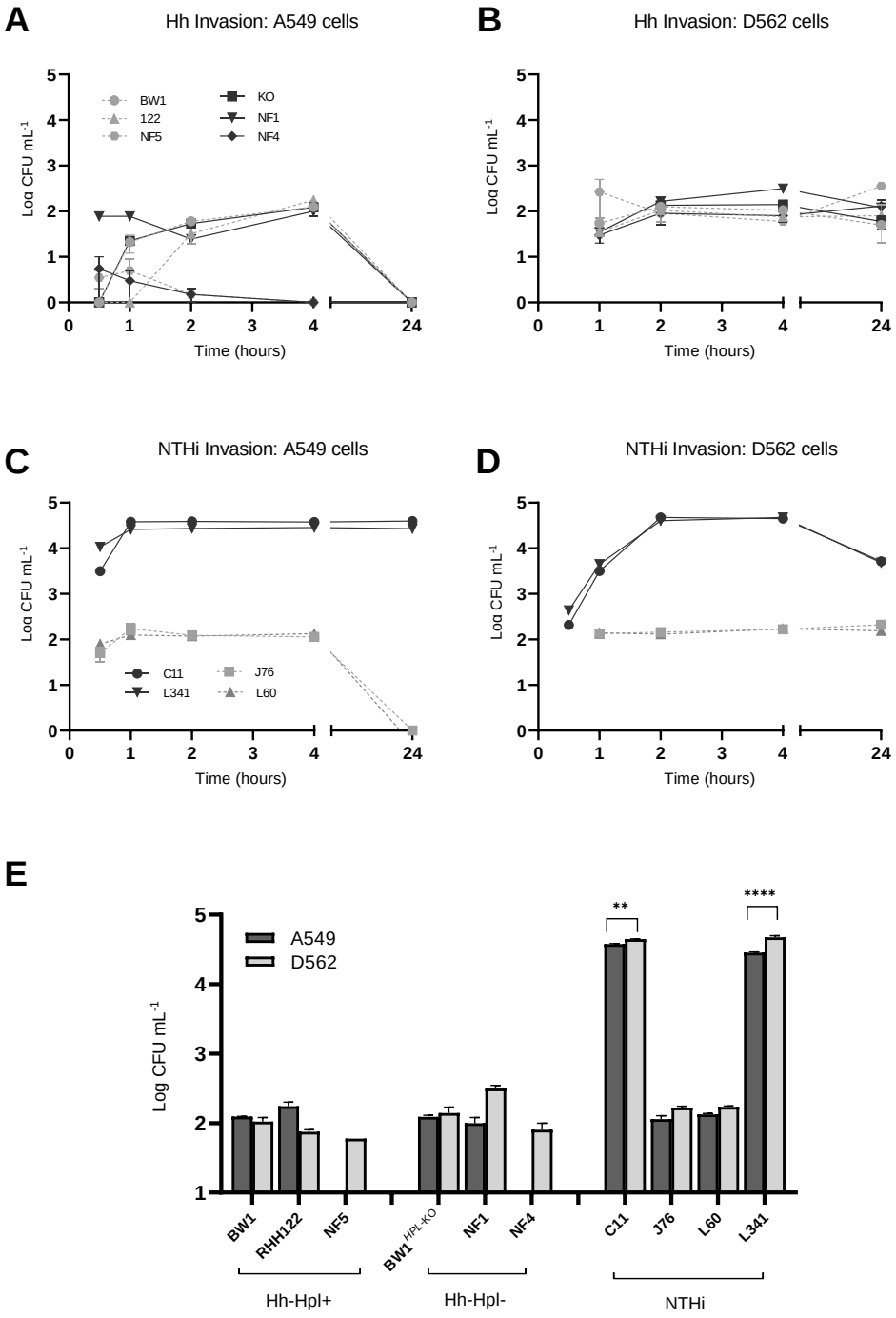
5.5.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism V7.04, 2017 (GraphPad Software, San Diego, California, USA). A two-way ANOVA with Dunnett's multiple comparisons test was used to compare baseline NTHi adherence and invasion to cells pre-treated with either Hh strains or native Hpl extracts. The minimum dose of Hh or concentration of native Hpl extracts required to significantly inhibit NTHi adherence or invasion was determined using a two-way ANOVA with Tukey's multiple comparisons test. Expression ratios and statistical significance were calculated with 2000 iterations by the Relative Expression Software Tool (REST; v 1.0, 2009) (362, 363).

5.6. Supplementary Materials



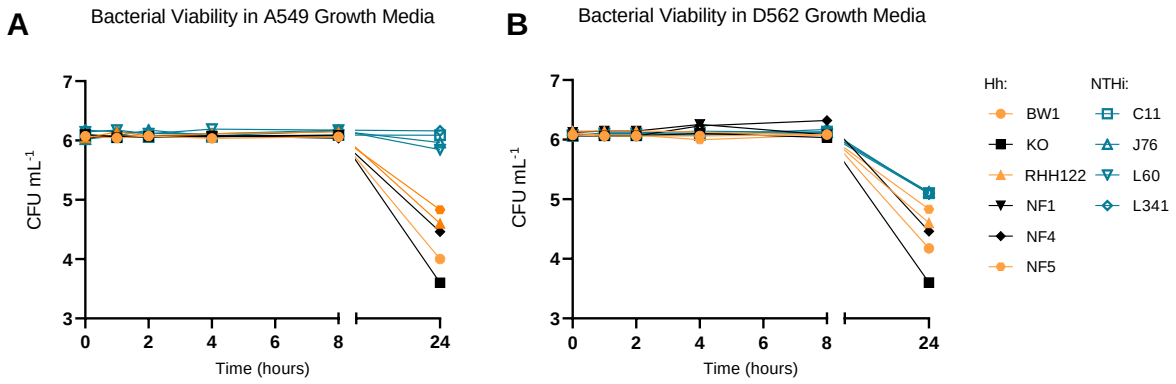
Supplementary Figure S5.1| Baseline bacterial attachment to epithelial cells. Colony counts (CFU mL^{-1}) of Hh attached to A549 **(A)**, and D562 **(B)** cell monolayers, showing significant variation between Hh strains. Colony counts (CFU mL^{-1}) of NTHi to A549 **(C)**, and D562 **(D)** during a 24-h incubation period. A summary of Hh and NTHi attachment to A549 and D562 cell monolayers at maximum attachment time points is shown **(E).** Error bars represent the ±SEM or three biological replicates. ^{ns} not significant, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.



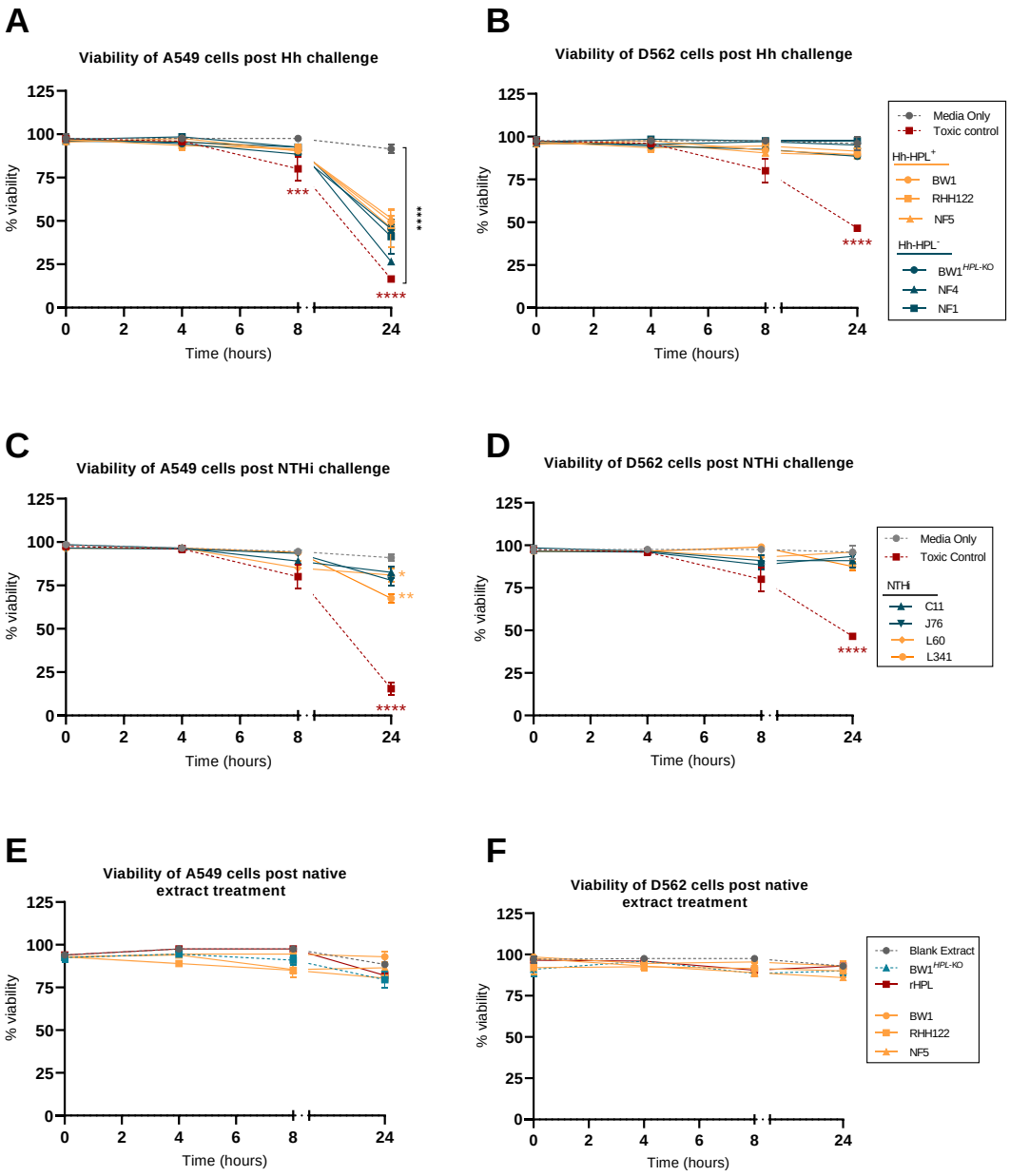
Supplementary Figure S5.2| Baseline bacterial invasion of epithelial cells. Colony counts (CFU mL^{-1}) of internalised Hh in A549 **(A)**, and D562 **(B)** cell monolayers, and NTHi in A549 **(C)**, and D562 **(D)** during a 24-h incubation period. A summary of Hh and NTHi invasion of A549 and D562 cell monolayers at maximum invasion time points is shown **(E).** Error bars represent the ±SEM or three biological replicates. ^{ns} not significant, * $p < 0.05$, **$p < 0.05$ *** $p < 0.001$, **** $p < 0.0001$.

Supplementary Table S5.1| Specific Hh treatment load (CFU mL^{-1}) for competitive colonisation experiments, standardised for differences in attachment capacity.

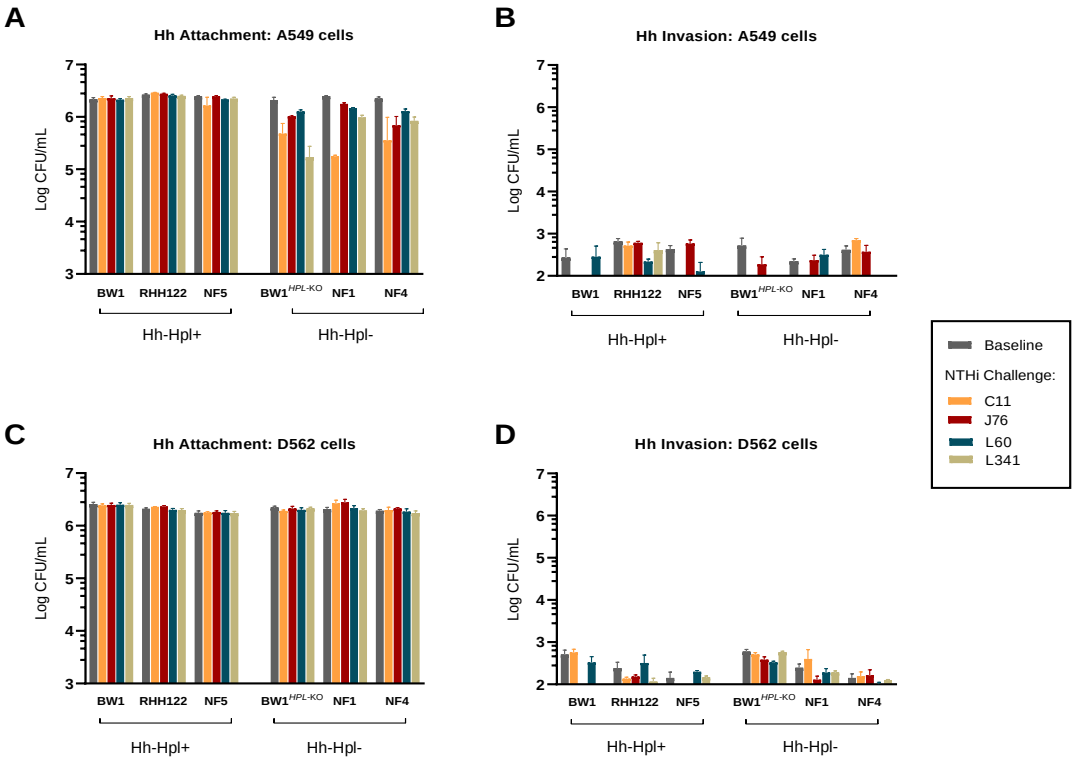
Hh strain	Treatment load for A549 cells (CFU mL^{-1})	Treatment load for D562 cells (CFU mL^{-1})
BW1	1.50×10^5	2.50×10^5
BW1$^{hpL\text{-}KO}$	3.00×10^5	5.00×10^5
RHH122	1.50×10^5	2.50×10^5
NF1	3.00×10^6	1.25×10^6
NF4	1.50×10^5	5.00×10^5
NF5	1.50×10^5	5.00×10^5



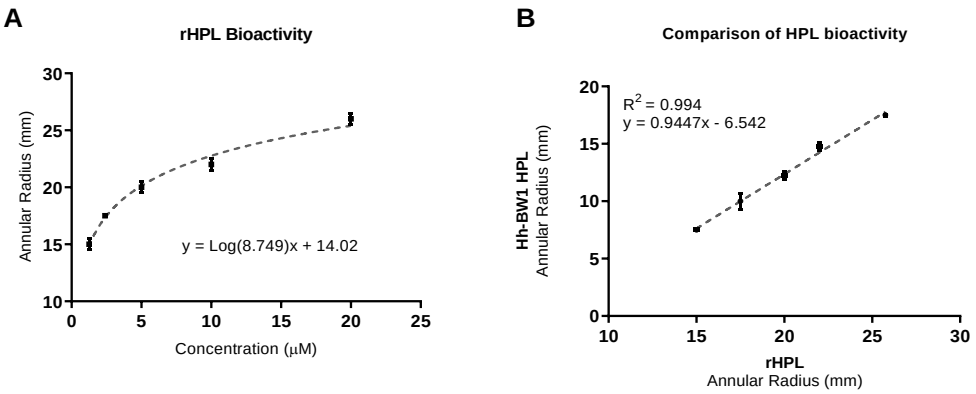
Supplementary Figure S5.3| Bacterial viability in cell culture media. Colony counts (CFU mL^{-1}) Hh and NTHi strains grown in RPMI (A549 growth media) **(A)**, and MEM (D562 growth media) **(B)** over 24 h. Error bars represent the ±SEM of two biological replicates.



Supplementary Figure S5.4| Viability of respiratory epithelium post bacterial challenge. Effect of 24-h Hh challenge on A549 **(A)**, and D562 **(B)** cell viability, compared to media alone. Effect of 24-h NTHi challenge on A549 **(C)**, and D562 **(D)** cell viability, compared to media alone. Effect of 24-h Hpl treatment on A549 **(E)**, and D562 **(F)** cell viability, compared to media alone. Error bars represent the ±SEM of three biological replicates. * $p < 0.05$, ** $p < 0.005$, **** $p < 0.0001$.



Supplementary Figure S5.5| Hh attachment and invasion of A549 and D652 cells post NTHi challenge. Colony counts (CFU mL^{-1}) of Hh attached to A549 **(A)** and D562 **(B)** cell monolayers post NTHi challenge. Colony counts (CFU mL^{-1}) of internalised Hh after exposure to A549 **(C)** and D562 **(D)** cell monolayers post NTHi challenge. Error bars represent the ±SEM of three biological replicates, measured triplicate. *p<0.05, **** p < 0.0001.



Supplementary Figure S5.6 | Bioactivity of recombinant and native Hpl. Annular radius of inhibitory zones produced by serial 2-fold dilutions of rHpl on agar containing indicator strain NTHi-252 **(A)**. Linear regression comparing the relationship between concentration and anti-NTHi inhibitory activity of rHPL and nHPL from Hh-BW1 **(B)**.

Chapter 6 | Manuscript 3

Oropharyngeal carriage of *hpl*-containing Hh predicts lower prevalence and density of NTHi colonisation in healthy adults

Brianna Atto [1], Dale Kunde [1], David A Gell [2] and Stephen Tristram [1],

[1] *School of Health Sciences, University of Tasmania, Launceston, Australia*

[2] *School of Medicine, University of Tasmania, Hobart, Australia*

The content of this chapter is reproduced as published in Pathogens MDPI as an original research article (Submitted 12 April 2021; Accepted: 7 May 2021; Published: 10 May 2021), however the text has been reformatted to meet the thesis submission requirements.

6.1. Abstract

Nontypeable *Haemophilus influenzae* (NTHi) is a major respiratory pathogen that initiates infection by colonising the upper airways. Strategies that interfere with this interaction may therefore have a clinically significant impact on the ability of NTHi to cause disease. We have previously shown that strains of the commensal bacterium *Haemophilus haemolyticus* (Hh) that produce a novel haem-binding protein, haemophilin, can prevent NTHi growth and interactions with host cells *in vitro*. We hypothesised that natural pharyngeal carriage of Hh strains with the *hpl* open reading frame (Hh-*hpl*$^+$) would be associated with a lower prevalence and/or density of NTHi colonisation in healthy individuals. Oropharyngeal swabs were collected from 257 healthy adults in Australia between 2018 and 2019. Real-time PCR was used to quantitatively compare the oropharyngeal carriage load of NTHi and Hh populations with the Hh-*hpl*$^+$ or Hh-*hpl*$^-$ genotype. The likelihood of acquiring/maintaining NTHi colonisation status over a two- to six-month period was assessed in individuals that carried either Hh-*hpl*$^-$ (*n* = 25) or Hh-*hpl*$^+$ (*n* = 25). Compared to carriage of Hh-*hpl*$^-$ strains, adult (18–65 years) and elderly (>65 years) participants that were colonised with Hh-*hpl*$^+$ were 2.43 or 2.67 times less likely to carry NTHi in their oropharynx, respectively. Colonisation with high densities of Hh-*hpl*$^+$ correlated with lower NTHi carriage loads and a 2.63 times lower likelihood of acquiring/maintaining NTHi colonisation status between visits. Together with supporting *in vitro* studies, these results encourage further investigation into the potential use of Hh-*hpl*$^+$ as a respiratory probiotic candidate for the prevention of NTHi infection.

6.2. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a major bacterial cause of opportunistic infections in the respiratory tract, most notably otitis media in infants and young children, community-acquired pneumonia in the elderly, and acute exacerbations in individuals with chronic obstructive pulmonary disease (COPD) (44, 87). Collectively, these infections and subsequent long-term health complications, such as hearing loss or decline in lung function, impart a significant global disease burden (2, 54). Asymptomatic pharyngeal colonisation occurs in 20–30% of healthy adults and 20–80% of children under the age of 5, and is characterised by the simultaneous carriage of multiple strains and a rapid turnover rate of constituent genotypes (22, 44, 87, 96, 395, 396). Week to week turnover rates of NTHi genotypes as high as 62% have been reported from healthy children attending day care (145). In the majority of cases, NTHi strains are typically replaced within three months of acquisition, but the persistent colonisation of a single strain up to six or seven months has been reported (22, 50).

Although the mechanisms that lead from colonisation to infection are poorly understood, the frequency of pharyngeal colonisation, especially with immunologically new NTHi strains, has been directly linked to an increased risk of developing acute otitis media (17, 145) and acute exacerbations of COPD (146, 396). Higher bacterial loads in the nasopharynx have also been correlated with an increased risk of developing acute otitis media (17, 148, 371) and a clinically significant increase in respiratory symptoms in COPD, even in the absence of a clinical exacerbation (149). Thus, eradicating or reducing the pharyngeal load of NTHi may have a clinically significant impact on the ability of NTHi to cause infection.

The need for alternative preventative therapies for NTHi infections has become apparent following ineffective vaccination attempts and a rapidly evolving antibiotic resistance profile that has resulted in treatment failure with first- and second-line antibiotic regimens (22, 97, 106, 369, 370, 397). One avenue gaining attention is the use of upper respiratory tract commensals, which may have utility in altering the pharyngeal habitability for pathogens such as NTHi (398). We have previously shown that some strains of the closely related respiratory tract commensal *Haemophilus haemolyticus* (Hh) produce a haem-binding protein, haemophilin (Hpl), that has been shown to inhibit NTHi growth *in vitro* (315, 386) and interactions with host cells in model respiratory cell lines (399). NTHi-inhibitory activity in these models was speculated to be mediated by Hpl haem binding that limited NTHi access to the essential nutrient haem (295), which is required for growth, survival, and pathogenicity (165).

We hypothesised that natural pharyngeal carriage of Hh strains with the *hpl* open reading frame (Hh-*hpl*$^{+}$) would be associated with a lower prevalence and/or density of NTHi colonisation in healthy individuals. To explore this hypothesis, real-time PCR was used to quantitatively compare the carriage load of NTHi and Hh populations with the Hh-*hpl*$^{+}$ or Hh-*hpl*$^{-}$ genotype from the oropharynx of 257 healthy adults in Australia collected between 2018 and 2019. Carriage of Hh-*hpl*$^{+}$ was associated with a significantly lower likelihood of concurrent NTHi carriage, long-term maintenance, or acquisition of NTHi colonisation status. Additionally, NTHi density was negatively correlated with Hh-*hpl*$^{+}$ carriage density. This work supports further investigation into the potential use of Hh-*hpl*$^{+}$ as a respiratory probiotic candidate for the prevention of NTHi colonisation and disease.

6.3. Results and Discussion

6.3.1. Carriage profile of NTHi and Hh varied between participant age groups

NTHi was detected by real-time PCR of the *siaT* gene target in 29% of adult (18–65 years) participants compared to 53% of elderly (> 65 years) participants (Supplementary Table S6.1). Conversely, the prevalence of Hh carriage was higher in adults (77%) compared to the elderly (52%) participants. Among Hh carriers, the frequency of detecting the Hh-*hpl*$^{+}$ genotype was slightly higher in the adults (55%) than in the elderly participants (39%). Together, the lower propensity for Hh and Hh-*hpl*$^{+}$ carriage in elderly participants may describe an environment that favours NTHi carriage.

NTHi carriage rates observed in this study are largely consistent with previous reports of nasopharyngeal carriage rates of 23–31% in healthy adults in the UK and Indigenous communities in Australia (35, 37-39, 395). However, carriage rates as low as 3–15% have also been reported in Kenya and Nepal (32, 34, 400), highlighting geographical differences in NTHi carriage. Despite their predisposition to NTHi-associated infections (42), information surrounding carriage rates in elderly demographics is limited to studies in Germany (2012–2013) and Brazil (2017), reporting rates of 1.9–2.5% (43, 44). It is unclear whether the substantially higher NTHi carriage rates detected in elderly participants is representative of the local population or of sampling bias. Although the *siaT* PCR target may also be detected in capsular *H. influenzae* (20), carriage of these strains is uncommon and collectively accounts for around 1.0% of *H.influenzae* isolates (20, 401). Thus, the false detection rate of NTHi strains is likely to be extremely low and unlikely to affect statistical findings presented in this study. This also does not compromise the clinical utility of Hh-*hpl*$^{+}$, as Hpl also exhibits inhibitory activity against capsular strains *in vitro* (295). This is the first study to assess Hh carriage prevalence among adults at a community level.

In addition to geographical and age-related variances, NTHi carriage rates vary considerably between studies, largely owing to the different culture- and molecular-based methods employed and the difficulty of distinguishing NTHi from Hh (14, 25). Although the nasopharynx is the preferred collection site for *H. influenzae* isolation in culture-based carriage studies (25), several studies have reported similar or improved detection of NTHi and Hh from oropharyngeal (OP) collections using qPCR-based methods (37, 402) that can reliably differentiate the two species,

such as the *hypD* and *siaT* targets employed in this study (20). Therefore, collection site is unlikely to impact carriage rates determined in this study.

6.3.2. Carriage of *hpl*-positive Hh is correlated with reduced prevalence and density of NTHi co-colonisation

Elderly participants were 2.43 times (95% CI, 1.95–2.61; $p \leq 0.0001$) and adult participants were 2.67 times (95% CI, 2.63–2.70; $p = 0.0036$) less likely to carry NTHi if Hh-*hpl*$^+$ strains were detected, compared to the carriage of Hh-*hpl*$^-$ strains. NTHi carriage prevalence was highest in adult (62%) and elderly (91%) participants that concurrently carried Hh strains that did not possess the *hpl* ORF (Hh-*hpl*$^-$) in their oropharynx. Among participants carrying Hh-*hpl*$^+$ strains, NTHi carriage rates were 25% (adults) and 14% (elderly) or 13% (adults) and 0% (elderly) in participants where Hh-*hpl*$^+$ represented the predominant Hh genotype (Figure 6.1A).

Comparison of Hh densities determined by qPCR of the *hpl* and *hypD* gene targets suggested concurrent carriage of multiple Hh genotypes, where not all possessed the *hpl* ORF. Simultaneous colonisation with multiple Hh and NTHi genotypes has previously been described in a longitudinal study of healthy adults (22). Hh-*hpl*$^+$ represented the predominating genotype in 49% (39/79) and 53% (8/15) of cases when the *hpl* gene was detected in adult and elderly participants, respectively (Supplementary Table S6.1). The proportionate density of Hh-*hpl*$^+$ (as a function of total Hh carriage) was negatively correlated with NTHi density among adult ($r_s = -0.16$; 95% CI, −0.314—−0.006; $p = 0.0366$) and, to a larger degree, elderly ($r_s = -0.53$; 95% CI, −0.7106–0.2851; $p < 0.0001$) participants that carried either species. In the adult age group, the average proportion of NTHi density decreased by 20% among individuals who concurrently carried Hh-*hpl*$^+$ as the non-predominant Hh genotype or by 47% if Hh-*hpl*$^+$ represented the predominant Hh genotype. This trend was more pronounced in the elderly age group where the average proportion of NTHi density decreased by 83% among individuals who concurrently carried Hh-*hpl*$^+$ or by 88% if Hh-*hpl*$^+$ represented the predominant Hh genotype (Figure 6.1B). Together these data suggest that carriage of Hh-*hpl*$^+$, but not Hh strains lacking the *hpl* ORF, lowers the incidence and density of concurrent NTHi carriage, particularly if Hh-*hpl*$^+$ represents the predominant Hh genotype.

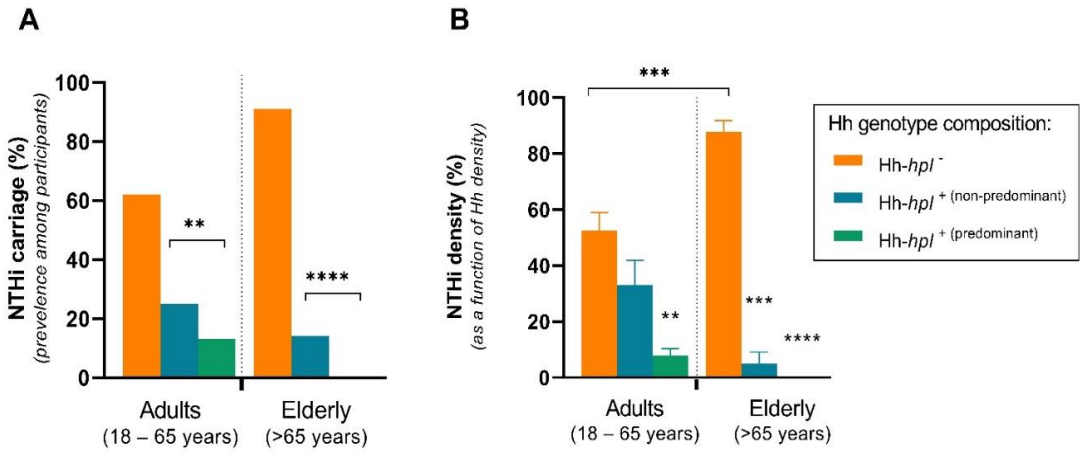


Figure 6.1 | NTHi dominance in oropharyngeal swabs of healthy adult (18–65 years) or elderly (> 65 years) participants co-colonised with Hh. NTHi oropharyngeal carriage prevalence **(A)** or proportion of NTHi (as a function of total Hh) **(B)** among participants concurrently carrying Hh strains that possess the *hpl* ORF (Hh-*hpl*$^{+}$) or do not possess the *hpl* ORF (Hh-*hpl*$^{-}$). Hh-*hpl*$^{+}$ (predominant) denotes instances where *hpl*$^{+}$ is the predominant Hh genotype (> 0.5 of total Hh). Error bars represent ±SEM; statistical significance was determined by simple logistic regression **(A)** or nonparametric Spearman correlation **(B)**; ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$.

6.3.3. Carriage of *hpl*-positive Hh prevents persistent colonisation or acquisition of NTHi carriage status

To investigate the risk of acquiring/maintaining NTHi colonisation status, follow-up OP swabs were collected from individuals that carried *hpl*$^{+}$ (n = 25) or *hpl*$^{-}$ (n = 25) strains of Hh. At visit 2, Hh-*hpl*$^{+}$ colonisation status was maintained in 19/25 of individuals, with an additional 11 participants gaining colonisation status, resulting in a total Hh-*hpl*$^{+}$ carriage rate of 60% (30/50) at visit 2. Maintenance or acquisition of NTHi carriage status at visit 2 was associated with the carriage of Hh-*hpl*$^{-}$ strains at visit 1 and at visit 2 in 75% (12/16) of cases (Table 6.1). The remaining four NTHi carriers were co-colonised with Hh-*hpl*$^{+}$ strains; however, in all cases, Hh-*hpl*$^{+}$ was not the predominant Hh genotype. In contrast, of participants who were not colonised with NTHi (either by loss of NTHi or who were never colonised), 88% (30/36) were carrying Hh-*hpl*$^{+}$ (Table 6.1). The likelihood of being colonised with NTHi at visit 2 (either by maintaining or acquiring NTHi colonisation status) was 2.63 times (95% CI, 2.56–2.70, p = 0.0112) lower in individuals colonised with Hh-*hpl*$^{+}$ strains at either visit 1 or visit 2. These results suggest that Hh-*hpl*$^{+}$ colonisation may have a protective effect against NTHi colonisation *in vivo*. However, the data can only account for total changes in carriage status between two visits and cannot assess the characteristically diverse and dynamic turnover of individual NTHi/Hh genotypes. Therefore, these findings may

underestimate the protective capacity of Hh-*hpl*$^{+}$ carriage and additional longitudinal studies with genotypic resolution are warranted. The findings presented by this study are only correlative and, as such, cannot rule out other unmeasured host-derived factors that may affect an individual's susceptibility to NTHi colonisation, such as smoking, airway dysbiosis, and underlying chronic respiratory diseases (150, 403-405).

Table 6.1 | NTHi colonisation status in participants between visit 1 and visit 2 (*n* = 50).

	NTHi Colonisation at Visit 1 -> Visit 2 Frequency (%)			
Hh Genotype	**NTHi + | NTHi - (Colonisation Loss)**	**NTHi-| NTHi- (Never Colonised)**	**NTHi + | NTHi + (Consistently Colonised)**	**NTHi-| NTHi + (Colonisation Gain)**
Total	8/50 (16)	26/50 (52)	4/50 (8)	12/50 (24)
***hpl*$^{+}$**	7/8 (88)	23/26 (88)	2/4 (50)	2/12 (17)
***hpl*$^{-}$**	1/8 (12)	3/26 (12)	2/4 (50)	10/12 (83)

$^{+/-}$ Detection by PCR for corresponding gene targets *siaT* (NTHi), *hypD* (Hh) and the *hpl* ORF.

6.3.4. Potential therapeutic utility of *hpl*-positive strains of Hh

In summary, the carriage of Hh-*hpl*$^{+}$ was associated with a significantly lower proportionate density and prevalence of concurrent NTHi carriage and long-term maintenance or acquisition of NTHi colonisation status. These findings suggest a potential protective role of Hh-*hpl*$^{+}$ strains against NTHi pharyngeal colonisation, particularly in an elderly population with a predisposition to NTHi colonisation in the absence of Hh-*hpl*$^{+}$. The frequency and density of NTHi colonisation or the acquisition of immunologically new strains are factors associated with an increased risk of disease onset and severity (146, 396). Thus, a commensal bacterium that can prevent NTHi pharyngeal colonisation incidence and/or bacterial load has compounded therapeutic utility and may be favourable over immunogenic approaches that are hampered by the highly variable expression of NTHi surface proteins and immunogenicity that does not protect against reinfection (406). However, clinical trials are required to determine if Hh-*hpl*$^{+}$ can eradicate or protect against direct NTHi challenge *in vivo,* particularly in populations predisposed to NTHi infections.

Production of the Hpl haemophore has previously been shown to mediate the *in vitro* inhibitory capacity of Hh against NTHi growth and the adherence/invasion of model respiratory cell lines by restricting NTHi access to the essential nutrient haem (315, 386, 399). Although we postulate that the same mechanism may be involved here, the study design reports on the presence of the *hpl* coding region and does not assess phenotypic production of the Hpl protein. We have previously shown that even among Hh strains containing identical *hpl* ORF sequences, some strains

lack the capacity to express *hpl* and produce the Hpl protein that mediates NTHi inhibitory activity *in vitro* (386, 399). However, the majority (16/17) of Hh-*hpl*$^{+}$ detectable by our PCR assay from our culture collection produce Hpl and elicit NTHi-inhibitory activity (Supplementary Table S6.4), and there are no incidences of Hpl production in strains that do not contain the *hpl* ORF. Further work is underway to determine the genetic determinants of *hpl* expression and Hpl production. The involvement of other host-mediated responses or interactions with other microbial communities in the oropharynx also cannot be excluded. Immune modulation, rather than physical competition, was shown to play an important role in the protective capacity of intranasal *Muribacter muris* (Hh surrogate) against NTHi colonisation and infection in a murine NTHi otitis media model (292). Additionally, other microbial upper respiratory tract commensals capable of producing bacteriocins against common pathogens have been reported (320, 407). The substantial effect sizes correlating Hh-*hpl*$^{+}$ and NTHi density despite potential confounders support a protective role of Hh-*hpl*$^{+}$ against NTHi pharyngeal colonisation in healthy adults. However, given the small sample population of this study, any correlations made in this study should be confirmed by studies involving a larger and more diverse study population that take into account environmental, geographical and host factors.

The *in vivo* correlations from the current study, together with previously published causative *in vitro* evidence, support further investigation into the potential use of *hpl*-positive strains of Hh as a respiratory probiotic candidate for the prevention of NTHi colonisation and disease.

6.4. Materials and Methods

6.4.1. Study population

Participants (*n* = 257) were comprised of community groups and university staff and students in Tasmania, Australia. Recruitment and sample collection was conducted between June 2018 and November 2019. All participants were briefed on study details and received written information prior to giving informed consent to participate. Participants were included in the study if they fulfilled the following criteria: (i) over 18 years of age, (ii) not currently taking antibiotics, and (iii) not experiencing respiratory-related symptoms. The study was approved by the Tasmanian Health and Medical Human Research Ethics Committee (Ref No: H0016835, approved 11 December 2017) in accordance with the National Statement on the Ethical Conduct in Human Research (NHMRC 2007, updated 2014).

6.4.2. Oropharyngeal swab collection

Oropharyngeal (OP) swabs were collected due to ease of collection and participant tolerance. Several studies have reported similar or improved qPCR detection of NTHi and Hh from OP swabs compared to nasopharyngeal swabs (25, 37, 44, 402). OP swabs were collected by two investigators by depressing the tongue and rolling the tip of a sterile cotton swab on the posterior wall of either side of the oropharynx for 2 seconds, avoiding contact with the surface of the mouth to minimise contamination with mouth flora. Follow-up swabs were collected 2–6 months following the initial visit from a randomly selected subset of this population (all ages) that carried either Hh-*hpl*$^{-}$ (*n* = 25) or Hh-*hpl*$^{+}$ (*n* = 25) at the first visit.

Immediately following collection, swabs were stored in 1 mL of room temperature transport media containing skim milk, tryptone, glucose, and glycerin (STGG) and transported to the laboratory within 2 h. STGG has previously been described and evaluated for optimal storage (408) and PCR detection of *Haemophilus influenzae* from pharyngeal swabs (409). Specimens were subject to a vigorous vortex for 1 min to disperse organisms from the swab tip, and the tip was removed from the media by pressing the swab against the wall. Media suspensions were frozen at −80 °C until analysis.

6.4.3. Real-time PCR quantification of NTHi, Hh, and Hh-*hpl*$^{+}$

Template gDNA was prepared from thawed 500 µL OP suspension aliquots using the DNeasy Blood & Tissue kit (Qiagen) following the standard proteinase K extraction protocol. NTHi and Hh strains containing the *hpl* ORF were simultaneously detected and quantified from OP swab gDNA by using a previously optimised and validated triplex real-time PCR assay (386). Briefly, this PCR assay adapted the use of previously validated genes for the discrimination of Hh (*hypD*) and *H.influenzae* (*siaT*) (20) as well as primers specific for the detection of the *hpl* ORF (GenBank MN720274) in Hh strains. This assay was validated for the detection of *hpl* sequences within 85–100% similarity to the PCR amplicon, which accounts for all known Hpl-producing (and thus NTHi-inhibitory) isolates. Hh strains with more divergent sequences have been isolated; however, none are known to produce Hpl based on bioactivity assays (data not shown). Further details of PCR validation, including the limits of detection and quantification of gene targets, are supplied in the Supplementary Materials. PCRs were performed using the CFX96 Touch™ real-time PCR system (Bio-Rad) in 96-well optical plates. The complete details of PCR thermocycling conditions, reagents, primer/probe design, and assay optimisation/validation have previously been described (386). Each run included a negative control (*H. parainfluenzae* ATCC 7901), no template

control, and 10-fold dilutions of a standard containing 2×10^{-8} ng NTHi ATCC 49247 and Hh-BW1 (known Hh-*hpl*$^{+}$ strain) gDNA. The quantification of NTHi and Hh was expressed as genome equivalents calculated from the appropriate standard, as previously described (20). Bacterial carriage status was considered negative for Ct values above 35 or if genome equivalents fell below the limit of quantification for the corresponding gene target. The Hh-*hpl*$^{+}$ genotype was considered predominant if genome equivalents calculated from the *hpl* target accounted for more than 50% of the total calculated Hh (from the *hypD* target).

6.4.4. Statistical analysis

Based on reported age-associated variation in the pharyngeal carriage rate and density of NTHi, analyses were stratified by age (18–65 years and > 65 years). Logistic regression models were used to assess whether Hh carriage status (either absolute presence of Hh-*hpl*$^{+}$ or Hh-*hpl*$^{-}$) or age predicted NTHi colonisation by measuring odds ratios (ORs) for each bacterium associated with the density of the other species. A simple logistic regression was also used to measure the OR between Hh-*hpl*$^{+}$ carriage and NTHi colonisation at follow up. A nonparametric Spearman correlation was conducted to determine the correlation between the proportion Hh-*hpl*$^{+}$ carriage density (as a function of total Hh) and the proportion of NTHi carriage in the swabs.

6.5. Supplementary Materials

Supplementary Table S6.1| Oropharyngeal colonisation frequencies of NTHi and Hh strains among 257 healthy adults in Australia.

	Colonisation frequency (%)	
Colonisation status	**Adults (18-65 years)**	**Elderly (>65 years)**
Total NTHi+	**54/184 (29)**	**39/73 (53)**
Total Hh-	**42/184 (23)**	**35/73 (48)**
Total Hh+	**142/184 (77)**	**38/73 (52)**
Total Hh-*hpl*$^{-}$	**63/184 (34)**	**23/73 (32)**
NTHi+	39/63 (62)	21/23 (91)
NTHi-	24/63 (38)	2/23 (9)
Total Hh-*hpl*$^{+}$ (non-predominant [a])	**40/184 (22)**	**7/73 (10)**
NTHi+	10/40 (25)	1/7 (14)
NTHi-	30/40 (65)	6/7 (86)
Total Hh-*hpl*$^{+}$ (predominant [b])	**39/184 (21)**	**8/73 (11)**
NTHi+	5/39 (13)	0/8 (0)
NTHi-	34/39 (87)	8/8 (100)

$^{+/-}$ Detection by PCR for corresponding gene targets *siaT* (NTHi), *hypD* (Hh) and the *hpl* ORF; [a] Hh-*hpl*$^{+}$ is not the predominant Hh genotype (<0.5 of total Hh); [b] Hh-*hpl*$^{+}$ is the predominant Hh genotype (>0.5 of total Hh).

Supplementary Table S6.2 | Average oropharyngeal colonisation density of NTHi and Hh strains among 257 healthy adults in Australia.

	Average density of NTHi and Hh genotypes (GE) (95% CI)		
Age group	**NTHi**	**Hh-*hpl*$^{-}$**	**Hh-*hpl*$^{+}$**
Adults	3.08×10^5 (1.82×10^5 -6.65×10^5)	2.25×10^5 (1.13×10^5 – 3.67×10^5)	1.62×10^5 (7.54×10^4 – 3.09×10^5)
Elderly	6.84×10^4 (3.18×10^4 – 1.30×10^5)	1.89×10^4 (1.05×10^4 – 8.87×10^4)	3.48×10^4 (1.97×10^3 - 8.12×10^4)

6.5.1. Triplex real-time PCR assay design, optimisation and validation

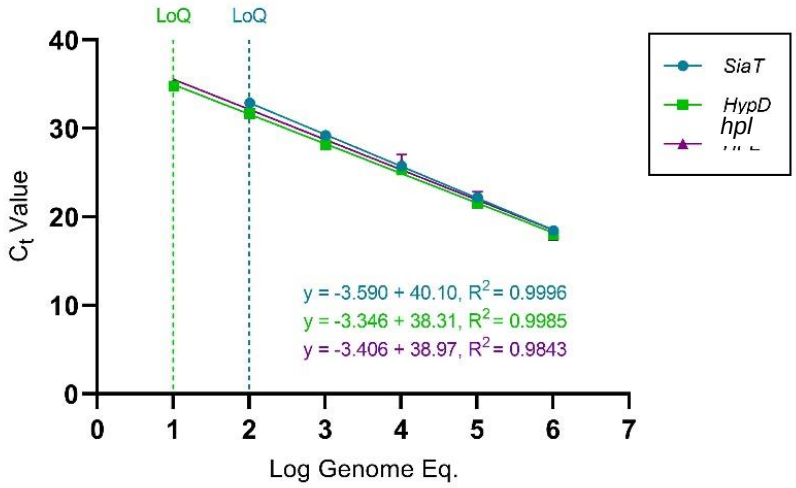
A triplex PCR assay was designed to detect and quantify Hi, Hh and the *hpl* ORF. The targets used for discrimination of Hh (*hypD*) and NTHi (*siaT*) have previously been described and validated (20). For detection of the *hpl* ORF, primers were designed using the ORF of the model Hpl-producer, Hh strain BW1 (GenBank MN720274) (365). Primer specificity was confirmed using discontiguous megaBLAST analysis performed across 115 complete and 862 draft genome assemblies for *Haemophilus.spp* available from Genbank. A nonredundant nucleotide (nr/nt) collection BLAST search was also conducted to determine amplicon specificity in non-*Haemophilus* genomes. Following optimisation in singleplex format, the three assays were merged into triplex format. Annealing temperature was optimised using an 8-step temperature gradient ranging from 53-63°C. Specificity of amplicons was determined by gel electrophoresis and the optimal temperature was selected based on highest yield of amplicons of the correct size in the absence of non-specific amplification. PCR specificity for Hi, Hh and *hpl* was determined using 13 Hh strains with varying *hpl* sequence similarity to Hh-BW1, 13 Hi strains and 9 other genera representing common upper respiratory tract flora. Reaction efficiency of triplex reaction was determined using 10-fold dilutions of control strains over the range of 2 to 2×10^{-8} ng (Hi ATCC 49247, Hh ATCC 33390 and Hh BW1). Limits of quantification (LoQ) values were determined for *hypD* and *siaT* targets in triplex format based on criteria where replicates at a given dilution with a cycles to threshold (Ct) standard deviation (σ) of ≥0.8 were considered to exceed the LoQ, with one or more amplification failures also deemed a LoQ failure. The upper LoQ value was not determined due to the unlikelihood of encountering such high DNA concentrations in clinical specimens. The lower limit of detection was also determined for the *hpl* target and defined from linearity data (Supplementary Figure S6.1).

In silico specificity for the *hpl* amplicon revealed 97-100% primer and probe nucleotide sequence identity for 26 of 61 complete or draft Hh genomes available in Genbank. Sequence similarity to the *hpl* amplicon was detected in 20 *H. influenzae* genomes out of 757 complete and draft genome assemblies. However, all alignments contained a minimum of 4 SNPs in the reverse primer and Taqman probe and did not contain any sequence homology with the forward primer. *hpl* was also detected in 3 genome assemblies available for *Haemopilus quentini*, which was expected based on previous analysis of the *hpl* ORF (365). Despite close relatedness to Hh, isolation of this strain has only been described in the genitourinary tract so is unlikely to be co-isolated from respiratory specimens (367). Comparison of 11 previously sequenced *hpl* ORFs (365) to these databases yielded the same results, indicating high sensitivity to known *hpl* sequence

variants (ranging from 85-100% homology to Hh-BW1). PCR of these isolates, and additional Hh, Hi and common upper respiratory tract colonisers confirmed specificity for each target.

Supplementary Table S6.3| Summary of primer and LNA-probe sequences, and expected amplicon size for the *hypD*, *siaT* and *hpl* targets.

Primers and Probes	Sequence	Amplicon Size (bp)
***hypD* Forward**	5'- GGCAATCAGATGGTTTACAACG	187
***hypD* Reverse**	5'- CAGCTTAAAGYAAGYAGTGAATG	
***hypD* LNA-probe**	/5HEX/CCA+C+AA+C+GA+G+AATTAG/3IABkFQ/	
***siaT* Forward**	5'- AATGCGTGATGCTGGTTATGAC	138
***siaT* Reverse**	5'- AATGCGTGATGCTGGTTATGAC	
***siaT* LNA-probe**	/56-FAM/A+GA+A+GCAGC+A+G+TAATT/3IABkFQ/	
***hpl* Forward**	5'- TATTCCTAATGATCCCGCT	120
***hpl* Reverse**	5' - TCTTTTTTCGCTACCCCT	
***hpl* LNA-probe**	/5Cy5/AT+CCATTTA+TCGG+CACGTTCT/3IAbRQSp/	



Supplementary Figure S6.1 |PCR efficiency and measure of LoQ and LoD. Shows PCR C_t values for the *siaT, hypD* and *hpl* targets measured from serial dilutions of Hi, Hh^{hpl-} and Hh^{hpl+} control strain gDNA over the range of 2 to 2 × 10^{-8} ng. Data points are represented as mean +/- SEM of two separate experiments, each conducted in duplicate.

Supplementary Table S6.4| Genotypic and phenotypic characteristics of Hh strains containing the *hpl* ORF. Sequences were determined by whole-genome sequencing, performed using the MiSeq (Illumina) platform. ORF similarities are compared to a model Hpl-producing strain (Hh-BW1) that has demonstrated high anti-NTHi activity *in vitro.*

Hh Strain	% Identity of the *hpl* ORF	Hpl production	Hh-*hpl*+ by tri-plex PCR
BW1	-	+	+
BW5	99% (807/819)	+	+
BW15	100% (819/819)	+	+
BW18	100% (819/819)	+	+
BW36	100% (819/819)	+	+
CF14	96% (786/819)	+	+
L19	85% (709/833)	-	-
L117	85% (709/833)	-	-
L152	85% (709/833)	-	-
L153	98% (805/819)	+	+
NF4	100% (819/819)	-	+
NF5	100% (819/819)	+	+
NF6	96% (786/819)	+	+
NF1	80% (636/794)	-	-
RHH122	100% (819/819)	+	+
L37	80% (636/794)	-	-
CF26	79% (630/794)	-	-
L52	98% (805/819)	+	+
L56	81% (621/768)	-	-
OP1	100% (819/819)	+	+
OP2	100% (819/819)	+	+
OP3	100% (819/819)	+	+
OP4	100% (819/819)	+	+
NF11	96% (788/819)	+	+

$^{+/-}$ Hpl-producing/non-producing (determined by detection of *hpl* expression and bioassay of culture supernatants).

Chapter 7 | General Discussion

The research projects and publications that comprise this thesis explore the probiotic potential of newly discovered strains of the upper respiratory commensal, *Haemophilus haemolyticus* (Hh), with the unique capacity to produce a novel haemophore (haemophilin; Hpl) and inhibit growth of the major respiratory pathogen nontypeable *Haemophilus influenzae* (NTHi).

In chapter 3, we provide structural, functional and bioinformatic evidence that characterises Hpl as a previously unrecognised haem uptake mechanism in Hh that blocks uptake of haem and subsequently inhibits the growth of NTHi. The unique protein structure of Hpl, and the high prevalence and exclusivity of the predicted *hpl* gene cassette among Hh strains indicates that Hpl may be important for the competitive acquisition of haem and survival amongst polymicrobial communities in the nasopharynx. Although *hpl* mRNA levels were predictive of the NTH-inhibitory phenotype, the regulatory elements that govern Hpl production are yet to be determined and are beyond the scope of this thesis.

Based on the findings from chapter 3, we proposed a model by which Hh strains with high-level expression of particular *hpl* alleles might starve local communities of haem and inhibit nasopharyngeal colonisation by NTHi. In chapters 4 and 5, competition models directly demonstrated the *in vitro* capacity of Hpl-producing Hh to prevent growth and host cell interactions by NTHi. The high potency of this NTHi-inhibitory activity was further demonstrated by the ability of Hpl-producing Hh to inhibit NTHi when present in relatively small numbers, and under haem conditions in excess of what is expected to be encountered *in vivo*. These characteristics are favourable in the context of a respiratory probiotic, as protection is not likely to be compromised by physiological fluctuations in haem availability or bacterial density.

In chapter 6, investigations progressed to an *in vivo* setting, in which epidemiological data strongly suggested that Hpl-producing Hh may offer protection against NTHi colonisation in healthy adults. However, this data is correlative in nature and the methods employed cannot differentiate between phenotypic production and possession of the *hpl* gene; thus, clinical trials are warranted to establish a causative role and rule out involvement of other host- or microbiome-mediated interactions.

In summary, these findings present a strong case for further development of Hpl-producing Hh as a respiratory probiotic to prevent NTHi infections. As a commensal of the upper respiratory tract, Hpl-producing Hh possess the necessary characteristics for probiotic applications; it is not a recognised cause of infection and is able to thrive in the nasopharyngeal niche among other microbial inhabitants.

Although the evidence presented in this thesis is pre-clinical by design, there are several factors that may predict favourable clinical outcomes using this probiotic approach. Firstly, higher frequencies and densities of NTHi colonisation, or acquisition of immunologically new strains are linked to an increased risk of disease onset, severity, and chronic airway inflammation. Secondly, the ability to sequester adequate sources of haem-iron is a major determinant of NTHi survival and ability to perform the bacterial-host cell interactions necessary for pathogenesis. Lastly, the presence of healthy carriers indicates that clinical efficacy is not predicated upon complete eradiation of NTHi from the nasopharynx and that small reductions in carriage load may be sufficient for beneficial clinical outcomes. Using a model designed to predict the risk of otitis media in children based on NTHi pharyngeal carriage load, we predict a ≈40% decrease in the risk of infection, provided the level of protection conferred by Hpl-producing Hh to model cell lines was preserved *in vivo*. However, translational studies are required to determine the clinical efficacy of these strains *in vivo* as the experiments described in this thesis cannot account for the complexities of the host environment. For example, NTHi is adept at acquiring haem from a variety of ligands, and thus, Hpl must also share affinity for these sources in order to adequately block NTHi access to haem-iron. Similarly, other communities which may compete for the same, limited pool of haem-iron may interfere with Hpl-mediated haem-sequestration.

The NTHi-inhibitory capacity of Hpl extracts observed in the absence of Hh also proposes the potential for development of a protein-based therapy. Although observations suggest that sustained production and high local concentrations of Hpl generated by adherent Hh may offer more consistent and longer-term protection than bolus applications of the protein alone, future development may benefit from formulations including adjuvant Hpl. In theory, a therapy combining the long-term protection of adherent Hh and the immediate NTHi-inhibitory activity of Hpl, may be used both as a preventative measure and as a treatment for active infection. However, the efficacy of either approach during an active infection was not assessed in this thesis and remains an avenue for future research. Additionally, elucidation of Hpl regulation may become important further into the development pipeline, as generation or selection of strains capable of Hpl hyper-production may have improved NTHi-inhibitory capacity, and thus be advantageous in the context of a respiratory probiotic. The inability of Hh-NF4 to express *hpl* despite possessing an identical sequence (including promotors) to Hh-BW1 is completely unique among the Hh strains tested in this study and remains unexplainable with our current knowledge of Hpl. Thus, future studies that elucidate the mechanisms of Hpl production, export and function may be paramount to maximising the efficacy of future probiotic applications.

Bacteriotherapy has several advantages over traditional antibiotic- or vaccine-based management. Probiotics have a narrow spectrum of activity that does not damage host tissue, provoke collateral

damage to the healthy microbiome or promote enrichment of resistant clones; properties which make them an asset against the emergence of antibiotic resistance. Probiotics may also be favourable over immunogenic approaches that are hampered by the highly variable expression of NTHi surface proteins and immunogenicity that does not protect against reinfection. However, probiotic applications face considerations associated with the unpredictability of living organisms. Although we showed that Hh-Hpl+ colonisation status is relatively stable over a 2-6 month period, the methods used could not detect individual strain turnover and may underestimate the transiency of Hh-Hpl+ colonisation (Chapter 6). Poor retention of Hh-Hpl+ colonisation in the nasopharynx may limit the protection offered by this probiotic approach. In the clinical setting, this may be addressed by periodic administration if the probiotic formulation, a common practice for probiotic therapies. Future clinical trials should therefore assess the retention of Hh strains, which are likely to differ between Hpl phenotypes.

In conclusion, the work presented in this thesis demonstrates the potential therapeutic value of Hpl-producing Hh as a respiratory probiotic to prevent infections caused by NTHi. Further development through clinical trials to determine the safety profile, effective dosage and *in vivo* efficacy, particularly in populations with a high propensity for NTHi infections, is strongly encouraged. If found to be effective *in vivo*, such a strategy could have a significant impact on the growing burden of NTHi-associated disease and antibiotic resistance. Evidence of *in vivo* efficacy may also encourage development of similar approaches that exploit the natural competition between other pathogens and their commensal relatives to combat infections otherwise resistant to routine management.

Chapter 8 | References

1. Tristram S, Jacobs MR, Appelbaum PC. Antimicrobial resistance in Haemophilus influenzae. Clinical microbiology reviews. 2007;20(2):368-89.
2. Van Eldere J, Slack MP, Ladhani S, Cripps AW. Non-typeable Haemophilus influenzae, an under-recognised pathogen. The Lancet infectious diseases. 2014;14(12):1281-92.
3. Organization WH. Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis. World Health Organization; 2017.
4. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. The Lancet Infectious Diseases. 2018;18(3):318-27.
5. Jalalvand F, Riesbeck K. Update on non-typeable Haemophilus influenzae-mediated disease and vaccine development. Expert review of vaccines. 2018;17(6):503-12.
6. Nørskov-Lauritsen N. Classification, identification, and clinical significance of Haemophilus and Aggregatibacter species with host specificity for humans. Clinical microbiology reviews. 2014;27(2):214-40.
7. Heath PT, Booy R, Azzopardi HJ, Slack MP, Fogarty J, MOLONEY AC, et al. Non-type b Haemophilus influenzae disease: clinical and epidemiologic characteristics in the Haemophilus influenzae type b vaccine era. The Pediatric infectious disease journal. 2001;20(3):300-5.
8. Maguire JE, Beard F, Méder K, Dey A, Macartney K, McIntyre P. Australian vaccine preventable disease epidemiological review series: invasive Haemophilus influenzae type b disease, 2000-2017. Communicable diseases intelligence (2018). 2020;44.
9. Soeters HM, Blain A, Pondo T, Doman B, Farley MM, Harrison LH, et al. Current epidemiology and trends in invasive Haemophilus influenzae disease—United States, 2009–2015. Clinical Infectious Diseases. 2018;67(6):881-9.
10. Anderson R, Wang X, Briere EC, Katz LS, Cohn AC, Clark TA, et al. Haemophilus haemolyticus isolates causing clinical disease. Journal of clinical microbiology. 2012;50(7):2462-5.
11. de Gier C, Kirkham L-AS, Nørskov-Lauritsen N. Complete deletion of the fucose operon in Haemophilus influenzae is associated with a cluster in multilocus sequence analysis-based phylogenetic Group II related to Haemophilus haemolyticus: implications for identification and typing. Journal of clinical microbiology. 2015;53(12):3773-8.
12. Hu F, Rishishwar L, Sivadas A, Mitchell GJ, Jordan IK, Murphy TF, et al. Comparative Genomic Analysis of Haemophilus haemolyticus and Nontypeable Haemophilus influenzae and a New Testing Scheme for Their Discrimination. Journal of Clinical Microbiology. 2016;54(12):3010-7.
13. Latham R, Zhang B, Tristram S. Identifying Haemophilus haemolyticus and Haemophilus influenzae by SYBR Green real-time PCR. Journal of microbiological methods. 2015;112:67-9.
14. Pickering J, Richmond PC, Kirkham L-AS. Molecular tools for differentiation of non-typeable Haemophilus influenzae from Haemophilus haemolyticus. Frontiers in microbiology. 2014;5:664.
15. Theodore MJ, Anderson RD, Wang X, Katz LS, Vuong JT, Bell ME, et al. Evaluation of new biomarker genes for differentiating Haemophilus influenzae from Haemophilus haemolyticus. Journal of clinical microbiology. 2012;50(4):1422-4.

16. Murphy TF, Brauer AL, Sethi S, Kilian M, Cai X, Lesse AJ. Haemophilus haemolyticus: a human respiratory tract commensal to be distinguished from Haemophilus influenzae. The Journal of infectious diseases. 2007;195(1):81-9.
17. Kirkham L-AS, Wiertsema SP, Mowe EN, Bowman JM, Riley TV, Richmond PC. Nasopharyngeal carriage of Haemophilus haemolyticus in otitis-prone and healthy children. Journal of clinical microbiology. 2010;48(7):2557-9.
18. McCrea KW, Xie J, LaCross N, Patel M, Mukundan D, Murphy TF, et al. Relationships of nontypeable Haemophilus influenzae strains to hemolytic and nonhemolytic Haemophilus haemolyticus strains. Journal of clinical microbiology. 2008;46(2):406-16.
19. Binks MJ, Temple B, Kirkham L-A, Wiertsema SP, Dunne EM, Richmond PC, et al. Molecular surveillance of true nontypeable Haemophilus influenzae: an evaluation of PCR screening assays. PloS one. 2012;7(3):e34083.
20. Price EP, Harris TM, Spargo J, Nosworthy E, Beissbarth J, Chang AB, et al. Simultaneous identification of Haemophilus influenzae and Haemophilus haemolyticus using real-time PCR. Future microbiology. 2017;12(7):585-93.
21. Chang A, Adlowitz DG, Yellamatty E, Pichichero M. Haemophilus influenzae outer membrane protein P6 molecular characterization may not differentiate all strains of H. influenzae from H. haemolyticus. Journal of clinical microbiology. 2010;48(10):3756-7.
22. Mukundan D, Ecevit Z, Patel M, Marrs CF, Gilsdorf JR. Pharyngeal colonization dynamics of Haemophilus influenzae and Haemophilus haemolyticus in healthy adult carriers. Journal of clinical microbiology. 2007;45(10):3207-17.
23. Faden H, Duffy L, Williams A, Pediatrics TW, Krystofik DA, Wolf J. Epidemiology of nasopharyngeal colonization with nontypeable Haemophilus influenzae in the first 2 years of life. Journal of Infectious Diseases. 1995;172(1):132-5.
24. Raymond J, Armand-Lefevre L, Moulin F, Dabernat H, Commeau A, Gendrel D, et al. Nasopharyngeal colonization by Haemophilus influenzae in children living in an orphanage. The Pediatric infectious disease journal. 2001;20(8):779-84.
25. Hare KM, Binks MJ, Grimwood K, Chang AB, Leach AJ, Smith-Vaughan H. Culture and PCR detection of Haemophilus influenzae and Haemophilus haemolyticus in Australian Indigenous children with bronchiectasis. Journal of clinical microbiology. 2012;50(7):2444-5.
26. Ortiz-Romero MdM, Espejo-García MP, Alfayate-Miguelez S, Ruiz-López FJ, Zapata-Hernandez D, Gonzalez-Pacanowska AJ. Epidemiology of Nasopharyngeal Carriage by Haemophilus influenzae in Healthy Children. The Pediatric infectious disease journal. 2017;36(10):919-23.
27. Navne J, Børresen M, Slotved H, Andersson M, Melbye M, Ladefoged K, et al. Nasopharyngeal bacterial carriage in young children in Greenland: a population at high risk of respiratory infections. Epidemiology & Infection. 2016;144(15):3226-36.
28. King PT, Sharma R. The lung immune response to nontypeable Haemophilus influenzae (lung immunity to NTHi). Journal of immunology research. 2015;2015.
29. Beissbarth J, Wilson N, Arrowsmith B, Binks MJ, Oguoma V, Lawrence K, et al. Nasopharyngeal carriage of otitis media pathogens in infants receiving 10-valent non-typeable Haemophilus influenzae protein D conjugate vaccine (PHiD-CV10), 13-valent pneumococcal conjugate vaccine (PCV13) or a mixed primary schedule of both vaccines: A randomised controlled trial. Vaccine. 2021.
30. Leach AJ, Wigger C, Hare K, Hampton V, Beissbarth J, Andrews R, et al. Reduced middle ear infection with non-typeable Haemophilus influenzae, but not Streptococcus pneumoniae, after transition to 10-valent pneumococcal non-typeable H. influenzae protein D conjugate vaccine. BMC pediatrics. 2015;15(1):1-13.
31. Lewnard JA, Huppert A, Givon-Lavi N, Pettigrew MM, Regev-Yochay G, Dagan R, et al. Density, serotype diversity, and fitness of Streptococcus pneumoniae in upper respiratory tract cocolonization with nontypeable Haemophilus influenzae. The Journal of infectious diseases. 2016;214(9):1411-20.

32. Abdullahi O, Nyiro J, Lewa P, Slack M, Scott JAG. The descriptive epidemiology of Streptococcus pneumoniae and Haemophilus influenzae nasopharyngeal carriage in children and adults in Kilifi district, Kenya. The Pediatric infectious disease journal. 2008;27(1):59.
33. Subramanya SH, Thapa S, Dwedi SK, Gokhale S, Sathian B, Nayak N, et al. Streptococcus pneumoniae and Haemophilus species colonization in health care workers: the launch of invasive infections? BMC research notes. 2016;9(1):66.
34. Adegbola RA, DeAntonio R, Hill PC, Roca A, Usuf E, Hoet B, et al. Carriage of Streptococcus pneumoniae and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis. PloS one. 2014;9(8):e103293.
35. Shak JR, Cremers AJ, Gritzfeld JF, de Jonge MI, Hermans PW, Vidal JE, et al. Impact of experimental human pneumococcal carriage on nasopharyngeal bacterial densities in healthy adults. PloS one. 2014;9(6):e98829.
36. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC infectious diseases. 2010;10(1):304.
37. Greenberg D, Broides A, Blancovich I, Peled N, Givon-Lavi N, Dagan R. Relative importance of nasopharyngeal versus oropharyngeal sampling for isolation of Streptococcus pneumoniae and Haemophilus influenzae from healthy and sick individuals varies with age. Journal of Clinical Microbiology. 2004;42(10):4604-9.
38. Rawlings BA, Higgins TS, Han JK. Bacterial pathogens in the nasopharynx, nasal cavity, and osteomeatal complex during wellness and viral infection. American journal of rhinology & allergy. 2013;27(1):39-42.
39. Chi DH, Hendley JO, French P, Arango P, Hayden FG, Winther B. Nasopharyngeal reservoir of bacterial otitis media and sinusitis pathogens in adults during wellness and viral respiratory illness. American journal of rhinology. 2003;17(4):209-14.
40. Coleman A, Wood A, Bialasiewicz S, Ware RS, Marsh RL, Cervin A. The unsolved problem of otitis media in indigenous populations: a systematic review of upper respiratory and middle ear microbiology in indigenous children with otitis media. Microbiome. 2018;6(1):1-15.
41. Jacoby P, Carville KS, Hall G, Riley TV, Bowman J, Leach AJ, et al. Crowding and other strong predictors of upper respiratory tract carriage of otitis media-related bacteria in Australian Aboriginal and non-Aboriginal children. The Pediatric infectious disease journal. 2011;30(6):480-5.
42. Blain A, MacNeil J, Wang X, Bennett N, Farley MM, Harrison LH, et al., editors. Invasive Haemophilus influenzae disease in adults≥ 65 years, United States, 2011. Open forum infectious diseases; 2014: Oxford University Press.
43. Drayß M, Claus H, Hubert K, Thiel K, Berger A, Sing A, et al. Asymptomatic carriage of Neisseria meningitidis, Haemophilus influenzae, Streptococcus pneumoniae, Group A Streptococcus and Staphylococcus aureus among adults aged 65 years and older. PloS one. 2019;14(2):e0212052.
44. Zanella RC, Brandileone MCdC, Almeida SCG, de Lemos APS, Sacchi CT, Gonçalves CR, et al. Nasopharyngeal carriage of Streptococcus pneumoniae, Haemophilus influenzae, and Staphylococcus aureus in a Brazilian elderly cohort. PloS one. 2019;14(8):e0221525.
45. Slack MP. A review of the role of Haemophilus influenzae in community-acquired pneumonia. Pneumonia. 2015;6(1):26.
46. Mukundan D, Ecevit Z, Patel M, Marrs CF, Gilsdorf JR. Pharyngeal colonization dynamics of Haemophilus influenzae and haemophilus haemolyticus in healthy adult carriers. Journal of clinical microbiology. 2008;46(4):1575.
47. Puig C, Marti S, Fleites A, Trabazo R, Calatayud L, Linares J, et al. Oropharyngeal colonization by nontypeable Haemophilus influenzae among healthy children attending day care centers. Microbial Drug Resistance. 2014;20(5):450-5.

48. Van Kempen M, Dhooge I, Vaneechoutte M, Claeys G. Turnover of Haemophilus influenzae isolates in otitis-prone children. Acta oto-rhino-laryngologica belgica. 2001;55(1):83.
49. Farjo RS, Foxman B, Patel MJ, Zhang L, Pettigrew MM, McCoy SI, et al. Diversity and sharing of Haemophilus influenzae strains colonizing healthy children attending day-care centers. The Pediatric infectious disease journal. 2004;23(1):41-6.
50. Kaur R, Chang A, Xu Q, Casey JR, Pichichero ME. Phylogenetic relatedness and diversity of non-typable Haemophilus influenzae in the nasopharynx and middle ear fluid of children with acute otitis media. Journal of medical microbiology. 2011;60(Pt 12):1841.
51. Murphy TF, Sethi S, Klingman KL, Brueggemann AB, Doern GV. Simultaneous respiratory tract colonization by multiple strains of nontypeable Haemophilus influenzae in chronic obstructive pulmonary disease: implications for antibiotic therapy. The journal of infectious diseases. 1999;180(2):404-9.
52. Bilton D, Pye A, Johnson M, Mitchell J, Dodd M, Webb A, et al. The isolation and characterization of non-typeable Haemophilus influenzae from the sputum of adult cystic fibrosis patients. European Respiratory Journal. 1995;8(6):948-53.
53. Román F, Cantón R, Pérez-Vázquez M, Baquero F, Campos J. Dynamics of long-term colonization of respiratory tract by Haemophilus influenzae in cystic fibrosis patients shows a marked increase in hypermutable strains. Journal of clinical microbiology. 2004;42(4):1450-9.
54. Cerquetti M, Giufrè M. Why we need a vaccine for non-typeable Haemophilus influenzae. Human vaccines & immunotherapeutics. 2016;12(9):2357-61.
55. Teele DW, Klein JO, Rosner B, Group GBOMS. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. Journal of infectious diseases. 1989;160(1):83-94.
56. Sierra A, Lopez P, Zapata MA, Vanegas B, Castrejon MM, DeAntonio R, et al. Non-typeable Haemophilus influenzae and Streptococcus pneumoniae as primary causes of acute otitis media in colombian children: a prospective study. BMC infectious diseases. 2011;11(1):4.
57. Monasta L, Ronfani L, Marchetti F, Montico M, Brumatti LV, Bavcar A, et al. Burden of disease caused by otitis media: systematic review and global estimates. PloS one. 2012;7(4):e36226.
58. Haggard M. Otitis media: prospects for prevention. Vaccine. 2008;26:G20-G4.
59. Barkai G, Leibovitz E, Givon-Lavi N, Dagan R. Potential contribution by nontypable Haemophilus influenzae in protracted and recurrent acute otitis media. The Pediatric infectious disease journal. 2009;28(6):466-71.
60. Mahadevan M, Navarro-Locsin G, Tan H, Yamanaka N, Sonsuwan N, Wang P-C, et al. A review of the burden of disease due to otitis media in the Asia-Pacific. International Journal of Pediatric Otorhinolaryngology. 2012;76(5):623-35.
61. Sun W, Jacoby P, Riley TV, Bowman J, Leach AJ, Coates H, et al. Association between early bacterial carriage and otitis media in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia: a cohort study. BMC infectious diseases. 2012;12(1):1-9.
62. Bakaletz LO. Immunopathogenesis of polymicrobial otitis media. Journal of leukocyte biology. 2010;87(2):213-22.
63. Leibovitz E. Complicated otitis media and its implications. Vaccine. 2008;26:G16-G9.
64. Arguedas A, Kvaerner K, Liese J, Schilder A, Pelton S. Otitis media across nine countries: disease burden and management. International journal of pediatric otorhinolaryngology. 2010;74(12):1419-24.
65. Welp AL, Bomberger JM. Bacterial community interactions during chronic respiratory disease. Frontiers in Cellular and Infection Microbiology. 2020;10:213.
66. Penido NdO, Chandrasekhar SS, Borin A, Maranhão ASdA, Testa JRG. Complications of otitis media-a potentially lethal problem still present. Brazilian journal of otorhinolaryngology. 2016;82(3):253-62.

67. Szelestey BR, Heimlich DR, Raffel FK, Justice SS, Mason KM. Haemophilus responses to nutritional immunity: epigenetic and morphological contribution to biofilm architecture, invasion, persistence and disease severity. PLoS pathogens. 2013;9(10):e1003709.
68. Quaderi S, Hurst J. The unmet global burden of COPD. Global health, epidemiology and genomics. 2018;3.
69. Finney LJ, Ritchie A, Pollard E, Johnston SL, Mallia P. Lower airway colonization and inflammatory response in COPD: a focus on Haemophilus influenzae. International journal of chronic obstructive pulmonary disease. 2014;9:1119.
70. Duell BL, Su YC, Riesbeck K. Host–pathogen interactions of nontypeable Haemophilus influenzae: from commensal to pathogen. FEBS letters. 2016;590(21):3840-53.
71. Chronic Respiratory Diseases in Australia. Australian Institute of Health and Welfare; 2005.
72. King P. Haemophilus influenzae and the lung (Haemophilus and the lung). Clinical and translational medicine. 2012;1(1):10.
73. Toews G. Impact of bacterial infections on airway diseases. European Respiratory Review. 2005;14(95):62-8.
74. Bandi V, Apicella MA, Mason E, Murphy TF, Siddiqi A, Atmar RL, et al. Nontypeable Haemophilus influenzae in the lower respiratory tract of patients with chronic bronchitis. American journal of respiratory and critical care medicine. 2001;164(11):2114-9.
75. Sethi S, Wrona C, Eschberger K, Lobbins P, Cai X, Murphy TF. Inflammatory profile of new bacterial strain exacerbations of chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2008;177(5):491-7.
76. Crisafulli E, Barbeta E, Ielpo A, Torres A. Management of severe acute exacerbations of COPD: an updated narrative review. Multidisciplinary respiratory medicine. 2018;13(1):1-15.
77. Sriram KB, Cox AJ, Clancy RL, Slack MP, Cripps AW. Nontypeable Haemophilus influenzae and chronic obstructive pulmonary disease: a review for clinicians. Critical Reviews in Microbiology. 2017:1-18.
78. Sethi S, Murphy TF. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state-of-the-art review. Clinical microbiology reviews. 2001;14(2):336-63.
79. Anderson E, Begg N, Crawshaw S, Hargreaves R, Howard A, Slack M. Epidemiology of invasive Haemophilus influenzae infections in England and Wales in the pre-vaccination era (1990–2). Epidemiology & Infection. 1995;115(1):89-100.
80. Peltola H. Worldwide Haemophilus influenzae type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. Clinical microbiology reviews. 2000;13(2):302-17.
81. Ladhani S, Slack MP, Heath PT, Von Gottberg A, Chandra M, Ramsay ME, et al. Invasive Haemophilus influenzae disease, Europe, 1996–2006. Emerging infectious diseases. 2010;16(3):455.
82. Whittaker R, Economopoulou A, Dias JG, Bancroft E, Ramliden M, Celentano LP. Epidemiology of invasive Haemophilus influenzae disease, Europe, 2007–2014. Emerging infectious diseases. 2017;23(3):396.
83. Cheong JWS, Smith H, Heney C, Robson J, Schlebusch S, Fu J, et al. Trends in the epidemiology of invasive Haemophilus influenzae disease in Queensland, Australia from 2000 to 2013: what is the impact of an increase in invasive non-typable H. influenzae (NTHi)? Epidemiology & Infection. 2015;143(14):2993-3000.
84. Giufrè M, Fabiani M, Cardines R, Riccardo F, Caporali MG, D'Ancona F, et al. Increasing trend in invasive non-typeable Haemophilus influenzae disease and molecular characterization of the isolates, Italy, 2012–2016. Vaccine. 2018;36(45):6615-22.

85. Behrouzi A, Vaziri F, Rahimi-Jamnani F, Afrough P, Rahbar M, Satarian F, et al. Vaccine candidates against nontypeable haemophilus influenzae: a review. Iranian Biomedical Journal. 2017;21(2):69.
86. Collins S, Litt DJ, Flynn S, Ramsay ME, Slack MP, Ladhani SN. Neonatal invasive Haemophilus influenzae disease in England and Wales: epidemiology, clinical characteristics, and outcome. Clinical Infectious Diseases. 2015;60(12):1786-92.
87. Slack MP. A review of the role of Haemophilus influenzae in community-acquired pneumonia. Pneumonia. 2015;6:26-43.
88. Tamir SO, Sibbald A, Rupa V, Marchisio P, Homøe P, Daniel SJ, et al. Guidelines for the Treatment of Acute Otitis Media: Why Are There Worldwide Differences? Current Otorhinolaryngology Reports. 2017;5(2):101-7.
89. Maddi S, Kolsum U, Jackson S, Barraclough R, Maschera B, Simpson KD, et al. Ampicillin resistance in Haemophilus influenzae from COPD patients in the UK. International journal of chronic obstructive pulmonary disease. 2017;12:1507-18.
90. Ni W, Shao X, Cai X, Wei C, Cui J, Wang R, et al. Prophylactic use of macrolide antibiotics for the prevention of chronic obstructive pulmonary disease exacerbation: a meta-analysis. PloS one. 2015;10(3):e0121257.
91. Dixit D, Bridgeman MB, Madduri RP, Kumar ST, Cawley MJ. Pharmacological Management and Prevention Of Exacerbations of Chronic Obstructive Pulmonary Disease in Hospitalized Patients. Pharmacy and Therapeutics. 2016;41(11):703.
92. Smith-Vaughan HC, Chang AB, Sarovich DS, Marsh RL, Grimwood K, Leach AJ, et al. Absence of an important vaccine and diagnostic target in carriage-and disease-related nontypeable Haemophilus influenzae. Clin Vaccine Immunol. 2014;21(2):250-2.
93. Wang H-J, Wang C-Q, Hua C-Z, Yu H, Zhang T, Zhang H, et al. Antibiotic resistance profiles of Haemophilus influenzae isolates from children in 2016: a Multicenter study in China. Canadian Journal of Infectious Diseases and Medical Microbiology. 2019;2019.
94. de Carvalho CX, Kipnis A, Thörn L, de Andrade JG, Pimenta F, de Cunto Brandileone MC, et al. Carriage of Haemophilus influenzae among Brazilian children attending day care centers in the era of widespread Hib vaccination. Vaccine. 2011;29(7):1438-42.
95. Resman F, Ristovski M, Forsgren A, Kaijser B, Kronvall G, Medstrand P, et al. Increase of β-lactam-resistant invasive Haemophilus influenzae in Sweden, 1997 to 2010. Antimicrobial agents and chemotherapy. 2012;56(8):4408-15.
96. Puig C, Tirado-Vélez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C, et al. Molecular characterization of fluoroquinolone resistance in nontypeable Haemophilus influenzae clinical isolates. Antimicrobial agents and chemotherapy. 2015;59(1):461-6.
97. Dabernat H, Delmas C. Epidemiology and evolution of antibiotic resistance of Haemophilus influenzae in children 5 years of age or less in France, 2001–2008: a retrospective database analysis. European journal of clinical microbiology & infectious diseases. 2012;31(10):2745-53.
98. Li X-X, Xiao S-Z, Gu F-F, He W-P, Ni Y-X, Han L-Z. Molecular Epidemiology and Antimicrobial Resistance of Haemophilus influenzae in Adult Patients in Shanghai, China. Frontiers in public health. 2020;8:95.
99. Dagan R, Abramson O, Leibovitz E, Greenberg D, Lang R, Goshen S, et al. Bacteriologic response to oral cephalosporins: are established susceptibility breakpoints appropriate in the case of acute otitis media? Journal of Infectious Diseases. 1997;176(5):1253-9.
100. Patel JA, Reisner B, Vizirinia N, Owen M, Chonmaitree T, Howie V. Bacteriologic failure of amoxicillin-clavulanate in treatment of acute otitis media caused by nontypeable Haemophilus influenzae. The Journal of pediatrics. 1995;126(5):799-806.
101. Andersson M, Resman F, Eitrem R, Drobni P, Riesbeck K, Kahlmeter G, et al. Outbreak of a beta-lactam resistant non-typeable Haemophilus influenzae sequence

type 14 associated with severe clinical outcomes. BMC infectious diseases. 2015;15(1):1-6.
102. Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natås OB, Steinbakk M, et al. Emergence of clonally related multidrug resistant Haemophilus influenzae with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. Eurosurveillance. 2014;19(49):20986.
103. Heliodoro CIM, Bettencourt CR, Bajanca-Lavado MP. Molecular epidemiology of invasive Haemophilus influenzae disease in Portugal: an update of the post-vaccine period, 2011–2018. European Journal of Clinical Microbiology & Infectious Diseases. 2020:1-10.
104. Yamada S, Seyama S, Wajima T, Yuzawa Y, Saito M, Tanaka E, et al. β-Lactamase-non-producing ampicillin-resistant Haemophilus influenzae is acquiring multidrug resistance. Journal of infection and public health. 2020;13(4):497-501.
105. Honda H, Sato T, Shinagawa M, Fukushima Y, Nakajima C, Suzuki Y, et al. Multiclonal expansion and high prevalence of β-lactamase-negative Haemophilus influenzae with high-level ampicillin resistance in Japan and susceptibility to quinolones. Antimicrobial agents and chemotherapy. 2018;62(9).
106. Ito M, Hotomi M, Maruyama Y, Hatano M, Sugimoto H, Yoshizaki T, et al. Clonal spread of β-lactamase-producing amoxicillin–clavulanate-resistant (BLPACR) strains of non-typeable Haemophilus influenzae among young children attending a day care in Japan. International journal of pediatric otorhinolaryngology. 2010;74(8):901-6.
107. Hegstad K, Mylvaganam H, Janice J, Josefsen E, Sivertsen A, Skaare D. Role of Horizontal Gene Transfer in the Development of Multidrug Resistance in Haemophilus influenzae. Msphere. 2020;5(1).
108. Pfeifer Y, Meisinger I, Brechtel K, Gröbner S. Emergence of a multidrug-resistant Haemophilus influenzae strain causing chronic pneumonia in a patient with common variable immunodeficiency. Microbial Drug Resistance. 2013;19(1):1-5.
109. Pérez-Vázquez M, Román F, Aracil B, Cantón R, Campos J. Laboratory detection of Haemophilus influenzae with decreased susceptibility to nalidixic acid, ciprofloxacin, levofloxacin, and moxifloxacin due to GyrA and ParC mutations. Journal of clinical microbiology. 2004;42(3):1185-91.
110. Phaff SJ, Tiddens HA, Verbrugh HA, Ott A. Macrolide resistance of Staphylococcus aureus and Haemophilus species associated with long-term azithromycin use in cystic fibrosis. Journal of Antimicrobial Chemotherapy. 2006;57(4):741-6.
111. Casey JR, Pichichero ME. Changes in frequency and pathogens causing acute otitis media in 1995–2003. The Pediatric infectious disease journal. 2004;23(9):824-8.
112. Su P-Y, Huang A-H, Lai C-H, Lin H-F, Lin T-M, Ho C-H. Extensively drug-resistant Haemophilus influenzae–emergence, epidemiology, risk factors, and regimen. BMC microbiology. 2020;20:1-10.
113. Vila J, Ruiz J, Sanchez F, Navarro F, Mirelis B, de Anta MTJ, et al. Increase in Quinolone Resistance in aHaemophilus influenzae Strain Isolated from a Patient with Recurrent Respiratory Infections Treated with Ofloxacin. Antimicrobial agents and chemotherapy. 1999;43(1):161-2.
114. Bastida T, Perez-Vazquez M, Campos J, Cortés-Lletget MC, Román F, Tubau F, et al. Levofloxacin treatment failure in Haemophilus influenzae pneumonia. 2003.
115. Desai S, Jamieson FB, Patel SN, Seo CY, Dang V, Fediurek J, et al. The epidemiology of invasive Haemophilus influenzae non-serotype B disease in Ontario, Canada from 2004 to 2013. PloS one. 2015;10(11):e0142179.
116. Pumarola F, Marès J, Losada I, Minguella I, Moraga F, Tarragó D, et al. Microbiology of bacteria causing recurrent acute otitis media (AOM) and AOM treatment failure in young children in Spain: shifting pathogens in the post-pneumococcal conjugate vaccination era. International journal of pediatric otorhinolaryngology. 2013;77(8):1231-6.
117. Pettigrew MM, Tsuji BT, Gent JF, Kong Y, Holden PN, Sethi S, et al. Effect of fluoroquinolones and macrolides on eradication and resistance of Haemophilus

influenzae in chronic obstructive pulmonary disease. Antimicrobial agents and chemotherapy. 2016;60(7):4151-8.
118. Venekamp RP, Sanders SL, Glasziou PP, Del Mar CB, Rovers MM. Antibiotics for acute otitis media in children. Cochrane database of systematic reviews. 2015(6).
119. Rothberg MB, Pekow PS, Lahti M, Brody O, Skiest DJ, Lindenauer PK. Antibiotic therapy and treatment failure in patients hospitalized for acute exacerbations of chronic obstructive pulmonary disease. Jama. 2010;303(20):2035-42.
120. Roede BM, Bresser P, Prins JM, Schellevis F, Verheij TJ, Bindels PJ. Reduced risk of next exacerbation and mortality associated with antibiotic use in COPD. European Respiratory Journal. 2009;33(2):282-8.
121. Casey JR, Adlowitz DG, Pichichero ME. New patterns in the otopathogens causing acute otitis media six to eight years after introduction of pneumococcal conjugate vaccine. The Pediatric infectious disease journal. 2010;29(4):304.
122. García-Cobos S, Moscoso M, Pumarola F, Arroyo M, Lara N, Pérez-Vázquez M, et al. Frequent carriage of resistance mechanisms to β-lactams and biofilm formation in Haemophilus influenzae causing treatment failure and recurrent otitis media in young children. Journal of Antimicrobial Chemotherapy. 2014;69(9):2394-9.
123. Miravitlles M, Anzueto A. Antibiotic prophylaxis in COPD: Why, when, and for whom? Pulmonary pharmacology & therapeutics. 2015;32:119-23.
124. Hariadi NI, Zhang L, Patel M, Sandstedt SA, Davis GS, Marrs CF, et al. Comparative profile of heme acquisition genes in disease-causing and colonizing nontypeable Haemophilus influenzae and Haemophilus haemolyticus. Journal of clinical microbiology. 2015;53(7):2132-7.
125. Tong S, Amand C, Kieffer A, Kyaw MH. Trends in healthcare utilization and costs associated with acute otitis media in the United States during 2008–2014. BMC health services research. 2018;18(1):1-10.
126. Angoulvant F, Cohen R, Doit C, Elbez A, Werner A, Béchet S, et al. Trends in antibiotic resistance of Streptococcus pneumoniae and Haemophilus influenzae isolated from nasopharyngeal flora in children with acute otitis media in France before and after 13 valent pneumococcal conjugate vaccine introduction. BMC infectious diseases. 2015;15(1):1-8.
127. Clarke C, Bakaletz LO, Ruiz-Guiñazú J, Borys D, Mrkvan T. Impact of protein D-containing pneumococcal conjugate vaccines on non-typeable Haemophilus influenzae acute otitis media and carriage. Expert review of vaccines. 2017;16(7):751-64.
128. Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, et al. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. The Lancet. 2006;367(9512):740-8.
129. Vesikari T, Forsten A, Seppä I, Kaijalainen T, Puumalainen T, Soininen A, et al. Effectiveness of the 10-valent pneumococcal nontypeable Haemophilus influenzae protein d–conjugated vaccine (PHiD-CV) against carriage and acute otitis media—a double-blind randomized clinical trial in Finland. Journal of the Pediatric Infectious Diseases Society. 2016;5(3):237-48.
130. Siggins MK, Gill SK, Langford PR, Li Y, Ladhani SN, Tregoning JS. PHiD-CV induces anti-Protein D antibodies but does not augment pulmonary clearance of nontypeable Haemophilus influenzae in mice. Vaccine. 2015;33(38):4954-61.
131. Ysebaert C, Denoël P, Weynants V, Bakaletz LO, Novotny LA, Godfroid F, et al. A protein E-PilA fusion protein shows vaccine potential against nontypeable Haemophilus influenzae in mice and chinchillas. Infection and immunity. 2019;87(8).
132. Knerer G, Ismaila A, Pearce D. Health and economic impact of PHiD-CV in Canada and the UK: a Markov modelling exercise. Journal of medical economics. 2012;15(1):61-76.

133. Murphy TF. Vaccines for nontypeable Haemophilus influenzae: the future is now. Clinical and Vaccine Immunology. 2015;22(5):459-66.
134. Novotny LA, Brockman KL, Mokrzan EM, Jurcisek JA, Bakaletz LO. Biofilm biology and vaccine strategies for otitis media due to nontypeable Haemophilus influenzae. Journal of pediatric infectious diseases. 2019;14(2):69.
135. Collins S, Vickers A, Ladhani SN, Flynn S, Platt S, Ramsay ME, et al. Clinical and molecular epidemiology of childhood invasive nontypeable Haemophilus influenzae disease in England and Wales. The pediatric infectious disease journal. 2016;35(3):e76-e84.
136. Parrow NL, Fleming RE, Minnick MF. Sequestration and scavenging of iron in infection. Infection and immunity. 2013;81(10):3503-14.
137. Hare SA. Diverse structural approaches to haem appropriation by pathogenic bacteria. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2017;1865(4):422-33.
138. Choby JE, Skaar EP. Heme synthesis and acquisition in bacterial pathogens. Journal of molecular biology. 2016;428(17):3408-28.
139. Clementi C, Murphy T. Non-typeable Haemophilus influenzae invasion and persistence in the human respiratory tract. Frontiers in cellular and infection microbiology. 2011;1:1.
140. Goyal M, Singh M, Ray P, Srinivasan R, Chakraborti A. Cellular interaction of nontypeable Haemophilus influenzae triggers cytotoxicity of infected type II alveolar cells via apoptosis. Pathogens and disease. 2015;73(2):1.
141. Moriyama S, Hotomi M, Shimada J, Billal DS, Fujihara K, Yamanaka N. Formation of biofilm by Haemophilus influenzae isolated from pediatric intractable otitis media. Auris Nasus Larynx. 2009;36(5):525-31.
142. Harrison A, Santana EA, Szelestey BR, Newsom DE, White P, Mason KM. Ferric uptake regulator and its role in the pathogenesis of nontypeable Haemophilus influenzae. Infection and immunity. 2013;81(4):1221-33.
143. Rodríguez-Arce I, Al-Jubair T, Euba B, Fernández-Calvet A, Gil-Campillo C, Martí S, et al. Moonlighting of Haemophilus influenzae heme acquisition systems contributes to the host airway-pathogen interplay in a coordinated manner. Virulence. 2019;10(1):315-33.
144. Hare KM, Grimwood K, Leach AJ, Smith-Vaughan H, Torzillo PJ, Morris PS, et al. Respiratory bacterial pathogens in the nasopharynx and lower airways of Australian indigenous children with bronchiectasis. The Journal of pediatrics. 2010;157(6):1001-5.
145. Harabuchi Y, Faden H, Yamanaka N, Duffy L, Wolf J, Krystofik D, et al. Nasopharyngeal colonization with nontypeable Haemophilus influenzae and recurrent otitis media. Journal of infectious diseases. 1994;170(4):862-6.
146. Sethi S, Evans N, Grant BJ, Murphy TF. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. New England Journal of Medicine. 2002;347(7):465-71.
147. Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, et al. Measuring nasal bacterial load and its association with otitis media. BMC Ear, Nose and Throat Disorders. 2006;6(1):10.
148. Smith-Vaughan HC, Binks MJ, Marsh RL, Kaestli M, Ward L, Hare KM, et al. Dominance of Haemophilus influenzae in ear discharge from Indigenous Australian children with acute otitis media with tympanic membrane perforation. BMC Ear, Nose and Throat Disorders. 2013;13(1):1-9.
149. Pettigrew MM, Laufer AS, Gent JF, Kong Y, Fennie KP, Metlay JP. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. Applied and environmental microbiology. 2012;78(17):6262-70.
150. Atto B, Eapen MS, Sharma P, Frey U, Ammit AJ, Markos J, et al. New therapeutic targets for the prevention of infectious acute exacerbations of COPD: role of epithelial adhesion molecules and inflammatory pathways. Clinical Science. 2019;133(14):1663-703.

151. Qu J, Lesse AJ, Brauer AL, Cao J, Gill SR, Murphy TF. Proteomic expression profiling of Haemophilus influenzae grown in pooled human sputum from adults with chronic obstructive pulmonary disease reveal antioxidant and stress responses. BMC microbiology. 2010;10(1):1-12.
152. Pidcock KA, Wooten JA, Daley BA, Stull TL. Iron acquisition by Haemophilus influenzae. Infection and immunity. 1988;56(4):721-5.
153. Hassan HM, Troxell B. Transcriptional regulation by Ferric Uptake Regulator (Fur) in pathogenic bacteria. Frontiers in cellular and infection microbiology. 2013;3:59.
154. Rempe KA, Porsch EA, Wilson JM, Geme JWS. The HMW1 and HMW2 adhesins enhance the ability of nontypeable Haemophilus influenzae to colonize the upper respiratory tract of rhesus macaques. Infection and immunity. 2016;84(10):2771-8.
155. Ikeda M, Enomoto N, Hashimoto D, Fujisawa T, Inui N, Nakamura Y, et al. Nontypeable Haemophilus influenzae exploits the interaction between protein-E and vitronectin for the adherence and invasion to bronchial epithelial cells. BMC microbiology. 2015;15(1):1-11.
156. Ronander E, Brant M, Eriksson E, Mörgelin M, Hallgren O, Westergren-Thorsson G, et al. Nontypeable Haemophilus influenzae adhesin protein E: characterization and biological activity. The Journal of infectious diseases. 2009;199(4):522-31.
157. Al Jubair T, Singh B, Fleury C, Blom AM, Mörgelin M, Thunnissen MM, et al. Haemophilus influenzae stores and distributes hemin by using protein E. International Journal of Medical Microbiology. 2014;304(5-6):662-8.
158. Mason KM, Raffel FK, Ray WC, Bakaletz LO. Heme utilization by nontypeable Haemophilus influenzae is essential and dependent on Sap transporter function. Journal of bacteriology. 2011;193(10):2527-35.
159. Vogel AR, Szelestey BR, Raffel FK, Sharpe SW, Gearinger RL, Justice SS, et al. SapF-mediated heme-iron utilization enhances persistence and coordinates biofilm architecture of Haemophilus. Frontiers in cellular and infection microbiology. 2012;2:42.
160. Raffel FK, Szelestey BR, Beatty WL, Mason KM. The Haemophilus influenzae Sap transporter mediates bacterium-epithelial cell homeostasis. Infection and immunity. 2013;81(1):43-54.
161. Ghio AJ. Disruption of iron homeostasis and lung disease. Biochimica et Biophysica Acta (BBA)-General Subjects. 2009;1790(7):731-9.
162. Mason KM, Munson RS, Bakaletz LO. A mutation in the sap operon attenuates survival of nontypeable Haemophilus influenzae in a chinchilla model of otitis media. Infection and immunity. 2005;73(1):599-608.
163. Morton DJ, Bakaletz LO, Jurcisek JA, VanWagoner TM, Seale TW, Whitby PW, et al. Reduced severity of middle ear infection caused by nontypeable Haemophilus influenzae lacking the hemoglobin/hemoglobin–haptoglobin binding proteins (Hgp) in a chinchilla model of otitis media. Microbial pathogenesis. 2004;36(1):25-33.
164. Maciver I, Latimer JL, Liem H, Muller-Eberhard U, Hrkal Z, Hansen EJ. Identification of an outer membrane protein involved in utilization of hemoglobin-haptoglobin complexes by nontypeable Haemophilus influenzae. Infection and immunity. 1996;64(9):3703-12.
165. Zhang L, Xie J, Patel M, Bakhtyar A, Ehrlich GD, Ahmed A, et al. Nontypeable Haemophilus influenzae genetic islands associated with chronic pulmonary infection. PLoS One. 2012;7(9):e44730.
166. Hardie K, Adams RA, Towner K. Transferrin-binding ability of invasive and commensal isolates of Haemophilus spp. Journal of medical microbiology. 1993;39(3):218-24.
167. Morton DJ, Seale TW, Bakaletz LO, Jurcisek JA, Smith A, VanWagoner TM, et al. The heme-binding protein (HbpA) of Haemophilus influenzae as a virulence determinant. International Journal of Medical Microbiology. 2009;299(7):479-88.
168. Seale TW, Morton DJ, Whitby PW, Wolf R, Kosanke SD, VanWagoner TM, et al. Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in Haemophilus influenzae virulence in the infant rat model of invasive infection. Infection and immunity. 2006;74(11):6213-25.

169. Ahearn CP, Gallo MC, Murphy TF. Insights on persistent airway infection by non-typeable Haemophilus influenzae in chronic obstructive pulmonary disease. Pathogens and Disease. 2017;75(4).
170. Morton DJ, Seale TW, Madore LL, VanWagoner TM, Whitby PW, Stull TL. The haem–haemopexin utilization gene cluster (hxuCBA) as a virulence factor of Haemophilus influenzae. Microbiology. 2007;153(1):215-24.
171. Gray-Owen SD, Schryvers AB. Characterization of transferrin binding proteins 1 and 2 in invasive type b and nontypeable strains of Haemophilus influenzae. Infection and immunity. 1995;63(10):3809-15.
172. LaCross NC, Marrs CF, Gilsdorf JR. Otitis media associated polymorphisms in the hemin receptor HemR of nontypeable Haemophilus influenzae. Infection, Genetics and Evolution. 2014;26:47-57.
173. Osanai A, Li SJ, Asano K, Sashinami H, Hu DL, Nakane A. Fibronectin‐binding protein, FbpA, is the adhesin responsible for pathogenesis of Listeria monocytogenes infection. Microbiology and immunology. 2013;57(4):253-62.
174. Morton DJ, Turman EJ, Hensley PD, Whitby PW, Stull TL, Seale TW, et al. Identification of a siderophore utilization locus in nontypeable Haemophilus influenzae. BMC microbiology. 2010;10(1):113.
175. Singh B, Brant M, Kilian M, Hallström B, Riesbeck K. Protein E of Haemophilus influenzae is a ubiquitous highly conserved adhesin. The Journal of infectious diseases. 2010;201(3):414-9.
176. Clementi CF, Håkansson AP, Murphy TF. Internalization and trafficking of nontypeable Haemophilus influenzae in human respiratory epithelial cells and roles of IgA1 proteases for optimal invasion and persistence. Infection and immunity. 2014;82(1):433-44.
177. Rosadini CV, Wong SM, Akerley BJ. The periplasmic disulfide oxidoreductase DsbA contributes to Haemophilus influenzae pathogenesis. Infection and immunity. 2008;76(4):1498-508.
178. Vergauwen B, Elegheert J, Dansercoer A, Devreese B, Savvides SN. Glutathione import in Haemophilus influenzae Rd is primed by the periplasmic heme-binding protein HbpA. Proceedings of the National Academy of Sciences. 2010;107(30):13270-5.
179. Tanaka KJ, Pinkett HW. Oligopeptide-binding protein from nontypeable Haemophilus influenzae has ligand-specific sites to accommodate peptides and heme in the binding pocket. Journal of Biological Chemistry. 2019;294(3):1070-82.
180. Hardison RL, Heimlich DR, Harrison A, Beatty WL, Rains S, Moseley MA, et al. Transient nutrient deprivation promotes macropinocytosis-dependent intracellular bacterial community development. MSphere. 2018;3(5).
181. Atack JM, Srikhanta YN, Fox KL, Jurcisek JA, Brockman KL, Clark TA, et al. A biphasic epigenetic switch controls immunoevasion, virulence and niche adaptation in non-typeable Haemophilus influenzae. Nature communications. 2015;6(1):1-12.
182. Jung WH, Do E. Iron acquisition in the human fungal pathogen Cryptococcus neoformans. Current opinion in microbiology. 2013;16(6):686-91.
183. Chen C, Pande K, French SD, Tuch BB, Noble SM. An iron homeostasis regulatory circuit with reciprocal roles in Candida albicans commensalism and pathogenesis. Cell host & microbe. 2011;10(2):118-35.
184. Marvig RL, Damkiær S, Khademi SH, Markussen TM, Molin S, Jelsbak L. Within-host evolution of Pseudomonas aeruginosa reveals adaptation toward iron acquisition from hemoglobin. MBio. 2014;5(3).
185. Ren Z, Jin H, Whitby PW, Morton DJ, Stull TL. Role of CCAA Nucleotide Repeats in Regulation of Hemoglobin and Hemoglobin-Haptoglobin Binding Protein Genes ofHaemophilus influenzae. Journal of bacteriology. 1999;181(18):5865-70.
186. Poole J, Foster E, Chaloner K, Hunt J, Jennings MP, Bair T, et al. Analysis of nontypeable Haemophilus influenzae phase-variable genes during experimental

human nasopharyngeal colonization. The Journal of infectious diseases. 2013;208(5):720-7.
187. Richardson AR, Stojiljkovic I. HmbR, a hemoglobin-binding outer membrane protein of Neisseria meningitidis, undergoes phase variation. Journal of Bacteriology. 1999;181(7):2067-74.
188. Rao VK, Krasan GP, Hendrixson DR, Dawid S, St. Geme III JW. Molecular determinants of the pathogenesis of disease due to non-typable Haemophilus influenzae. FEMS microbiology reviews. 1999;23(2):99-129.
189. Brockman KL, Jurcisek JA, Atack JM, Srikhanta YN, Jennings MP, Bakaletz LO. ModA2 phasevarion switching in nontypeable Haemophilus influenzae increases the severity of experimental otitis media. The Journal of infectious diseases. 2016;214(5):817-24.
190. Brockman KL, Branstool MT, Atack JM, Robledo-Avila F, Partida-Sanchez S, Jennings MP, et al. The ModA2 phasevarion of nontypeable Haemophilus influenzae regulates resistance to oxidative stress and killing by human neutrophils. Scientific reports. 2017;7(1):1-11.
191. Brockman KL, Azzari PN, Branstool MT, Atack JM, Schulz BL, Jen FE-C, et al. Epigenetic regulation alters biofilm architecture and composition in multiple clinical isolates of nontypeable Haemophilus influenzae. MBio. 2018;9(5).
192. Atack JM, Winter LE, Jurcisek JA, Bakaletz LO, Barenkamp SJ, Jennings MP. Selection and counterselection of Hia expression reveals a key role for phase-variable expression of Hia in infection caused by nontypeable Haemophilus influenzae. The Journal of infectious diseases. 2015;212(4):645-53.
193. Ahearn CP, Gallo MC, Murphy TF. Insights on persistent airway infection by non-typeable Haemophilus influenzae in chronic obstructive pulmonary disease. Pathogens and disease. 2017;75(4):ftx042.
194. Murphy TF, Brauer AL, Schiffmacher AT, Sethi S. Persistent colonization by Haemophilus influenzae in chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2004;170(3):266-72.
195. Morton DJ, Hempel RJ, Whitby PW, Seale TW, Stull TL. An invasive Haemophilus haemolyticus isolate. Journal of clinical microbiology. 2012;50(4):1502-3.
196. Vogel AR, Szelestey BR, Raffel FK, Sharpe SW, Gearinger RL, Justice SS, et al. SapF-mediated heme-iron utilization enhances persistence and coordinates biofilm architecture of Haemophilus. Frontiers in cellular and infection microbiology. 2012;2.
197. Clifton MC, Corrent C, Strong RK. Siderocalins: siderophore-binding proteins of the innate immune system. Biometals. 2009;22(4):557-64.
198. Nelson AL, Barasch JM, Bunte RM, Weiser JN. Bacterial colonization of nasal mucosa induces expression of siderocalin, an iron‐sequestering component of innate immunity. Cellular microbiology. 2005;7(10):1404-17.
199. Holden VI, Bachman MA. Diverging roles of bacterial siderophores during infection. Metallomics. 2015;7(6):986-95.
200. Geitani R, Moubareck CA, Xu Z, Karam Sarkis D, Touqui L. Expression and Roles of Antimicrobial Peptides in Innate Defense of Airway Mucosa: Potential Implication in Cystic Fibrosis. Frontiers in Immunology. 2020;11:1198.
201. Shelton CL, Raffel FK, Beatty WL, Johnson SM, Mason KM. Sap transporter mediated import and subsequent degradation of antimicrobial peptides in Haemophilus. PLoS Pathog. 2011;7(11):e1002360.
202. Vergauwen B, Pauwels F, Van Beeumen JJ. Glutathione and catalase provide overlapping defenses for protection against respiration-generated hydrogen peroxide in Haemophilus influenzae. Journal of bacteriology. 2003;185(18):5555-62.
203. Mason KM, Bruggeman ME, Munson RS, Bakaletz LO. The non‐typeable Haemophilus influenzae Sap transporter provides a mechanism of antimicrobial

peptide resistance and SapD‐dependent potassium acquisition. Molecular microbiology. 2006;62(5):1357-72.
204. Persson LJ, Aanerud M, Hardie JA, Nilsen RM, Bakke PS, Eagan TM, et al. Antimicrobial peptide levels are linked to airway inflammation, bacterial colonisation and exacerbations in chronic obstructive pulmonary disease. European Respiratory Journal. 2017;49(3).
205. Hardison RL, Harrison A, Wallace RM, Heimlich DR, O'Bryan ME, Sebra RP, et al. Microevolution in response to transient heme-iron restriction enhances intracellular bacterial community development and persistence. PLoS pathogens. 2018;14(10):e1007355.
206. Harrison A, Hardison RL, Fullen AR, Wallace RM, Gordon DM, White P, et al. Continuous microevolution accelerates disease progression during sequential episodes of infection. Cell reports. 2020;30(9):2978-88. e3.
207. Kaya E, Dag I, Incesulu A, Gurbuz MK, Acar M, Birdane L. Investigation of the presence of biofilms in chronic suppurative otitis media, nonsuppurative otitis media, and chronic otitis media with cholesteatoma by scanning electron microscopy. The scientific world journal. 2013;2013.
208. Gu X, Keyoumu Y, Long L, Zhang H. Detection of bacterial biofilms in different types of chronic otitis media. European Archives of Oto-Rhino-Laryngology. 2014;271(11):2877-83.
209. Sgheiza V, Novick B, Stanton S, Pierce J, Kalmeta B, Holmquist MF, et al. Covalent bonding of heme to protein prevents heme capture by nontypeable Haemophilus influenzae. FEBS open bio. 2017;7(11):1778-83.
210. White DC, Granick S. Hemin biosynthesis in Haemophilus. Journal of bacteriology. 1963;85(4):842-50.
211. Morton DJ, Smith A, Ren Z, Madore LL, VanWagoner TM, Seale TW, et al. Identification of a haem-utilization protein (Hup) in Haemophilus influenzae. Microbiology. 2004;150(12):3923-33.
212. Barber MF, Elde NC. Buried treasure: evolutionary perspectives on microbial iron piracy. Trends in Genetics. 2015;31(11):627-36.
213. Cassat JE, Skaar EP. Iron in infection and immunity. Cell host & microbe. 2013;13(5):509-19.
214. Ganz T. Iron and infection. International journal of hematology. 2018;107(1):7-15.
215. Cloonan SM, Mumby S, Adcock IM, Choi AM, Chung KF, Quinlan GJ. The "iron"-y of iron overload and iron deficiency in chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2017;196(9):1103-12.
216. Ali MK, Kim RY, Karim R, Mayall JR, Martin KL, Shahandeh A, et al. Role of iron in the pathogenesis of respiratory disease. The international journal of biochemistry & cell biology. 2017;88:181-95.
217. Smith A, McCulloh RJ. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. Frontiers in physiology. 2015;6:187.
218. Whitby PW, Seale TW, VanWagoner TM, Morton DJ, Stull TL. The iron/heme regulated genes of Haemophilus influenzae: comparative transcriptional profiling as a tool to define the species core modulon. BMC genomics. 2009;10(1):1-19.
219. Fournier C, Smith A, Delepelaire P. Haem release from haemopexin by HxuA allows Haemophilus influenzae to escape host nutritional immunity. Molecular microbiology. 2011;80(1):133-48.
220. Williams P, Morton DJ, Towner KJ, Stevenson P, Griffiths E. Utilization of enterobactin and other exogenous iron sources by Haemophilus influenzae, H. parainfluenzae and H. paraphrophilus. Microbiology. 1990;136(12):2343-50.
221. Morton DJ, VanWagoner TM, Seale TW, Whitby PW, Stull TL. Differential utilization by Haemophilus influenzae of haemoglobin complexed to the three human haptoglobin phenotypes. FEMS Immunology & Medical Microbiology. 2006;46(3):426-32.

222. Langlois MR, Delanghe JR. Biological and clinical significance of haptoglobin polymorphism in humans. Clinical chemistry. 1996;42(10):1589-600.
223. Bullen J, Griffiths E, Edmiston CE. Iron and infection: molecular, physiological and clinical aspects. LWW; 1999.
224. Morton DJ, Turman EJ, Hensley PD, VanWagoner TM, Seale TW, Whitby PW, et al. Identification of a siderophore utilization locus in nontypeable Haemophilus influenzae. BMC microbiology. 2010;10(1):1-12.
225. Noinaj N, Guillier M, Barnard TJ, Buchanan SK. TonB-dependent transporters: regulation, structure, and function. Annual review of microbiology. 2010;64:43-60.
226. Kortman GA, Boleij A, Swinkels DW, Tjalsma H. Iron availability increases the pathogenic potential of Salmonella typhimurium and other enteric pathogens at the intestinal epithelial interface. PloS one. 2012;7(1):e29968.
227. Reid DW, Anderson GJ, Lamont IL. Role of lung iron in determining the bacterial and host struggle in cystic fibrosis. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2009;297(5):L795-L802.
228. Nairz M, Schroll A, Haschka D, Dichtl S, Tymoszuk P, Demetz E, et al. Genetic and dietary iron overload differentially affect the course of Salmonella typhimurium infection. Frontiers in cellular and infection microbiology. 2017;7:110.
229. Rahav G, Volach V, Shapiro M, Rund D, Rachmilewitz EA, Goldfarb A. Severe infections in thalassaemic patients: prevalence and predisposing factors. British journal of haematology. 2006;133(6):667-74.
230. Teawtrakul N, Jetsrisuparb A, Sirijerachai C, Chansung K, Wanitpongpun C. Severe bacterial infections in patients with non-transfusion-dependent thalassemia: prevalence and clinical risk factors. International Journal of Infectious Diseases. 2015;39:53-6.
231. Kontoghiorghes GJ, Kolnagou A, Skiada A, Petrikkos G. The role of iron and chelators on infections in iron overload and non iron loaded conditions: prospects for the design of new antimicrobial therapies. Hemoglobin. 2010;34(3):227-39.
232. Wright AC, SimpSOn LM, Oliver JD. Role of iron in the pathogenesis of Vibrio vulnificus infections. Infection and immunity. 1981;34(2):503-7.
233. Quenee LE, Hermanas TM, Ciletti N, Louvel H, Miller NC, Elli D, et al. Hereditary hemochromatosis restores the virulence of plague vaccine strains. The Journal of infectious diseases. 2012;206(7):1050-8.
234. Weinberg E. Microbial pathogens with impaired ability to acquire host iron. Biometals. 2000;13(1):85-9.
235. Ballart IJ, Estevez ME, Sen L, Diez RA, Giuntoli J, De Miani SA, et al. Progressive dysfunction of monocytes associated with iron overload and age in patients with thalassemia major. 1986.
236. Cantinieaux B, Hariga C, Ferster A, De Maertelaere E, Toppet M, Fondu P. Neutrophil dysfunctions in thalassaemia major: the role of cell iron overload. European journal of haematology. 1987;39(1):28-34.
237. Cherayil BJ. Iron and immunity: immunological consequences of iron deficiency and overload. Archivum immunologiae et therapiae experimentalis. 2010;58(6):407-15.
238. Kutukculer N, Kutlu O, Nisli G, Oztop S, Cetingul N, Caglayan S. Assessment of neutrophil chemotaxis and random migration in children with thalassemia major. Pediatric hematology and oncology. 1996;13(3):239-45.
239. Porto G, De Sousa M. Iron overload and immunity. World journal of gastroenterology: WJG. 2007;13(35):4707.
240. Ashrafian H. Hepcidin: the missing link between hemochromatosis and infections. Infection and immunity. 2003;71(12):6693-700.
241. Kao J-K, Wang S-C, Ho L-W, Huang S-W, Chang S-H, Yang R-C, et al. Chronic iron overload results in impaired bacterial killing of THP-1 derived macrophage through the inhibition of lysosomal acidification. PloS one. 2016;11(5):e0156713.
242. Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. Increased concentrations of iron and isoferritins in the lower respiratory tract of patients with

stable cystic fibrosis. American journal of respiratory and critical care medicine. 1999;160(3):796-801.
243. Mateos F, Brock JH, Pérez-Arellano JL. Iron metabolism in the lower respiratory tract. Thorax. 1998;53(7):594-600.
244. Fenker DE, McDaniel CT, Panmanee W, Panos RJ, Sorscher EJ, Sabusap C, et al. A comparison between two pathophysiologically different yet microbiologically similar lung diseases: cystic fibrosis and chronic obstructive pulmonary disease. International journal of respiratory and pulmonary medicine. 2018;5(2).
245. Michels KR, Zhang Z, Bettina AM, Cagnina RE, Stefanova D, Burdick MD, et al. Hepcidin-mediated iron sequestration protects against bacterial dissemination during pneumonia. JCI insight. 2017;2(6).
246. Minandri F, Imperi F, Frangipani E, Bonchi C, Visaggio D, Facchini M, et al. Role of iron uptake systems in Pseudomonas aeruginosa virulence and airway infection. Infection and immunity. 2016;84(8):2324-35.
247. Brown KA, Ratledge C. The effect of p-aminosalicyclic acid on iron transport and assimilation in mycobacteria. Biochimica et Biophysica Acta (BBA)-General Subjects. 1975;385(2):207-20.
248. Ribeiro M, Simões M. Advances in the antimicrobial and therapeutic potential of siderophores. Environmental Chemistry Letters. 2019;17(4):1485-94.
249. Imperi F, Massai F, Facchini M, Frangipani E, Visaggio D, Leoni L, et al. Repurposing the antimycotic drug flucytosine for suppression of Pseudomonas aeruginosa pathogenicity. Proceedings of the National Academy of Sciences. 2013;110(18):7458-63.
250. Kurnatowski P, Kurnatowska AK. Treatment of fungal infections of upper respiratory tract and ear. Otolaryngologia polska= The Polish otolaryngology. 2007;61(3):280-5.
251. Kirienko DR, Kang D, Kirienko NV. Novel pyoverdine inhibitors mitigate Pseudomonas aeruginosa pathogenesis. Frontiers in microbiology. 2019;9:3317.
252. Raffatellu M, George MD, Akiyama Y, Hornsby MJ, Nuccio S-P, Paixao TA, et al. Lipocalin-2 resistance confers an advantage to Salmonella enterica serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe. 2009;5(5):476-86.
253. Abergel RJ, Wilson MK, Arceneaux JE, Hoette TM, Strong RK, Byers BR, et al. Anthrax pathogen evades the mammalian immune system through stealth siderophore production. Proceedings of the National Academy of Sciences. 2006;103(49):18499-503.
254. Murphy-Benenato KE, Dangel B, Davis HE, Durand-Réville TF, Ferguson AD, Gao N, et al. SAR and structural analysis of siderophore-conjugated monocarbam inhibitors of Pseudomonas aeruginosa PBP3. ACS medicinal chemistry letters. 2015;6(5):537-42.
255. Murphy-Benenato KE, Bhagunde PR, Chen A, Davis HE, Durand-Reville TF, Ehmann DE, et al. Discovery of efficacious Pseudomonas aeruginosa-targeted siderophore-conjugated monocarbams by application of a semi-mechanistic pharmacokinetic/pharmacodynamic model. Journal of medicinal chemistry. 2015;58(5):2195-205.
256. Page MG, Dantier C, Desarbre E. In vitro properties of BAL30072, a novel siderophore sulfactam with activity against multiresistant gram-negative bacilli. Antimicrobial agents and chemotherapy. 2010;54(6):2291-302.
257. Brown MF, Mitton-Fry MJ, Arcari JT, Barham R, Casavant J, Gerstenberger BS, et al. Pyridone-conjugated monobactam antibiotics with gram-negative activity. Journal of medicinal chemistry. 2013;56(13):5541-52.
258. McPherson CJ, Aschenbrenner LM, Lacey BM, Fahnoe KC, Lemmon MM, Finegan SM, et al. Clinically relevant Gram-negative resistance mechanisms have no effect on the efficacy of MC-1, a novel siderophore-conjugated monocarbam. Antimicrobial agents and chemotherapy. 2012;56(12):6334-42.

259. Pramanik A, Stroeher UH, Krejci J, Standish AJ, Bohn E, Paton JC, et al. Albomycin is an effective antibiotic, as exemplified with Yersinia enterocolitica and Streptococcus pneumoniae. International Journal of Medical Microbiology. 2007;297(6):459-69.
260. Ji C, Miller MJ. Chemical syntheses and in vitro antibacterial activity of two desferrioxamine B-ciprofloxacin conjugates with potential esterase and phosphatase triggered drug release linkers. Bioorganic & medicinal chemistry. 2012;20(12):3828-36.
261. Page MG. Siderophore conjugates. Annals of the New York Academy of Sciences. 2013;1277(1):115-26.
262. Matsumoto S, Singley CM, Hoover J, Nakamura R, Echols R, Rittenhouse S, et al. Efficacy of cefiderocol against carbapenem-resistant Gram-negative bacilli in immunocompetent-rat respiratory tract infection models recreating human plasma pharmacokinetics. Antimicrobial agents and chemotherapy. 2017;61(9).
263. Ito A, Sato T, Ota M, Takemura M, Nishikawa T, Toba S, et al. In vitro antibacterial properties of cefiderocol, a novel siderophore cephalosporin, against Gram-negative bacteria. Antimicrobial agents and chemotherapy. 2018;62(1).
264. El-Lababidi RM, Rizk JG. Cefiderocol: A Siderophore Cephalosporin. Annals of Pharmacotherapy. 2020;54(12):1215-31.
265. Leal Jr SM, Roy S, Vareechon C, Carrion Sd, Clark H, Lopez-Berges MS, et al. Targeting iron acquisition blocks infection with the fungal pathogens Aspergillus fumigatus and Fusarium oxysporum. PLoS Pathog. 2013;9(7):e1003436.
266. Page MG. The role of iron and siderophores in infection, and the development of siderophore antibiotics. Clinical Infectious Diseases. 2019;69(Supplement_7):S529-S37.
267. Kohira N, Hackel MA, Ishioka Y, Kuroiwa M, Sahm DF, Sato T, et al. Reduced susceptibility mechanism to cefiderocol, a siderophore cephalosporin, among clinical isolates from a global surveillance programme (SIDERO-WT-2014). Journal of Global Antimicrobial Resistance. 2020;22:738-41.
268. Mobarra N, Shanaki M, Ehteram H, Nasiri H, Sahmani M, Saeidi M, et al. A review on iron chelators in treatment of iron overload syndromes. International journal of hematology-oncology and stem cell research. 2016;10(4):239.
269. Choon-Mee K, Yong-Jin P, Sung-Heui S. A widespread deferoxamine-mediated iron-uptake system in Vibrio vulnificus. The Journal of infectious diseases. 2007;196(10):1537-45.
270. Arifin AJ, Hannauer M, Welch I, Heinrichs DE. Deferoxamine mesylate enhances virulence of community-associated methicillin resistant Staphylococcus aureus. Microbes and infection. 2014;16(11):967-72.
271. Lal A, Porter J, Sweeters N, Ng V, Evans P, Neumayr L, et al. Combined chelation therapy with deferasirox and deferoxamine in thalassemia. Blood Cells, Molecules, and Diseases. 2013;50(2):99-104.
272. Kim C-M, Shin S-H. Effect of iron-chelator deferiprone on the in vitro growth of staphylococci. Journal of Korean medical science. 2009;24(2):289.
273. Thompson MG, Corey BW, Si Y, Craft DW, Zurawski DV. Antibacterial activities of iron chelators against common nosocomial pathogens. Antimicrobial agents and chemotherapy. 2012;56(10):5419-21.
274. Stojiljkovic I, Kumar V, Srinivasan N. Non‐iron metalloporphyrins: potent antibacterial compounds that exploit haem/Hb uptake systems of pathogenic bacteria. Molecular microbiology. 1999;31(2):429-42.
275. Choi S-r, Britigan BE, Narayanasamy P. Iron/heme metabolism-targeted gallium (III) nanoparticles are active against extracellular and intracellular Pseudomonas aeruginosa and Acinetobacter baumannii. Antimicrobial agents and chemotherapy. 2019;63(4).
276. Tripathi A, Schofield MM, Chlipala GE, Schultz PJ, Yim I, Newmister SA, et al. Baulamycins A and B, broad-spectrum antibiotics identified as inhibitors of

siderophore biosynthesis in Staphylococcus aureus and Bacillus anthracis. Journal of the American Chemical Society. 2014;136(4):1579-86.

277. Hartzen SH, Frimodt‐MØLler N, Thomsen VF. The antibacterial activity of a siderophore: 3. The activity of deferoxamine in vitro and its influence on the effect of antibiotics against Escherichia coli, Proteus mirabilis and coagulase‐negative staphylococci. APMIS. 1994;102(1‐6):219-26.
278. Arivett BA, Fiester SE, Ohneck EJ, Penwell WF, Kaufman CM, Relich RF, et al. Antimicrobial activity of gallium protoporphyrin IX against Acinetobacter baumannii strains displaying different antibiotic resistance phenotypes. Antimicrobial agents and chemotherapy. 2015;59(12):7657-65.
279. Hijazi S, Visaggio D, Pirolo M, Frangipani E, Bernstein L, Visca P. Antimicrobial activity of gallium compounds on ESKAPE pathogens. Frontiers in cellular and infection microbiology. 2018;8:316.
280. Hijazi S, Visca P, Frangipani E. Gallium-protoporphyrin IX inhibits Pseudomonas aeruginosa growth by targeting cytochromes. Frontiers in cellular and infection microbiology. 2017;7:12.
281. Furci LM, Lopes P, Eakanunkul S, Zhong S, MacKerell AD, Wilks A. Inhibition of the bacterial heme oxygenases from Pseudomonas aeruginosa and Neisseria meningitidis: novel antimicrobial targets. Journal of medicinal chemistry. 2007;50(16):3804-13.
282. Behrouzi A, Bouzari S, Vaziri F, Fateh A, Afrough P, Motlagh ADV, et al. Recombinant truncated E protein as a new vaccine candidate against nontypeable H. influenzae: its expression and immunogenic evaluation. Microbial pathogenesis. 2017;110:431-8.
283. Wilkinson TM, Schembri S, Brightling C, Bakerly ND, Lewis K, MacNee W, et al. Non-typeable Haemophilus influenzae protein vaccine in adults with COPD: a phase 2 clinical trial. Vaccine. 2019;37(41):6102-11.
284. Mike LA, Smith SN, Sumner CA, Eaton KA, Mobley HL. Siderophore vaccine conjugates protect against uropathogenic Escherichia coli urinary tract infection. Proceedings of the National Academy of Sciences. 2016;113(47):13468-73.
285. Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HL. Mucosal immunization with iron receptor antigens protects against urinary tract infection. PLoS Pathog. 2009;5(9):e1000586.
286. Kim HK, DeDent A, Cheng AG, McAdow M, Bagnoli F, Missiakas DM, et al. IsdA and IsdB antibodies protect mice against Staphylococcus aureus abscess formation and lethal challenge. Vaccine. 2010;28(38):6382-92.
287. Deriu E, Liu JZ, Pezeshki M, Edwards RA, Ochoa RJ, Contreras H, et al. Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron. Cell host & microbe. 2013;14(1):26-37.
288. Manti S, Parisi GF, Papale M, Licari A, Salpietro C, Miraglia del Giudice M, et al. Bacteriotherapy with Streptococcus salivarius 24SMB and Streptococcus oralis 89a nasal spray for treatment of upper respiratory tract infections in children: a pilot study on short-term efficacy. Italian journal of pediatrics. 2020;46:1-7.
289. Andaloro C, Santagati M, Stefani S, La Mantia I. Bacteriotherapy with Streptococcus salivarius 24SMB and Streptococcus oralis 89a oral spray for children with recurrent streptococcal pharyngotonsillitis: a randomized placebo-controlled clinical study. European Archives of Oto-Rhino-Laryngology. 2019;276(3):879-87.
290. Marchisio P, Santagati M, Scillato M, Baggi E, Fattizzo M, Rosazza C, et al. Streptococcus salivarius 24SMB administered by nasal spray for the prevention of acute otitis media in otitis-prone children. European Journal of Clinical Microbiology & Infectious Diseases. 2015;34(12):2377-83.
291. Pickering JL, Prosser A, Corscadden KJ, de Gier C, Richmond PC, Zhang G, et al. Haemophilus haemolyticus interaction with host cells is different to nontypeable

Haemophilus influenzae and prevents NTHi association with epithelial cells. Frontiers in cellular and infection microbiology. 2016;6.
292. Granland CM, Scott NM, Lauzon-Joset J-F, Langereis JD, De Gier C, Sutherland KM, et al. Nasal delivery of a commensal Pasteurellaceae species inhibits nontypeable Haemophilus influenzae colonization and delays onset of otitis media in mice. Infection and immunity. 2020;88(4).
293. Di Pierro F, Colombo M, Giuliani M, Danza M, Basile I, Bollani T, et al. Effect of administration of Streptococcus salivarius K12 on the occurrence of streptococcal pharyngo-tonsillitis, scarlet fever and acute otitis media in 3 years old children. Eur Rev Med Pharmacol Sci. 2016;20(21):4601-6.
294. Zipperer A, Konnerth MC, Laux C, Berscheid A, Janek D, Weidenmaier C, et al. Human commensals producing a novel antibiotic impair pathogen colonization. Nature. 2016;535(7613):511-6.
295. Latham RD, Gell DA, Fairbairn RL, Lyons AB, Shukla SD, Cho KY, et al. An isolate of Haemophilus haemolyticus produces a bacteriocin-like substance that inhibits the growth of nontypeable Haemophilus influenzae. International Journal of Antimicrobial Agents. 2017;49(4):503-6.
296. Kruszewska D, Sahl H-G, Bierbaum G, Pag U, Hynes SO, Ljungh Å. Mersacidin eradicates methicillin-resistant Staphylococcus aureus (MRSA) in a mouse rhinitis model. Journal of Antimicrobial Chemotherapy. 2004;54(3):648-53.
297. Niu W-W, Neu H. Activity of mersacidin, a novel peptide, compared with that of vancomycin, teicoplanin, and daptomycin. Antimicrobial agents and chemotherapy. 1991;35(5):998-1000.
298. Richter K, Thomas N, Claeys J, McGuane J, Prestidge CA, Coenye T, et al. A topical hydrogel with deferiprone and gallium-protoporphyrin targets bacterial iron metabolism and has antibiofilm activity. Antimicrobial agents and chemotherapy. 2017;61(6).
299. Richter K, Thomas N, Zhang G, Prestidge CA, Coenye T, Wormald P-J, et al. Deferiprone and gallium-protoporphyrin have the capacity to potentiate the activity of antibiotics in Staphylococcus aureus small colony variants. Frontiers in cellular and infection microbiology. 2017;7:280.
300. Leroux-Roels G, Van Damme P, Haazen W, Shakib S, Caubet M, Aris E, et al. Phase I, randomized, observer-blind, placebo-controlled studies to evaluate the safety, reactogenicity and immunogenicity of an investigational non-typeable Haemophilus influenzae (NTHi) protein vaccine in adults. Vaccine. 2016;34(27):3156-63.
301. Hare SA. Diverse structural approaches to haem appropriation by pathogenic bacteria. Biochimica et Biophysica Acta (BBA)-Proteins and Proteomics. 2017.
302. Bagnoli F, Bertholet S, Grandi G. Inferring reasons for the failure of Staphylococcus aureus vaccines in clinical trials. Frontiers in cellular and infection microbiology. 2012;2:16.
303. Fowler VG, Allen KB, Moreira ED, Moustafa M, Isgro F, Boucher HW, et al. Effect of an investigational vaccine for preventing Staphylococcus aureus infections after cardiothoracic surgery: a randomized trial. Jama. 2013;309(13):1368-78.
304. De Boeck I, Spacova I, Vanderveken OM, Lebeer S. Lactic acid bacteria as probiotics for the nose? Microbial Biotechnology. 2021.
305. Martín R, Langella P. Emerging health concepts in the probiotics field: streamlining the definitions. Frontiers in microbiology. 2019;10:1047.
306. Cohen R, Martin E, de La Rocque F, Thollot F, Pecquet S, Werner A, et al. Probiotics and prebiotics in preventing episodes of acute otitis media in high-risk children: a randomized, double-blind, placebo-controlled study. The Pediatric infectious disease journal. 2013;32(8):810-4.
307. Hao Q, Dong BR, Wu T. Probiotics for preventing acute upper respiratory tract infections. Cochrane Database of Systematic Reviews. 2015(2).

308. Kang E-J, Kim SY, Hwang I-H, Ji Y-J. The effect of probiotics on prevention of common cold: a meta-analysis of randomized controlled trial studies. Korean journal of family medicine. 2013;34(1):2.
309. Vouloumanou EK, Makris GC, Karageorgopoulos DE, Falagas ME. Probiotics for the prevention of respiratory tract infections: a systematic review. International journal of antimicrobial agents. 2009;34(3):197. e1-. e10.
310. Falagas ME, Rafailidis PI, Makris GC. Bacterial interference for the prevention and treatment of infections. International journal of antimicrobial agents. 2008;31(6):518-22.
311. Li Y, Zhang Q, Winterbotham M, Mowe E, Gorringe A, Tang CM. Immunization with live Neisseria lactamica protects mice against meningococcal challenge and can elicit serum bactericidal antibodies. Infection and immunity. 2006;74(11):6348-55.
312. Shekhar S, Khan R, Schenck K, Petersen FC. Intranasal immunization with the commensal Streptococcus mitis confers protective immunity against pneumococcal lung infection. Applied and environmental microbiology. 2019;85(6).
313. Van den Broek M, De Boeck I, Claes I, Nizet V, Lebeer S. Multifactorial inhibition of lactobacilli against the respiratory tract pathogen Moraxella catarrhalis. Beneficial microbes. 2018;9(3):429-39.
314. Burton JP, Drummond BK, Chilcott CN, Tagg JR, Thomson WM, Hale JD, et al. Influence of the probiotic Streptococcus salivarius strain M18 on indices of dental health in children: a randomized double-blind, placebo-controlled trial. Journal of medical microbiology. 2013;62(6):875-84.
315. Latham RD, Torrado M, Atto B, Walshe JL, Wilson R, Guss JM, et al. A heme‐binding protein produced by Haemophilus haemolyticus inhibits non‐typeable Haemophilus influenzae. Molecular microbiology. 2020;113(2):381-98.
316. Gillor O, Ghazaryan L. Recent advances in bacteriocin application as antimicrobials. Recent patents on anti-infective drug discovery. 2007;2(2):115-22.
317. Drutz DJ, Van Way MH, Schaffner W, Koenig MG. Bacterial interference in the therapy of recurrent staphylococcal infections: multiple abscesses due to the implantation of the 502A strain of Staphylococcus. New England Journal of Medicine. 1966;275(21):1161-5.
318. Tiwari SK, Noll KS, Cavera VL, Chikindas ML. Improved antimicrobial activities of synthetic-hybrid bacteriocins designed from enterocin E50-52 and pediocin PA-1. Applied and environmental microbiology. 2015;81(5):1661-7.
319. Field D, Cotter PD, Hill C, Ross RP. Bioengineering lantibiotics for therapeutic success. Frontiers in microbiology. 2015;6:1363.
320. Hols P, Ledesma-García L, Gabant P, Mignolet J. Mobilization of microbiota commensals and their bacteriocins for therapeutics. Trends in microbiology. 2019;27(8):690-702.
321. Rupnik M, Wilcox MH, Gerding DN. Clostridium difficile infection: new developments in epidemiology and pathogenesis. Nature Reviews Microbiology. 2009;7(7):526-36.
322. Lemon KP, Klepac-Ceraj V, Schiffer HK, Brodie EL, Lynch SV, Kolter R. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. MBio. 2010;1(3):e00129-10.
323. Atack JM, Murphy TF, Pettigrew MM, Seib KL, Jennings MP. Non-typeable Haemophilus influenzae isolates from patients with chronic obstructive pulmonary disease contain new phase-variable modA methyltransferase alleles controlling phasevarions. Scientific reports. 2019;9(1):1-10.
324. Herriott RM, Meyer EY, Vogt M, Modan M. Defined medium for growth of Haemophilus influenzae. Journal of Bacteriology. 1970;101(2):513-6.
325. Schillinger U, Lücke FK. Antibacterial activity of Lactobacillus sake isolated from meat. Applied and environmental microbiology. 1989;55(8):1901-6.
326. Darling AC, Mau B, Blattner FR, Perna NT. Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome research. 2004;14(7):1394-403.

327. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: molecular evolutionary genetics analysis across computing platforms. Molecular biology and evolution. 2018;35(6):1547.
328. Alikhan N-F, Petty NK, Zakour NLB, Beatson SA. BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. BMC genomics. 2011;12(1):1-10.
329. Norskov-Lauritsen N. Classification, identification, and clinical significance of *Haemophilus* and *Aggregatibacter* species with host specificity for humans. Clin Microbiol Rev. 2014;27(2):214-40.
330. Vogel AR, Szelestey BR, Raffel FK, Sharpe SW, Gearinger RL, Justice SS, et al. SapF-mediated heme-iron utilization enhances persistence and coordinates biofilm architecture of *Haemophilus*. Front Cell Infect Microbiol. 2012;2:42.
331. Mason KM, Raffel FK, Ray WC, Bakaletz LO. Heme utilization by nontypeable *Haemophilus influenzae* is essential and dependent on Sap transporter function. J Bacteriol. 2011;193(10):2527-35.
332. Hooda Y, Lai CC, Moraes TF. Identification of a large family of slam-dependent surface lipoproteins in Gram-negative bacteria. Frontiers in cellular and infection microbiology. 2017;7:207.
333. Jin H, Ren Z, Whitby PW, Morton DJ, Stull TL. Characterization of hgpA, a gene encoding a haemoglobin/haemoglobin-haptoglobin-binding protein of Haemophilus influenzae. Microbiology. 1999;145 (Pt 4)(Pt 4):905-14.
334. Morton DJ, Whitby PW, Jin H, Ren Z, Stull TL. Effect of multiple mutations in the hemoglobin- and hemoglobin-haptoglobin-binding proteins, HgpA, HgpB, and HgpC, of Haemophilus influenzae type b. Infect Immun. 1999;67(6):2729-39.
335. Ren Z, Jin H, Morton DJ, Stull TL. hgpB, a gene encoding a second Haemophilus influenzae hemoglobin- and hemoglobin-haptoglobin-binding protein. Infect Immun. 1998;66(10):4733-41.
336. Ghigo JM, Letoffe S, Wandersman C. A new type of hemophore-dependent heme acquisition system of *Serratia marcescens* reconstituted in Escherichia coli. J Bacteriol. 1997;179(11):3572-9.
337. Izadi N, Henry Y, Haladjian J, Goldberg ME, Wandersman C, Delepierre M, et al. Purification and characterization of an extracellular heme-binding protein, HasA, involved in heme iron acquisition. Biochemistry. 1997;36(23):7050-7.
338. Ochsner UA, Johnson Z, Vasil ML. Genetics and regulation of two distinct haem-uptake systems, phu and has, in Pseudomonas aeruginosa. Microbiology. 2000;146 (Pt 1)(Pt 1):185-98.
339. Rossi MS, Fetherston JD, Letoffe S, Carniel E, Perry RD, Ghigo JM. Identification and characterization of the hemophore-dependent heme acquisition system of Yersinia pestis. Infect Immun. 2001;69(11):6707-17.
340. Wojtowicz H, Guevara T, Tallant C, Olczak M, Sroka A, Potempa J, et al. Unique structure and stability of HmuY, a novel heme-binding protein of *Porphyromonas gingivalis*. PLoS Pathog. 2009;5(5):e1000419.
341. Gao JL, Kwan AH, Yammine A, Zhou X, Trewhella J, Hugrass BM, et al. Structural properties of a haemophore facilitate targeted elimination of the pathogen *Porphyromonas gingivalis*. Nat Commun. 2018;9(1):4097.
342. Wong CT, Xu Y, Gupta A, Garnett JA, Matthews SJ, Hare SA. Structural analysis of haemoglobin binding by HpuA from the Neisseriaceae family. Nature communications. 2015;6(1):1-11.
343. Wheeler R. Outer membrane proteomics of Pasteurella multocida isolates to identify putative host-specificity determinants. Bioscience Horizons. 2009;2(1):1-12.
344. Hooda Y, Lai C, Judd A, Buckwalter C, Shin H, Gray-Owen S. Slam is an outer membrane protein that is required for the surface display of lipidated virulence factors in Neisseria. Nat Microbiol. 2016; 1: 16009. 2016.
345. Mozzi A, Forni D, Clerici M, Cagliani R, Sironi M. The diversity of mammalian hemoproteins and microbial heme scavengers is shaped by an arms race for iron piracy. Frontiers in immunology. 2018;9:2086.

346. Langereis JD, de Jonge MI. Invasive disease caused by nontypeable Haemophilus influenzae. Emerging infectious diseases. 2015;21(10):1711.
347. Wilson R, Sethi S, Anzueto A, Miravitlles M. Antibiotics for treatment and prevention of exacerbations of chronic obstructive pulmonary disease. Journal of Infection. 2013;67(6):497-515.
348. Vila J, Ruiz J, Sanchez F, Navarro F, Mirelis B, De Anta MTJ, et al. Increase in quinolone resistance in a Haemophilus influenzae strain isolated from a patient with recurrent respiratory infections treated with ofloxacin. Antimicrobial agents and chemotherapy. 1999;43(1):161-2.
349. Skaar EP. The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog. 2010;6(8):e1000949.
350. Fenger MG, Ridderberg W, Olesen HV, Nørskov-Lauritsen N. Low occurrence of 'non-haemolytic Haemophilus haemolyticus' misidentified as Haemophilus influenzae in cystic fibrosis respiratory specimens, and frequent recurrence of persistent H. influenzae clones despite antimicrobial treatment. International Journal of Medical Microbiology. 2012;302(7-8):315-9.
351. Hotomi M, Kono M, Togawa A, Arai J, Takei S, Ikeda Y, et al. Haemophilus influenzae and Haemophilus haemolyticus in tonsillar cultures of adults with acute pharyngotonsillitis. Auris Nasus Larynx. 2010;37(5):594-600.
352. Zhang B, Kunde D, Tristram S. Haemophilus haemolyticus is infrequently misidentified as Haemophilus influenzae in diagnostic specimens in Australia. Diagnostic microbiology and infectious disease. 2014;80(4):272-3.
353. Freschi CR, Carvalho LFdO, Oliveira CJBd. Comparison of DNA-extraction methods and selective enrichment broths on the detection of Salmonella Typhimurium in swine feces by polymerase chain reaction (PCR). Brazilian Journal of Microbiology. 2005;36:363-7.
354. Reischl U, Linde H-Jr, Metz M, Leppmeier B, Lehn N. Rapid identification of methicillin-resistant Staphylococcus aureus and simultaneous species confirmation using real-time fluorescence PCR. Journal of clinical microbiology. 2000;38(6):2429-33.
355. Reischl U, Pulz M, Ehret W, Wolf HJ. PCR-based detection of mycobacteria in sputum samples using a simple and reliable DNA extraction protocol. BioTechniques. 1994;17(5):844-5.
356. Sweeney RW, Whitlock RH, McAdams SC. Comparison of three DNA preparation methods for real-time polymerase chain reaction confirmation of Mycobacterium avium subsp. paratuberculosis growth in an automated broth culture system. Journal of veterinary diagnostic investigation. 2006;18(6):587-90.
357. Van Tongeren S, Degener J, Harmsen H. Comparison of three rapid and easy bacterial DNA extraction methods for use with quantitative real-time PCR. European journal of clinical microbiology & infectious diseases. 2011;30(9):1053-61.
358. Wilson DA, Yen-Lieberman B, Reischl U, Gordon SM, Procop GW. Detection of Legionella pneumophila by real-time PCR for the mip gene. Journal of clinical microbiology. 2003;41(7):3327-30.
359. Coyne SR, Craw PD, Norwood DA, Ulrich MP. Comparative analysis of the Schleicher and Schuell IsoCode Stix DNA isolation device and the Qiagen QIAamp DNA mini kit. Journal of clinical microbiology. 2004;42(10):4859-62.
360. Rantakokko-Jalava K, Jalava J. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. Journal of clinical microbiology. 2002;40(11):4211-7.
361. Wiser MJ, Lenski RE. A comparison of methods to measure fitness in Escherichia coli. PloS one. 2015;10(5):e0126210.
362. Pfaffl MW. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic acids research. 2001;29(9):e45-e.
363. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research. 2002;30(9):e36-e.

364. Price EP, Harris TM, Spargo J, Nosworthy E, Beissbarth J, Chang AB, et al. Simultaneous identification of Haemophilus influenzae and Haemophilus haemolyticus using real-time PCR. Future Microbiology. 2017(0).
365. Latham RD, Torrado M, Atto B, Walshe JL, Wilson R, Guss JM, et al. A heme-binding protein produced by Haemophilus haemolyticus inhibits non-typeable Haemophilus influenzae. Molecular Microbiology, In press. 2019:626416.
366. Waiblinger H-U, Grohmann L. Guidelines for validation of DNA extraction methods applied in subsequent PCR analysis of food and feed products for the presence of genetically modified material. Journal für Verbraucherschutz und Lebensmittelsicherheit. 2014;9(2):183-90.
367. Glover WA, Suarez CJ, Clarridge III J. Genotypic and phenotypic characterization and clinical significance of 'Haemophilus quentini'isolated from the urinary tract of adult men. Journal of medical microbiology. 2011;60(11):1689-92.
368. Atkinson CT, Kunde DA, Tristram SG. Expression of acquired macrolide resistance genes in Haemophilus influenzae. Journal of Antimicrobial Chemotherapy. 2017;72(12):3298-301.
369. Hare KM, Leach AJ, Morris PS, Smith-Vaughan H, Torzillo P, Bauert P, et al. Impact of recent antibiotics on nasopharyngeal carriage and lower airway infection in Indigenous Australian children with non-cystic fibrosis bronchiectasis. International journal of antimicrobial agents. 2012;40(4):365-9.
370. Witherden EA, Tristram SG. Prevalence and mechanisms of β-lactam resistance in Haemophilus haemolyticus. Journal of Antimicrobial Chemotherapy. 2013;68(5):1049-53.
371. Smith-Vaughan H, Byun R, Nadkarni M, Jacques NA, Hunter N, Halpin S, et al. Measuring nasal bacterial load and its association with otitis media. BMC Ear, Nose and Throat Disorders. 2006;6(1):1-9.
372. Desai H, Eschberger K, Wrona C, Grove L, Agrawal A, Grant B, et al. Bacterial colonization increases daily symptoms in patients with chronic obstructive pulmonary disease. Annals of the American Thoracic Society. 2014;11(3):303-9.
373. Hotomi M, Arai J, Billal DS, Takei S, Ikeda Y, Ogami M, et al. Nontypeable Haemophilus influenzae isolated from intractable acute otitis media internalized into cultured human epithelial cells. Auris Nasus Larynx. 2010;37(2):137-44.
374. Morey P, Cano V, Martí-Lliteras P, Lopez-Gomez A, Regueiro V, Saus C, et al. Evidence for a non-replicative intracellular stage of nontypable Haemophilus influenzae in epithelial cells. Microbiology. 2011;157(1):234-50.
375. Singh NK, Kunde DA, Tristram SG. Effect of epithelial cell type on in vitro invasion of non-typeable Haemophilus influenzae. Journal of microbiological methods. 2016;129:66-9.
376. Swords WE, Buscher BA, Ver Steeg Ii K, Preston A, Nichols WA, Weiser JN, et al. Non‐typeable Haemophilus influenzae adhere to and invade human bronchial epithelial cells via an interaction of lipooligosaccharide with the PAF receptor. Molecular microbiology. 2000;37(1):13-27.
377. van Schilfgaarde M, Eijk P, Regelink A, van Ulsen P, Everts V, Dankert J, et al. Haemophilus influenzaelocalized in epithelial cell layers is shielded from antibiotics and antibody-mediated bactericidal activity. Microbial pathogenesis. 1999;26(5):249-62.
378. Groeneveld K, van Alphen L, Eijk PP, Visschers G, Jansen HM, Zanen H. Endogenous and exogenous reinfections by Haemophilus influenzae in patients with chronic obstructive pulmonary disease: the effect of antibiotic treatment on persistence. Journal of Infectious Diseases. 1990;161(3):512-7.
379. Moller LV, Timens W, Van der Bij W, Kooi K, de Wever B, Dankert J, et al. Haemophilus influenzae in lung explants of patients with end-stage pulmonary disease. American journal of respiratory and critical care medicine. 1998;157(3):950-6.

380. Su Y-C, Jalalvand F, Thegerström J, Riesbeck K. The interplay between immune response and bacterial infection in COPD: focus upon non-typeable Haemophilus influenzae. Frontiers in immunology. 2018;9:2530.
381. Contreras H, Chim N, Credali A, Goulding CW. Heme uptake in bacterial pathogens. Current opinion in chemical biology. 2014;19:34-41.
382. Richard KL, Kelley BR, Johnson JG. Heme uptake and utilization by gram-negative bacterial pathogens. Frontiers in cellular and infection microbiology. 2019;9:81.
383. Loeb MR. Ferrochelatase activity and protoporphyrin IX utilization in Haemophilus influenzae. Journal of Bacteriology. 1995;177(12):3613-5.
384. Whitby PW, Sim KE, Morton DJ, Patel JA, Stull TL. Transcription of genes encoding iron and heme acquisition proteins of Haemophilus influenzae during acute otitis media. Infection and Immunity. 1997;65(11):4696-700.
385. Whitby PW, VanWagoner TM, Seale TW, Morton DJ, Stull TL. Comparison of transcription of the Haemophilus influenzae iron/heme modulon genes in vitro and in vivo in the chinchilla middle ear. BMC genomics. 2013;14(1):1-12.
386. Atto B, Latham R, Kunde D, Gell DA, Tristram S. In vitro anti-NTHi activity of haemophilin-producing strains of Haemophilus haemolyticus. Pathogens. 2020;9(4):243.
387. Mokrzan EM, Johnson TJ, Bakaletz LO. Expression of the nontypeable Haemophilus influenzae type IV pilus is stimulated by coculture with host respiratory tract epithelial cells. Infection and immunity. 2019;87(12):e00704-19.
388. Hibbing ME, Fuqua C, Parsek MR, Peterson SB. Bacterial competition: surviving and thriving in the microbial jungle. Nature reviews microbiology. 2010;8(1):15-25.
389. Leinweber A, Weigert M, Kümmerli R. The bacterium Pseudomonas aeruginosa senses and gradually responds to interspecific competition for iron. Evolution. 2018;72(7):1515-28.
390. Trejo-Hernández A, Andrade-Domínguez A, Hernández M, Encarnacion S. Interspecies competition triggers virulence and mutability in Candida albicans–Pseudomonas aeruginosa mixed biofilms. The ISME journal. 2014;8(10):1974-88.
391. Yang Y-p, Loosmore SM, Underdown BJ, Klein MH. Nasopharyngeal colonization with nontypeable Haemophilus influenzae in chinchillas. Infection and immunity. 1998;66(5):1973-80.
392. De Gier C, Granland CM, Pickering JL, Walls T, Bhuiyan M, Mills N, et al. PCV7-and PCV10-vaccinated otitis-prone children in New Zealand have similar pneumococcal and haemophilus influenzae densities in their nasopharynx and middle ear. Vaccines. 2019;7(1):14.
393. Hill AT, Campbell EJ, Hill SL, Bayley DL, Stockley RA. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. The American journal of medicine. 2000;109(4):288-95.
394. Hood D, Moxon R, Purnell T, Richter C, Williams D, Azar A, et al. A new model for non-typeable Haemophilus influenzae middle ear infection in the Junbo mutant mouse. Disease models & mechanisms. 2016;9(1):69-79.
395. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. BMC infectious diseases. 2010;10(1):1-10.
396. Dhooge I, Vaneechoutte M, Claeys G, Verschraegen G, Van Cauwenberge P. Turnover of Haemophilus influenzae isolates in otitis-prone children. International journal of pediatric otorhinolaryngology. 2000;54(1):7-12.
397. Atkinson C, Tristram S. Antimicrobial resistance in cystic fibrosis isolates of Haemophilus influenzae. British journal of biomedical science. 2016;73(2):87-9.
398. Clark SE. Commensal bacteria in the upper respiratory tract regulate susceptibility to infection. Current opinion in immunology. 2020;66:42-9.

399. Atto B, Kunde D, Gell DA, Tristram S. Haemophilin-Producing Strains of Haemophilus haemolyticus Protect Respiratory Epithelia from NTHi Colonisation and Internalisation. Pathogens. 2021;10(1):29.
400. Subramanya SH, Thapa S, Dwedi SK, Gokhale S, Sathian B, Nayak N, et al. Streptococcus pneumoniae and Haemophilus species colonization in health care workers: the launch of invasive infections? BMC research notes. 2016;9(1):1-6.
401. Dabernat H, Plisson-Sauné M-A, Delmas C, Séguy M, Faucon G, Pélissier R, et al. Haemophilus influenzae carriage in children attending French day care centers: a molecular epidemiological study. Journal of clinical microbiology. 2003;41(4):1664-72.
402. Lieberman D, Shleyfer E, Castel H, Terry A, Harman-Boehm I, Delgado J, et al. Nasopharyngeal versus oropharyngeal sampling for isolation of potential respiratory pathogens in adults. Journal of clinical microbiology. 2006;44(2):525-8.
403. Pickering J, Smith-Vaughan H, Beissbarth J, Bowman J, Wiertsema S, Riley T, et al. Diversity of nontypeable Haemophilus influenzae strains colonizing Australian Aboriginal and non-Aboriginal children. Journal of clinical microbiology. 2014;52(5):1352-7.
404. Voss M, Wonnenberg B, Honecker A, Kamyschnikow A, Herr C, Bischoff M, et al. Cigarette smoke-promoted acquisition of bacterial pathogens in the upper respiratory tract leads to enhanced inflammation in mice. Respiratory research. 2015;16(1):1-11.
405. Zhang J, Zhu Z, Zuo X, Pan H, Gu Y, Yuan Y, et al. The role of NTHi colonization and infection in the pathogenesis of neutrophilic asthma. Respiratory Research. 2020;21(1):1-12.
406. Sethi S, Wrona C, Grant BJ, Murphy TF. Strain-specific immune response to Haemophilus influenzae in chronic obstructive pulmonary disease. American journal of respiratory and critical care medicine. 2004;169(4):448-53.
407. De Boeck I, van den Broek MF, Allonsius CN, Spacova I, Wittouck S, Martens K, et al. Lactobacilli have a niche in the human nose. Cell Reports. 2020;31(8):107674.
408. Kaijalainen T, Ruokokoski E, Ukkonen P, Herva E. Survival of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis frozen in skim milk-tryptone-glucose-glycerol medium. Journal of clinical microbiology. 2004;42(1):412-4.
409. Hare KM, Smith-Vaughan HC, Beissbarth J, Leach AJ. Haemophilus influenzae isolates survive for up to 20 years at− 70 C in skim milk tryptone glucose glycerol broth (STGGB) if thawing is avoided during re-culture. Journal of microbiological methods. 2015;119:132-3.