

1 ***In Vitro* Probiotic Potential of Hemophilin-producing Strains of *Haemophilus***
2 ***haemolyticus***

3

4 Brianna Atto ^{a#}, Roger Latham ^b, Dale Kunde ^a, David Gell ^b, Stephen Tristram ^{a#}

5 ^aSchool of Health Sciences, University of Tasmania, Launceston, TAS, Australia

6 ^bSchool of Medicine, University of Tasmania, Hobart, TAS, Australia

7

8 Running Head: Probiotic potential of *Haemophilus haemolyticus*.

9

10 #Address correspondence to Brianna Atto, brianna.atto@utas.edu.au and Stephen Tristram,
11 stephen.tristram@utas.edu.au.

12

13 **ABSTRACT**

14 Non-typeable *Haemophilus influenzae* (NTHi) is a leading causative organism of opportunistic
15 respiratory tract infections, including otitis media and acute exacerbations of chronic
16 obstructive pulmonary disease. Despite the enormous disease burden associated with NTHi
17 infections, there are currently no effective prevention strategies, and the rapid development of
18 antibiotic resistance is compromising treatment.

19 We previously discovered *Haemophilus haemolyticus* (Hh) strains capable of producing
20 haemophilin (HPL), a heme-binding protein that restricts NTHi growth by limiting its access
21 to an essential growth factor, heme. Thus, these strains may have utility as a probiotic therapy
22 against NTHi infection by limiting colonization, migration and subsequent infection in
23 susceptible individuals. Here, we have assessed the feasibility of this approach by *in vitro*
24 competition assays between NTHi and Hh strains with varying capacity to produce HPL. HPL-
25 producing strains of Hh exhibited enhanced growth and consistently outcompeted NTHi
26 compared to Hh strains unable to produce the protein. This competitive advantage was
27 maintained over a period of six days, culminating in the complete eradication of NTHi.

28 Expression analysis of *HPL* during competition coincided with the NTHi-inhibitory capacity
29 of HPL-producers, confirming that inhibition was mediated by the presence of HPL.

30 Together, results suggest that natural levels of HPL production by Hh are sufficient to limit
31 NTHi's access to heme, even under excess heme conditions unlikely to be encountered *in vivo*.
32 Further investigation is required to determine the protective capacity of HPL-producers *in vivo*
33 and their ability to interrupt NTHi colonization of host cells.

34

35 INTRODUCTION

36 The bacterium non-typeable *Haemophilus influenzae* (NTHi) is commonly associated with
37 upper respiratory tract (URT) colonization in healthy adults (1). However, migration to other
38 sites in the respiratory tract frequently occur in children, the elderly and individuals with
39 underlying respiratory diseases; making NTHi a leading cause of mucosal infections (2-4). In
40 particular, enormous global morbidity is attributed to otitis media (OM) and exacerbations of
41 chronic obstructive pulmonary disease (COPD), which are accompanied by long-term health
42 complications and considerable mortality, respectively (5, 6). NTHi has also gained attention
43 as an increasingly important cause of invasive infections (7-9).

44 There are currently no effective vaccination strategies for the prevention of NTHi infections
45 and treatment has been complicated by the rapid development of antibiotic resistance to first-
46 and second-line antibiotics. Resistance is predominantly mediated by β -lactamase production
47 (10); however, the emergence and spread of β -lactamase-negative, ampicillin-resistant strains
48 in many regions of the world is of substantial concern with treatment failure also being reported
49 in response to macrolides (11-14) and fluoroquinolones (15-17).

50 NTHi infection is preceded by successful colonization of the URT and survival in this
51 environment relies on the bacterium's ability to acquire the vital growth factor, heme (18).
52 There is also evidence to suggest heme-acquisition genes are important modulators of NTHi
53 virulence factors (19), demonstrated by the increased prevalence in disease-causing strains
54 from the middle ear, compared to colonizing throat strains (18). Deletion of multiple genes
55 related to haem-iron scavenging, utilization and regulation has been shown to significantly
56 reduce NTHi virulence, disease severity and duration in animal models of OM (20, 21).
57 Similarly, an isogenic mutant of two haem-acquisition pathways was unable to sustain
58 bacteraemia or produce meningitis in a rat model of invasive disease (22). Thus, haem-

59 acquisition pathways represent potentially high value targets for the development of novel
60 therapies for the eradication NTHi from the respiratory tract (23, 24).

61 NTHi is particularly susceptible to heme restriction as it is incapable of heme synthesis and
62 relies solely on scavenging heme from the host, either in the form of free-heme or bound to
63 host carrier molecules (20, 25-27). Evidence from our laboratory suggests that closely related
64 commensals may present a competitive challenge for heme acquisition in the URT. Previously,
65 we discovered *Haemophilus haemolyticus* (Hh) strains that exhibited inhibitory activity against
66 NTHi (28, 29). Further investigation revealed this inhibition was mediated by the production
67 of a heme-binding protein, haemophilin (HPL) that restricted NTHi growth by limiting its
68 access to heme (29). Thus, these strains may have utility as a probiotic therapy against NTHi
69 infection by limiting colonization, migration and subsequent infection in susceptible
70 individuals. Here, we aim to determine the feasibility of the probiotic approach by assessing *in*
71 *vitro* competition between NTHi and HPL-producing strains of Hh.

72

73 **METHODS**

74 **Bacterial growth conditions**

75 *Bacterial Strains*

76 Hh strains used in this study have previously been isolated and screened for the *HPL* ORF (28,
77 29) An *HPL* knockout (BW1^{HPL-}) of a model HPL-producing strain of Hh (Hh-BW1) was
78 constructed using insertional inactivation as previously described (29).

79 NTHi and Hh isolates were propagated from liquid nitrogen frozen glycerol stock, followed by
80 two overnight passages on chocolate agar (CA) at 37°C with 5–10% CO₂ prior to
81 experimentation. Strains were grown in supplemented Tryptone Soy Broth (sTSB), which
82 consisted of tryptone soy broth (TSB) (Oxoid Ltd., Basingstoke, UK) supplemented with 2%
83 (v/v) Vitox® (Oxoid Ltd) and 15 µg mL⁻¹ of porcine haematin (ferriprotoporphyrin IX
84 hydroxide, Sigma-Aldrich). Exposure to non-growth conditions was minimized by maintaining
85 suspensions and diluents at 37°C in heat block with sand or benchtop incubator.

86 *Propagation of heme-replete populations for growth experiments*

87 Strains were also propagated under heme-replete conditions prior to competition to replenish
88 bacterial heme stores and minimise external stressors that may influence the outcome of

89 competitive studies (29-31). Bacterial suspensions of ~1.0 OD₆₀₀ were made in TSB from 8–
90 10 hr growth on CA and diluted 1:10 in 5 pre-warmed sTSB (5 mL). Broths were incubated
91 for 12 hr at 37°C aerobically with shaking (220 RPM), centrifuged at 4000 × g for 5 min at
92 37°C and resuspended in fresh, pre-warmed TSB to an OD₆₀₀ of 1.0 prior to use in growth
93 experiments.

94

95 **Determination of NTHi-inhibitory activity**

96 A well diffusion assay of the extracted HPL protein was used to categorise Hh strains
97 containing the *HPL* ORF as either HPL-producers (Hh-HPL⁺) or non-producers (Hh-HPL⁻), as
98 previously described (28). This assay was also used to establish the relative inhibitory activity
99 of each strain. Testing was conducted on two indicator NTHi strains (ATCC 49427 and clinical
100 isolate NTHi-L15). Two Hh strains were included as additional *HPL* ORF negative controls
101 for HPL extraction: Hh ATCC 33390 and BW1^{HPL-}.

102

103 **Triplex real-time PCR for the quantification of NTHi, Hh and detection of *HPL***

104 A real-time quantitative triplex PCR assay was designed to quantify NTHi, Hh and detect the
105 *HPL* open reading frame (ORF). The targets used for discrimination of Hh (*hypD*) and NTHi
106 (*siaT*) have previously been described and validated (32). For detection of the *HPL* ORF,
107 primers were designed based on the *HPL* ORF of Hh-BW1 (29) (GenBank MN720274). The
108 FAM, HEX and TET channels were used for simultaneous fluorescence detection of *siaT*, *hypD*
109 and *HPL*, respectively. Primer and probe sequences are detailed in Table 1. Primer specificity
110 was confirmed by discontinuous megaBLAST analysis and PCR of a panel of *Haemophilus*
111 *spp.* and multiple genera representing common upper respiratory tract flora. PCR assays were
112 extensively optimised and evaluated for detection/quantification limits in triplex format.

113 PCRs were performed using the CFX96 TouchTM real-time PCR system (Bio-Rad) in 96-well
114 optical plates. Polymerase activation was performed at 95°C for 3 minutes, followed by 40
115 amplification cycles of denaturation at 95°C for 15 seconds, and annealing at 62°C for 1
116 minute. Each reaction contained 0.25 μM of *hypD*, *siaT* and *HPL* primers, 0.1 μM LNA-probes,
117 1× PrimeTime master mix (Integrated DNA Technologies) and 5 μl genomic DNA and
118 molecular-grade water, to a total volume of 20 μl. Template DNA was prepared by a thermal
119 extraction protocol and tested in duplicate. Each run included a positive control for the *HPL*

120 ORF (Hh-BW1), negative control (*H. parainfluenzae* ATCC 7901), no-template control and
121 10-fold dilutions of a standard containing 2×10^{-8} ng NTHi ATCC 49247 and Hh ATCC 33390
122 DNA. Quantification of NTHi and Hh was expressed as genome equivalents (GE) calculated
123 from the standard, as previously described (32). Bacterial quantification from thermally
124 extracted DNA was validated against conventional quantification by optical density and colony
125 counts. Complete details of PCR primer design, assay optimisation and DNA extraction
126 protocol evaluation are available in supplementary material.

127

129 **Competition Assays**

130 *Short-term broth competition*

131 Culture mixes were prepared by adding 100 μ L of heme-replete preparations of Hh-HPL⁺ or
132 Hh-HPL⁻ and 100 μ L of NTHi ATCC 49247 to 5 mL pre-warmed sTSB containing 0.0, 0.9,
133 3.8 or 15.0 μ g mL⁻¹ porcine hematin. Broths containing single strains were also prepared in
134 parallel to determine baseline growth. Broths were incubated aerobically on an incubator
135 shaker at 37°C (220 RPM) for 16 hours. At different time intervals, aliquots of 50 μ L were
136 taken for boiled gDNA extraction and subsequent triplex PCR quantification of GE. Aliquots
137 of 500 μ L were taken at 8 hours for quantification of *HPL* expression. Purity of broth growth
138 was checked by plating on CA after 16 hours incubation.

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

$$142 \quad \ln \frac{N_t}{N_0} = \alpha(t - t_0)$$

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

145 *Fitness assay*

146 Culture mixes were prepared by adding 100 μ L of heme-replete preparations of Hh-HPL⁺ or
147 Hh-HPL⁻ and 100 μ L of NTHi (ATCC 49247, ATCC 49766 or NCTC 11315) to 5 mL of pre-
148 warmed sTSB containing 0.0, 0.9, 3.8 or 15.0 μ g mL⁻¹ porcine hematin. Broths were incubated
149 aerobically on an incubator shaker at 37°C (220 RPM) for 12 hours prior to sub-culture (200
150 μ L) in fresh sTSB (2 mL) containing the same concentration of heme as the inoculum. The

151 process of 12-hourly incubation followed by sub-culture into fresh broth was repeated until 6
152 days had elapsed. After each 12-hour incubation, aliquots of 50 μL were taken for boiled gDNA
153 extraction and subsequent triplex PCR quantification of GE. Purity of broth growth was
154 confirmed by plating on CA after each 12-hour incubation. Fitness of NTHi at each time point
155 was determined using the following equation (33):

$$156 \quad w = \frac{\ln\left(\frac{A_t}{A_{t_0}}\right)}{\ln\left(\frac{B_t}{B_{t_0}}\right)}$$

157 Where w is fitness, \mathbf{A} and \mathbf{B} are the population sizes of the two competitors, subscripts t_0 and t
158 indicate the initial and final time points in the assay. Growth after the first 12-hour culture was
159 used as baseline for fitness determination (t_0).

160

161 **Expression analysis**

162 *RNA extraction, purification and quantification*

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

175 *Expression validation*

176 Expression analysis was employed to determine baseline expression and suitability of
177 prospective competitive test conditions for *HPL* expression. Given the kinetics of bacterial
178 growth and the heme-binding capacity of HPL, time and heme availability were targeted as

179 factors that may influence *HPL* expression. The *hypD* target was selected as a potential
180 housekeeper gene and validated for test conditions.

181 Heme-starved preparations of Hh-BW1 and the Hh-BW1^{*HPL*-} (100 µL) were added to 5 mL
182 pre-warmed sTSB containing either 0.0 or 15.0 µg ml⁻¹ of porcine haematin. Broths were
183 incubated for 8 hours and aliquots of 500 µL were removed for RNA extraction and purification
184 at 0, 4 and 8 hours.

185

186 **Statistical analysis**

187 Statistical analysis was performed using GraphPad Prism V7.04, 2017. Statistical significance
188 was determined by comparison of growth data (growth rate or fitness) between strains grown
189 with a competitor and baseline growth. Data were tested for normality using the Shapiro-Wilk
190 test, followed by a two-way ANOVA with Dunnett's multiple comparison test. Expression
191 ratios and statistical significance were calculated with 2000 iterations by the Relative
192 Expression Software Tool (REST; v 1.0, 2009) (34, 35).

193

194 **RESULTS AND DISCUSSION**

195 *Validation of a triplex real-time PCR for quantification of NTHi, Hh and detection* 196 *of HPL*

197 The *HPL* amplicon was confirmed to be specific and sensitive for the detection of the five
198 previously identified *HPL* sequence variants (29) by *in silico* investigations and by PCR.
199 Specificity of the *HypD* and *SiatT* targets was also confirmed by PCR. Complete results of
200 PCR assay validation is detailed in supplementary materials. The low limit of quantification
201 values for the *HypD* and *SiatT* assays in triplex format were 2×10^{-5} ng and 2×10^{-4} ng,
202 corresponding to 10 and 100 GE respectively. The lower limit of detection for the *HPL* assay
203 was 10 GE (Figure S1). The upper limits of detection/quantification were not explicitly
204 determined as expected DNA levels from sample were unlikely to exceed the maximum 2 ng
205 tested.

206 Given the high volume of samples generated from growth experiments, a cheap and high-
207 throughput DNA extraction method was required to reliably distinguish and quantify NTHi
208 and Hh in co-culture. Extraction utilizing thermal lysis has previously been shown to be an

209 efficient and cost-effective method to harvest bacterial DNA for quantitative real-time PCR
210 from suspensions of several bacterial species in a range of sample matrices (36-41). Crude
211 DNA extraction methods are also prone to contamination with PCR inhibitors originating from
212 sample matrices (39, 42). There are also reports of intra- and inter-species differences in DNA
213 extractions efficiencies (39, 42, 43). PCR quantification of gDNA extracted by thermal lysis
214 was validated and found to be comparable to quantification by OD₆₀₀ and colony counts. Full
215 details of thermal extraction validation are available in supplementary materials.

216 ***Baseline NTHi-inhibitory activity of Hh-HPL⁺ strains***

217 We previously discovered a number of distinct genetic variations of the *HPL* ORF with varying
218 NTHi-inhibitory activity, as determined by functional screening (29). The two Hh-HPL⁺
219 clinical isolates (Hh-BW1 and Hh-RHH122) that exhibited the highest inhibitory capacity
220 share 100% sequence similarity in the *HPL* ORF. Thus, strains containing this sequence variant
221 were selected for further investigation.

222 A well diffusion assay was employed to confirm inhibitory capacity and establish relative
223 baseline NTHi-inhibitory activity, normalised for population density. Hh-RHH122 and Hh-
224 BW1 demonstrated the highest inhibitory activity, while Hh-NF5 and Hh-NF4 served as
225 intermediate (~50%) and non-inhibitory respectively (Figure S5).

226 ***HPL-production mediates NTHi growth inhibition and a competitive advantage in*** 227 ***Hh***

228 The growth rate of NTHi was significantly impaired during competition with all Hh-HPL⁺
229 strains, compared to growth without competition ($p < 0.0001$) (Figure 1A). This inhibitory
230 effect was more pronounced during competition with highly bioactive strains (Hh-BW1 and
231 Hh-RHH122), compared to the intermediate (Hh-NF5). The growth rate of NTHi during
232 competition with Hh-HPL⁻ was not significantly affected (Figure 1B, suggesting that the
233 inhibitory effect observed was a characteristic of Hh-HPL⁺ strains.

234 For commensals and pathogens living in or invading human tissues, iron-containing heme is
235 often a limiting nutrient, particularly in the respiratory tract where concentrations are
236 considered to be low (44). This is particularly true for heme auxotrophs including NTHi and
237 Hh; for these species survival in the URT niche is dependent on their ability to outcompete host
238 proteins and co-existing bacterial populations for heme (20). We previously demonstrated that
239 the NTHi-inhibitory mechanism of HPL is associated with its ability to bind heme in a form

240 inaccessible to NTHi and that inhibitory activity is lost in conditions where heme concentration
241 exceeds the binding capacity of HPL (29). While levels of heme/iron are considered to be low
242 in the respiratory tract, there is indirect evidence for increased heme/iron levels in airways of
243 smokers, COPD and CF which may contribute to increased susceptibility to infection in these
244 individuals (44). Thus, it was important to assess the effectiveness of HPL with varying
245 concentrations of heme to ensure probiotic effectiveness in a range of *in vitro* conditions
246 reflecting possible *in vivo* scenarios. The NTHi-inhibitory capacity of HPL was maintained
247 even in conditions of high heme-availability ($15 \mu\text{g mL}^{-1}$), albeit to a lesser degree than lower
248 heme concentrations ($0.0\text{-}3.8 \mu\text{g/mL}$) (Figure 1A). This suggests that levels of HPL produced
249 by Hh are sufficient to limit NTHi's access to heme in a dynamic *in vitro* system, even under
250 excess heme conditions unlikely to be encountered *in vivo* (44).

251 Interestingly, Hh-HPL⁺ strains exhibited a pattern of enhanced growth in response to NTHi
252 competition ($p < 0.0001$) (Figure 1B). This effect was observed in all heme concentrations and
253 was more pronounced in the highly inhibitory Hh-HPL⁺ strains. The converse was observed in
254 Hh-HPL⁻ strains, where they exhibited poorer growth in response to competition with NTHi
255 (Figure 1D). This may be a reflection of the highly efficient set of heme-scavenging systems
256 possessed by NTHi that outcompete Hh in the absence of HPL.

257

258 ***NTHi-inhibitory capacity is associated with expression of HPL***

259 To further test whether the observed competitive advantage of Hh-HPL⁺ strains could be
260 attributed to HPL production, *HPL* expression was quantified by reverse transcription and real-
261 time PCR during competitive growth with NTHi. The *hypD* target was validated as the
262 housekeeper gene (Figure S6A) and the optimal growth phase for *HPL* expression analysis was
263 determined (Figure S6B).

264 Baseline expression of *HPL* was highest in the highly bioactive strains (Hh-BW1 and Hh-
265 RHH122), significantly lower in Hh-NF5 ($p < 0.0001$), and completely absent in Hh-NF4
266 (Figure 2A). These results establish a connection between expression of HPL and NTHi-
267 inhibitory activity as demonstrated by well-diffusion and short-term competition studies
268 (Figure 1). Upregulation of *HPL* was observed in all Hh-HPL⁺ in response to competition with
269 NTHi, an effect that was more pronounced in Hh-BW1 and Hh-RHH122 (Figure 2B). This
270 may explain the enhanced growth of Hh-HPL⁺ strains in response to NTHi during the short-
271 term competition assays (Figure 1C). These results show that expression of *HPL* has a

272 significant impact on the NTHi-inhibitory capacity of Hh-HPL⁺ strains and therapeutic utility
273 in an *in vivo* setting. Therefore, the huge differential expression of *HPL* amongst Hh-HPL⁺
274 strains must be considered when selecting a probiotic candidate. However, our understanding
275 of HPL regulation is still rudimentary. Further investigation into potential upstream regulatory
276 components or post-translational modification is needed to elucidate the inter-strain differences
277 in HPL production and/or biological activity despite complete ORF sequence homology.

278

279 *NTHi fitness dramatically decreases in competition with HPL-producers*

280 Short-term competition may highlight the potency of HPL-mediated inhibition but is not
281 representative of *in vivo* competition dynamics. Thus, a longer-term study was employed to
282 assess the competition between NTHi and Hh-HPL⁺ over a period of 6 days (12 generations).
283 The competitive advantage of Hh-HPL⁺ strains was evident within the 2nd (24 hours) and 4th
284 generations (48 hours) with highly bioactive Hh-HPL⁺, or the intermediate Hh-HPL⁺,
285 respectively (Figure 3A). The stunted inhibitory activity exhibited by Hh-NF5 may be
286 attributed to lower levels of HPL production over the course of the assay. The fitness of NTHi
287 over subsequent generations decreases significantly until complete loss of fitness during the
288 final generations. Although, competition with Hh-HPL⁻ did not result in a significant loss of
289 fitness over the 6-day period, there was decrease in fitness of all Hh-HPL strains at 24 hours,
290 followed by complete recovery (Figure 3A). This may have arisen from competition for heme
291 prior to the onset of maximum HPL production.

292 To show that loss of fitness of NTHi was not unique to NTHi strain ATCC 49247, additional
293 reference strains NCTC 11315 and ATCC 49766 were tested in competition with Hh-BW1.
294 All three NTHi strains responded in the same manner, culminating in a total loss of fitness at
295 the end of the 6-day period (Figure 3B).

296

297 **CONCLUSION**

298 Previously, we identified an uncharacterized hemophore, designated hemophilin, produced by
299 Hh which is able to inhibit NTHi growth by heme starvation (29). The current study aimed to
300 further test the inhibitory capacity of Hh-HPL⁺ by direct *in vitro* competition with NTHi, for
301 the purpose of determining probiotic potential.

302 The unique inhibitory capacity and growth advantage exhibited by Hh-HPL⁺ strains, together
303 with expression analysis during competitive growth confirmed that inhibition was mediated by
304 the presence of HPL. These results demonstrate the enormous probiotic potential Hh-HPL⁺
305 strains against NTHi colonization in the URT. Reduction or elimination of NTHi carriage from
306 the URT and subsequent migration to the lower airways would be an effective means of
307 preventing infection with the organism. Further investigation is required to determine the
308 protective capacity of HPL-producers *in vivo* and their ability to interrupt NTHi colonization
309 of host cells.

310 **FUNDING**

311 This work was funded by a grant from the Clifford Craig Foundation, Launceston, Tasmania
312 (Grant number CCF 170).

313

314 **REFERENCES**

- 315 1. Mukundan D, Ecevit Z, Patel M, Marrs CF, Gilsdorf JR. 2007. Pharyngeal colonization
316 dynamics of *Haemophilus influenzae* and *Haemophilus haemolyticus* in healthy adult
317 carriers. *Journal of clinical microbiology* 45:3207-3217.
- 318 2. Puig C, Grau I, Marti S, Tubau F, Calatayud L, Pallares R, Liñares J, Ardanuy C. 2014. Clinical
319 and molecular epidemiology of *Haemophilus influenzae* causing invasive disease in adult
320 patients. *PLoS One* 9:e112711.
- 321 3. Murphy TF, Faden H, Bakaletz LO, Kyd JM, Forsgren A, Campos J, Virji M, Pelton SI. 2009.
322 Nontypeable *Haemophilus influenzae* as a pathogen in children. *The Pediatric infectious
323 disease journal* 28:43-48.
- 324 4. Slack MP. 2015. A review of the role of *Haemophilus influenzae* in community-acquired
325 pneumonia. *Pneumonia* 6:26-43.
- 326 5. Murphy TF. 2015. Vaccines for nontypeable *Haemophilus influenzae*: the future is now. *Clin
327 Vaccine Immunol* 22:459-466.
- 328 6. King P. 2012. *Haemophilus influenzae* and the lung (*Haemophilus* and the lung). *Clinical and
329 translational medicine* 1:1-9.
- 330 7. Langereis JD, de Jonge MI. 2015. Invasive disease caused by nontypeable *Haemophilus
331 influenzae*. *Emerging infectious diseases* 21:1711.
- 332 8. van Wessel K, Rodenburg GD, Veenhoven RH, Spanjaard L, van der Ende A, Sanders EA.
333 2011. Nontypeable *Haemophilus influenzae* invasive disease in The Netherlands: a
334 retrospective surveillance study 2001–2008. *Clinical Infectious Diseases* 53:e1-e7.
- 335 9. Giufrè M, Fabiani M, Cardines R, Riccardo F, Caporali MG, D'Ancona F, Pezzotti P, Cerquetti
336 M. 2018. Increasing trend in invasive non-typeable *Haemophilus influenzae* disease and
337 molecular characterization of the isolates, Italy, 2012–2016. *Vaccine* 36:6615-6622.
- 338 10. Sriram KB, Cox AJ, Clancy RL, Slack MP, Cripps AW. 2017. Nontypeable *Haemophilus
339 influenzae* and chronic obstructive pulmonary disease: a review for clinicians. *Critical
340 Reviews in Microbiology*:1-18.

- 341 11. Maddi S, Kolsum U, Jackson S, Barraclough R, Maschera B, Simpson KD, Pascal TG, Durviaux
342 S, Hessel EM, Singh D. 2017. ampicillin resistance in *Haemophilus influenzae* from COPD
343 patients in the UK. *International journal of chronic obstructive pulmonary disease* 12:1507.
- 344 12. Desai H, Richter S, Doern G, Heilmann K, Dohrn C, Johnson A, Brauer A, Murphy T, Sethi S.
345 2010. Antibiotic resistance in sputum isolates of *Streptococcus pneumoniae* in chronic
346 obstructive pulmonary disease is related to antibiotic exposure. *COPD: Journal of Chronic*
347 *Obstructive Pulmonary Disease* 7:337-344.
- 348 13. Pettigrew MM, Tsuji BT, Gent JF, Kong Y, Holden PN, Sethi S, Murphy TF. 2016. Effect of
349 fluoroquinolones and macrolides on eradication and resistance of *Haemophilus influenzae* in
350 chronic obstructive pulmonary disease. *Antimicrobial agents and chemotherapy* 60:4151-
351 4158.
- 352 14. Wilson R, Sethi S, Anzueto A, Miravittles M. 2013. Antibiotics for treatment and prevention
353 of exacerbations of chronic obstructive pulmonary disease. *Journal of Infection* 67:497-515.
- 354 15. Puig C, Tirado-Vélez JM, Calatayud L, Tubau F, Garmendia J, Ardanuy C, Marti S, Adela G,
355 Liñares J. 2015. Molecular characterization of fluoroquinolone resistance in nontypeable
356 *Haemophilus influenzae* clinical isolates. *Antimicrobial agents and chemotherapy* 59:461-
357 466.
- 358 16. Vila J, Ruiz J, Sanchez F, Navarro F, Mirelis B, de Anta MTJ, Prats G. 1999. Increase in
359 Quinolone Resistance in a *Haemophilus influenzae* Strain Isolated from a Patient with
360 Recurrent Respiratory Infections Treated with Ofloxacin. *Antimicrobial agents and*
361 *chemotherapy* 43:161-162.
- 362 17. Bastida T. 2003. Levofloxacin treatment failure in *Haemophilus influenzae* pneumonia.
- 363 18. Hariadi NI, Zhang L, Patel M, Sandstedt SA, Davis GS, Marrs CF, Gilsdorf JR. 2015.
364 Comparative profile of heme acquisition genes in disease-causing and colonizing
365 nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus*. *Journal of clinical*
366 *microbiology* 53:2132-2137.
- 367 19. Szelestey BR, Heimlich DR, Raffel FK, Justice SS, Mason KM. 2013. *Haemophilus* responses to
368 nutritional immunity: epigenetic and morphological contribution to biofilm architecture,
369 invasion, persistence and disease severity. *PLoS pathogens* 9:e1003709.
- 370 20. Morton DJ, Bakaletz LO, Jurcisek JA, VanWagoner TM, Seale TW, Whitby PW, Stull TL. 2004.
371 Reduced severity of middle ear infection caused by nontypeable *Haemophilus influenzae*
372 lacking the hemoglobin/hemoglobin-haptoglobin binding proteins (Hgp) in a chinchilla
373 model of otitis media. *Microbial pathogenesis* 36:25-33.
- 374 21. Morton DJ, Seale TW, Bakaletz LO, Jurcisek JA, Smith A, VanWagoner TM, Whitby PW, Stull
375 TL. 2009. The heme-binding protein (HbpA) of *Haemophilus influenzae* as a virulence
376 determinant. *International Journal of Medical Microbiology* 299:479-488.
- 377 22. Seale TW, Morton DJ, Whitby PW, Wolf R, Kosanke SD, VanWagoner TM, Stull TL. 2006.
378 Complex role of hemoglobin and hemoglobin-haptoglobin binding proteins in *Haemophilus*
379 *influenzae* virulence in the infant rat model of invasive infection. *Infection and immunity*
380 74:6213-6225.
- 381 23. Ahearn CP, Gallo MC, Murphy TF. 2017. Insights on persistent airway infection by non-
382 typeable *Haemophilus influenzae* in chronic obstructive pulmonary disease. *Pathogens and*
383 *Disease* 75.
- 384 24. Stites SW, Plautz MW, Bailey K, O'Brien-Ladner AR, Wesselius LJ. 1999. Increased
385 concentrations of iron and isoferitins in the lower respiratory tract of patients with stable
386 cystic fibrosis. *American journal of respiratory and critical care medicine* 160:796-801.
- 387 25. White DC, Granick S. 1963. Hemin biosynthesis in *Haemophilus*. *Journal of bacteriology*
388 85:842-850.
- 389 26. Sgheiza V, Novick B, Stanton S, Pierce J, Kalmeta B, Holmquist MF, Grimaldi K, Bren KL,
390 Michel LV. 2017. Covalent bonding of heme to protein prevents heme capture by
391 nontypeable *Haemophilus influenzae*. *FEBS open bio* 7:1778-1783.

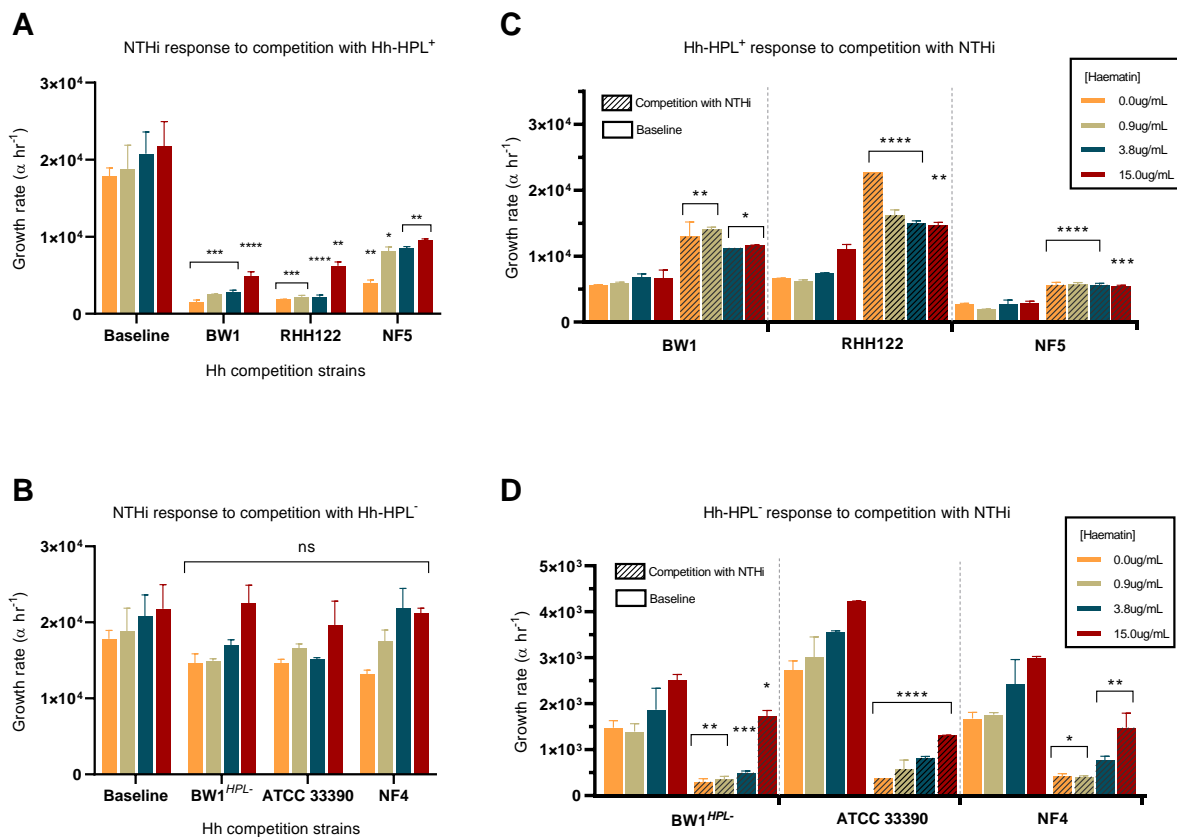
- 392 27. Skaar EP. 2010. The battle for iron between bacterial pathogens and their vertebrate hosts.
393 PLoS pathogens 6:e1000949.
- 394 28. Latham RD, Gell DA, Fairbairn RL, Lyons AB, Shukla SD, Cho KY, Jones DA, Harkness NM,
395 Tristram SG. 2017. An isolate of *Haemophilus haemolyticus* produces a bacteriocin-like
396 substance that inhibits the growth of nontypeable *Haemophilus influenzae*. *International*
397 *journal of antimicrobial agents* 49:503-506.
- 398 29. Latham RD, Torrado M, Atto B, Walshe JL, Wilson R, Guss JM, Mackay JP, Tristram S, Gell DA.
399 2019. A heme-binding protein produced by *Haemophilus haemolyticus* inhibits non-typeable
400 *Haemophilus influenzae*. *Molecular Microbiology*, In press doi:10.1101/626416:626416.
- 401 30. Vogel AR, Szelestey BR, Raffel FK, Sharpe SW, Gearinger RL, Justice SS, Mason KM. 2012.
402 SapF-mediated heme-iron utilization enhances persistence and coordinates biofilm
403 architecture of *Haemophilus*. *Front Cell Infect Microbiol* 2:42.
- 404 31. Mason KM, Raffel FK, Ray WC, Bakaletz LO. 2011. Heme utilization by nontypeable
405 *Haemophilus influenzae* is essential and dependent on Sap transporter function. *J Bacteriol*
406 193:2527-35.
- 407 32. Price EP, Harris TM, Spargo J, Nosworthy E, Beissbarth J, Chang AB, Smith-Vaughan HC,
408 Sarovich DS. 2017. Simultaneous identification of *Haemophilus influenzae* and *Haemophilus*
409 *haemolyticus* using real-time PCR. *Future Microbiology*.
- 410 33. Wisner MJ, Lenski RE. 2015. A comparison of methods to measure fitness in *Escherichia coli*.
411 *PLoS One* 10:e0126210.
- 412 34. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR.
413 *Nucleic acids research* 29:e45-e45.
- 414 35. Pfaffl MW, Horgan GW, Dempfle L. 2002. Relative expression software tool (REST©) for
415 group-wise comparison and statistical analysis of relative expression results in real-time PCR.
416 *Nucleic acids research* 30:e36-e36.
- 417 36. Reischl U, Linde H-J, Metz M, Leppmeier B, Lehn N. 2000. Rapid identification of methicillin-
418 resistant *Staphylococcus aureus* and simultaneous species confirmation using real-time
419 fluorescence PCR. *Journal of Clinical Microbiology* 38:2429-2433.
- 420 37. Reischl U, Pulz M, Ehret W, Wolf HJ. 1994. PCR-based detection of mycobacteria in sputum
421 samples using a simple and reliable DNA extraction protocol. *BioTechniques* 17:844-845.
- 422 38. Sweeney RW, Whitlock RH, McAdams SC. 2006. Comparison of three DNA preparation
423 methods for real-time polymerase chain reaction confirmation of *Mycobacterium avium*
424 subsp. *paratuberculosis* growth in an automated broth culture system. *Journal of veterinary*
425 *diagnostic investigation* 18:587-590.
- 426 39. Van Tongeren S, Degener J, Harmsen H. 2011. Comparison of three rapid and easy bacterial
427 DNA extraction methods for use with quantitative real-time PCR. *European journal of clinical*
428 *microbiology & infectious diseases* 30:1053-1061.
- 429 40. Wilson DA, Yen-Lieberman B, Reischl U, Gordon SM, Procop GW. 2003. Detection of
430 *Legionella pneumophila* by real-time PCR for the *mip* gene. *Journal of clinical microbiology*
431 41:3327-3330.
- 432 41. Freschi CR, Oliveira CJBd. 2005. Comparison of DNA-extraction methods and selective
433 enrichment broths on the detection of *Salmonella Typhimurium* in swine feces by
434 polymerase chain reaction (PCR). *Brazilian Journal of Microbiology* 36:363-367.
- 435 42. Coyne SR, Craw PD, Norwood DA, Ulrich MP. 2004. Comparative analysis of the Schleicher
436 and Schuell IsoCode Stix DNA isolation device and the Qiagen QIAamp DNA mini kit. *Journal*
437 *of clinical microbiology* 42:4859-4862.
- 438 43. Rantakokko-Jalava K, Jalava J. 2002. Optimal DNA isolation method for detection of bacteria
439 in clinical specimens by broad-range PCR. *Journal of clinical microbiology* 40:4211-4217.
- 440 44. Ali MK, Kim RY, Karim R, Mayall JR, Martin KL, Shahandeh A, Abbasian F, Starkey MR,
441 Loustaud-Ratti V, Johnstone D. 2017. Role of iron in the pathogenesis of respiratory disease.
442 *The international journal of biochemistry & cell biology* 88:181-195.

443 **FIGURES AND TABLES**

444

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

445 **Table 1** | Summary of primer and LNA-probe sequences, and expected amplicon size for the *hypD*,
446 *siaT* and *HPL* targets.

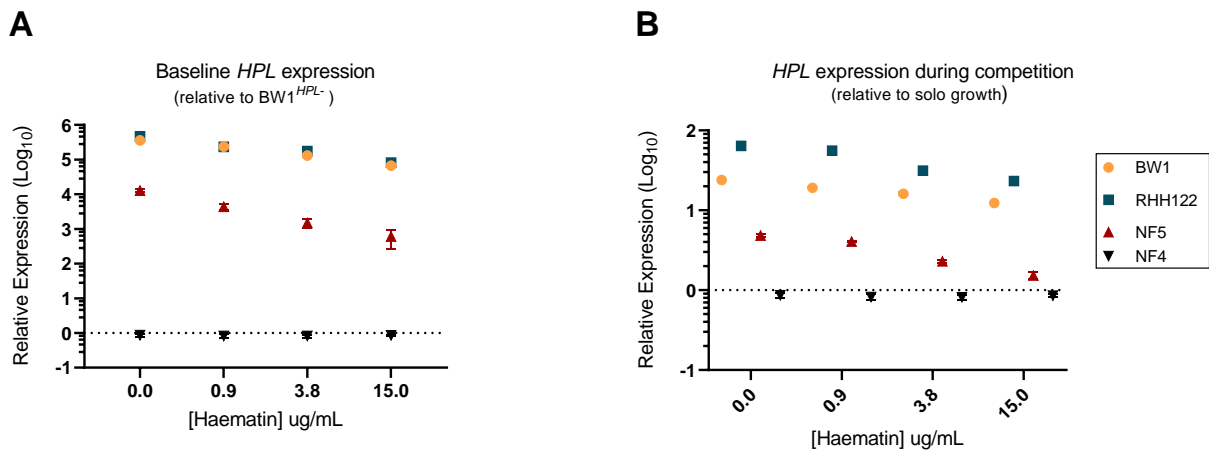


447

448 **Figure 1** | Short-term competition between NTHi and Hh. Calculated growth rates of
449 NTHi in response to competition with (A) Hh-HPL⁺ or (C) Hh-HPL⁻. The growth rate for
450 each (B) Hh-HPL⁺ and (D) Hh-HPL⁻ strain was also determined. Data points represented as

451 mean \pm SEM of three separate experiments, performed in triplicate; $P < 0.05^*$, $p < 0.005^{**}$,
 452 $p < 0.0005^{***}$, $p < 0.0001^{****}$.

453



454

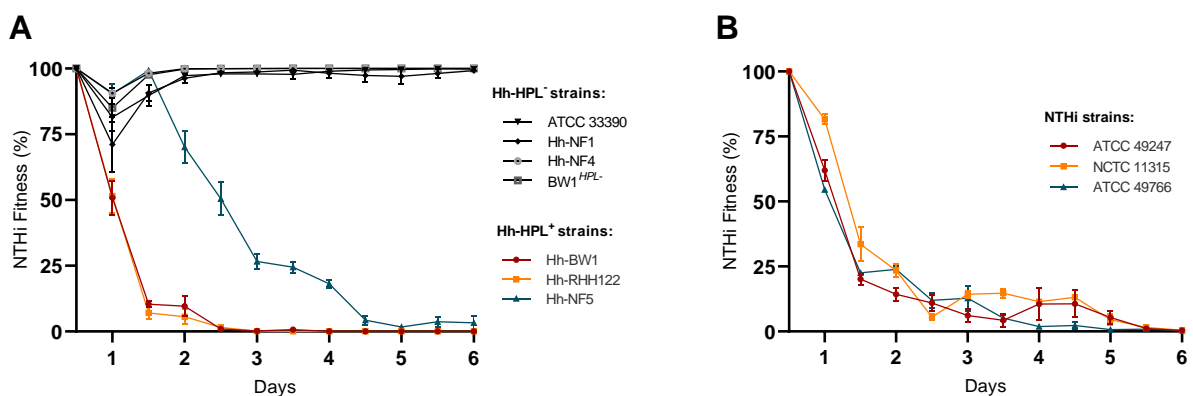
455 **Figure 2 | *HPL* expression among Hh strains during competition with NTHi.** PCR-

456 quantified expression of *HPL* (**A**) for baseline expression (compared to Hh- BW1^{HPL-}) or (**B**)

457 during competition with NTHi relative to individual growth. Data points represented as mean

458 \pm SEM of four biological replicates, performed from duplicate RNA extractions.

459



460

461 **Figure 3 | Fitness of NTHi strains during co-culture with Hh.** Calculated fitness of NTHi

462 in response to competition with Hh-HPL⁺ or Hh-HPL⁻ relative to growth of the competitor

463 strain. (**A**) Competition between a single NTHi strain and multiple Hh, or (**B**) multiple NTHi

464 against Hh-BW1. Data points represented as mean \pm SEM of three separate experiments,

465 performed in quadruplicate.