Molecular signatures of non-typeable *Haemophilus influenzae* lung adaptation in paediatric chronic lung disease

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Abstract

Non-typeable *Haemophilus influenzae* (NTHi), an opportunistic pathogen of the upper airways of healthy children, can infect the lower airways, driving chronic lung disease. However, the molecular basis underpinning NTHi transition from a commensal to a pathogen is not clearly understood. Here, we performed comparative genomic and transcriptomic analyses of 12 paired, isogenic NTHi strains, isolated from the nasopharynx (NP) and bronchoalveolar lavage (BAL) of 11 children with chronic lung disease, to identify convergent molecular signatures associated with lung adaptation. Comparative genomic analyses of the 12 NP-BAL pairs demonstrated that five were genetically identical, with the remaining seven differing by only 1 to 3 mutations. Within-patient transcriptomic analyses identified between 2 and 58 differentially expressed genes in 8 of the 12 NP-BAL pairs, including pairs with no observable genomic changes. Whilst no convergence was observed at the gene level, functional enrichment analysis revealed significant under-representation of differentially expressed genes belonging to *Coenzyme metabolism, Function unknown, Translation, ribosomal structure and biogenesis* Cluster of Orthologous Groups categories. In contrast, *Carbohydrate transport and metabolism*, *Cell motility and secretion*, *Intracellular trafficking and secretion*, and *Energy production* categories were over-represented. This observed trend amongst genetically-unrelated NTHi strains provides evidence of convergent transcriptional adaptation of NTHi to paediatric airways that deserves further exploration. Understanding the pathoadaptative mechanisms that NTHi employs to infect and persist in the lower paediatric airways is essential for devising targeted diagnostics and treatments aimed at minimising disease severity, and ultimately, preventing NTHi lung infections and subsequent chronic lung disease in children.

Introduction

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative bacterium that frequently colonises the respiratory tract mucosa of humans. NTHi is part of the upper airway flora and is found in 20-50% of healthy children and 20-30% of healthy adults (Slack, 2015). The upper airways may act as a reservoir for seeding the lung environment (Fothergill et al., 2014), where NTHi switches to an opportunistic pathogenic lifestyle, a transition driven by multiple, poorly understood factors (Duell et al., 2016).

NTHi facilitates the colonisation of other bacterial species in the lower airways (Van Eldere et al., 2014) and is associated with exacerbations in several respiratory diseases (Duell et al., 2016). Indeed, its presence in the lower airways is associated with increased risk of future development of bronchiectasis (Wurzel et al., 2016). In children with chronic suppurative lung disease (CSLD) or bronchiectasis, NTHi is the most commonly detected bacterium in the lower airways (Byun et al., 2017). In adults, the bacterium can chronically and repeatedly colonise the lungs of patients with chronic obstructive pulmonary disease (COPD), bronchiectasis, or COPD with bronchiectasis (Martinez-Garcia et al., 2011; Sriram et al., 2018), resulting in increased airway inflammation (Tufvesson et al., 2015). NTHi also has the capacity to form biofilms (Murphy and Kirkham, 2002; Swords, 2012) resulting in a decreased susceptibility to antibiotics and increased inflammatory and defence responses in the host (Starner et al., 2006). Unsurprisingly, NTHi is a pathogen with increasing public health recognition (Van Eldere et al., 2014; Slack, 2017).

NTHi has evolved several adaptive mechanisms that enable the bacterium to survive and persist in the human host, including modification of its genome via homologous recombination and phase variation (Erwin et al., 2005; Power et al., 2012). Modulation of gene expression through phase variation enables NTHi to rapidly adapt to and colonise new environments (Wong and Akerley, 2012). Analysis of the NTHi genome has identified several phase-variable virulence factors involved in persistence, nutrient uptake, cellular adherence, and host immune response evasion (Pettigrew et al., 2018), with direct modulation of gene expression in response to environmental stimuli and host milieu (Baddal et al., 2015). NTHi is naturally competent and can undergo high rates of recombination, resulting in greater genetic and phenotypic diversity than serotypeable *H. influenzae* (Connor et al., 2012); however, unlike other chronic respiratory infections (Smith et al., 2006; Price et al., 2013), no significant gene loss or gain has yet been observed in chronic *H. influenzae* infections (Pettigrew et al., 2018).

Identifying signals of NTHi adaptation to the lung would aid in unmasking the mechanisms facilitating pathogenesis, which is critical for improving diagnosis, treatment and prevention of NTHi-associated lower airway diseases. The mechanisms involved in the transition from a commensal state in the nasopharynx (NP) to a pathogenic lifestyle in the lower airways is not well understood (De Chiara et al., 2014; Pettigrew et al., 2018). In this study, we investigated genome- and transcriptome-wide differences between 12 isogenic NTHi pairs obtained from the NP and lower airways (via bronchoalveolar lavage; BAL) of patients with bronchiectasis or CSLD. We hypothesised that NTHi isolated from the airways of patients with lung disease would exhibit characteristic genetic or transcriptional profiles that signalled a change from a commensal to a pathogen.

Methods

Isolate Collection. NP swabs and BAL specimens were collected concurrently from Australian children (mean, 3.2 years; range: 1.1-13.9 years) diagnosed with bronchiectasis or CSLD (Table 1) at participating hospitals in the Northern Territory and Queensland (Hare et al., 2010). Following collection, specimens were transported on ice in 1 mL skim milk, tryptone, glucose and glycerol broth (STGGB) (Gibson and Khoury, 1986; Hare et al., 2010) and stored at -80°C before processing. All subsequent cultures were also stored in STGGB at -80°C. Following selective culture on chocolate blood agar (CBA) supplemented with bacitracin, vancomycin, and clindamycin (Oxoid, Scoresby, VIC, Australia), NTHi was isolated and confirmed as previously described (Hare et al., 2010; Price et al., 2017). Antibiotic sensitivity was determined by disc diffusion as per the Calibrated Dichotomous Susceptibility (CDS) testing guidelines (8th Edition) (Bell et al., 2016) for ampicillin, amoxicillin-clavulanate, cefaclor, ceftriaxone, chloramphenicol, tetracycline and azithromycin.

Whole-Genome Sequencing and Comparative Genomics. DNA extraction was performed as previously described (Price et al., 2015). Whole-genome sequencing (WGS) was carried out at the Australian Genome Research Facility (Parkville, VIC, Australia) using the Illumina (San Diego, CA, USA) HiSeq 2500 platform. As a criterion for our study, all isolate pairs were required to be highly genetically similar (i.e. isogenic). To identify isogenic NP-BAL pairs from each patient, multilocus sequence typing (MLST) (Jolley and Maiden, 2010) followed by phylogenetic analysis was performed. Further details on isolate selection is described in Text S1. Based on this analysis, we selected 12 paired NP-BAL isolates from 11 patients for further investigation; two genetically distinct NP-BAL pairs were selected from one patient (designated 60373_P1 and 60373_P2).

To catalogue the mutations separating each NP-BAL pair, SPANDx v3.2 (default settings) (Sarovich and Price, 2014) was used. Where possible, genetic variants were also confirmed using transcriptomic (RNA-seq) data. For each analysis, the NP isolate draft assembly was used as the reference for variant calling. Further details are provided in Text S1.

NTHi Liquid Media Growth Conditions for RNA Harvest. Due to the reliance of NTHi growth on the presence of X (haemin/haematin) and V (nicotinamide-adenine-dinucleotide) blood factors (Holt, 1962), we initially attempted to culture isolates in brain heart infusion (BHI) broth supplemented with Haemophilus Test Medium (HTM; Oxoid), and subsequently, a HTM-supplemented artificial sputum medium formulated to mimic the mucosal environment in the airways of cystic fibrosis patients (Fung et al., 2010; Price et al., 2017). However, due to inconsistent optical density (OD) readings and poor growth or flocculation of some or all isolates in these media, we investigated an alternative liquid medium for the growth of NTHi for RNA-seq (see Text S1 for further details). CBA is widely used for culturing NTHi and other fastidious bacteria (Artman et al., 1983). Thus, we used this medium as the basis for formulating liquid chocolate medium (LCM). Formulation of LCM and growth conditions are provided in Text S1.

RNA Extraction. All isolates were cultured in LCM and in duplicate to account for any technical replicability issues. RNA was extracted using TRIzol® as described in Text S1. After DNA digest, absence was verified using real-time PCR assays that detect Hi DNA (Aziz et al.,

2017) and defibrinated horse blood DNA (Humair et al., 2007). For further details see Text S1.

Differential Expression (DE) Analysis. RNA-seq processing and DE analysis was performed as described in Text S1. Breifly, RNA-seq was carried out using the Illumina HiSeq 2500 platform. In total, 24 isolates were sequenced in duplicate, generating 48 transcriptomes.

To identify molecular signatures of lung adaptation on a within-patient basis, DE analysis was first conducted by comparing BAL with NP isolates from individual patients. Subsequently, convergence analysis was performed by comparing BAL with NP isolates from all 12 isolate pairs. The following additional analyses with different sample sets were performed to ensure robustness: a) those NP-BAL pairs with DE detected in the within-patient analysis (eight in total), and b) the three NP-BAL pairs with >45 DE genes (60051, 60068, 60295). For all convergence analyses, the two NP-BAL pairs from patient 60373 were treated either as independent NP-BAL pairs or grouped as a single patient. DE was conducted using edgeR (v3.18.1) (Robinson et al., 2010), implemented in R (v3.4.1) (R Core Team, 2014). For all analyses, DE was determined using the quasi-likelihood method (*glmQLFit* function).

Genes with significant DE were annotated using Clusters of Orthologous Groups (COG) (Galperin et al., 2015), with categories retrieved from a previous study (Santana et al., 2014) with minor corrections (Table S1). For genes with multiple COG categories, all assigned categories were included in the analysis. To assess for significant enrichment of functional groups, COG categories of the reference genome 86-028NP were compared with those identified in the within-patient DE analyses. Statistical assessment comparing the proportion of categories was performed in R using a two-tailed Fisher's exact test with a false discovery rate corrected *p*-value of ≤0.05.

Data Availability. All genomic and transcriptomic data generated as part of this study are publicly available under NCBI BioProject PRJNA484075. Genome assemblies for the 24 NTHi strains can be found in NCBI GenBank (QWLW00000000-QWMT00000000), and Illumina WGS and RNA-Seq data are available on the Sequence Read Database (SRR7719256-SRR7719303).

Results

Comparative Genomic Analysis of Paired Isolates. To assess diversity and to identify potential clustering of NP or BAL isolates, a maximum parsimony phylogenetic tree was constructed using 124,262 biallelic, core-genome SNPs identified among the paired isolates and a global NTHi isolate set comprising 157 strains (Figure S1). Phylogenomic analysis confirmed that pairs from individual patients clustered closely together yet were distinct from other NP-BAL pairs. Consistent with previous studies (De Chiara et al., 2014; Price et al., 2015; Pettigrew et al., 2018), there was no evidence of NP- or BAL-specific clades. Isolate pairs were identical sequence types (STs), with 5 of the 12 pairs possessing novel STs (ST-1906 to ST-1910; Table S2). Patient 60373 had two distinct isolate pairs (NP-BAL pairs 60373_P1 and 60373_P2) that were separated by >17,000 SNPs, demonstrating the presence of at least two NTHi lineages colonising this patient. This finding was also reflected in the MLST data, with 60373_P1 isolates belonging to ST-1910 whereas 60373_P2 isolates were ST-139 (Table S2). Antibiotics are routinely used to treat exacerbation events in paediatric chronic lung disease, and resistance to clinically-relevant antibiotics can arise during such infections. In the current work, no NTHi isolate exhibited resistance towards any of the tested antibiotics, ruling out antibiotic resistance-driven mutations arising between isolate pairs.

Next, within-patient comparative genomic analyses were performed to identify variants separating the paired NP-BAL isolates. Pairs were highly genetically similar, with a maximum of three mutations observed between any one pair (Table 1). In total, two non-synonymous SNPs and seven indels (six in open-reading frames) were identified among seven NP-BAL pairs, with the remaining five NP-BAL pairs being genetically identical (Table 2). No copy-number variants, large deletions or inversions were observed between any of the pairs. One non-synonymous SNP occurred in the exogenous haem acquisition-encoding gene, *hgpB* ($HgpB_{Arg407Lys}$; 60295 BAL Hi1), and the second affected the outer membrane protein assembly factor, *bamA* ($BamA_{Glu508Gly}$; 65290 NP Hi3). Of the seven indels, four were simple sequence repeats (SSRs) that were predicted to result in frameshift mutations, leading to truncated proteins in phase-variable genes ($HsdM3_{Glu9fs}$, $HsdM3_{Glu12fs}$, $HgpB_{Thr35fs}$, and $Lic3A_{Ser18fs}$ in 60051 BAL Hi1, 60373_P1 BAL Hi1, 60068 BAL Hi1, and 65001 BAL Hi1, respectively). The remaining three indels were in: *ompP2* ($OmpP2_{Ala269_Gly270insValGlyAla}$; 65001 BAL Hi1), *hsdS2* ($HsdS2_{Thr173_Leu176del}$; 65001 BAL Hi1), and an intergenic region 62bp upstream of *hxuC* (60069 BAL Hi1). Notably, *hsdM3* (in 60051 and 60373_P1) and *hgpB* (in 60068 and 60295) were mutated in isolates from two patients. When compared with 86-028NP, all mutations were shown to deleteriously affect the BAL isolates (Table 2).

The *hsdM3* indels in both the 60051 and 60373_P1 BAL isolates occurred in the same pentanucleotide SSR tract, which comprises variable copy number of a 'CGAGA' motif at the 5' end of this gene. Phase variation in *hsdM3* is driven by changes in this highly mutable SSR tract and is influenced by a lack of adenine methylation (Zaleski et al., 2005). A 5bp AGACG deletion in this SSR tract in 60051 BAL Hi1 (Figure 1A and 1C) is predicted to produce a truncated protein of just 13 residues. Similarly, a 5bp CGAGA deletion was observed in 60373_P1 BAL Hi1; however, it was only supported by ~60% of aligned reads. Upon closer inspection, a 10bp deletion (CGAGACGAGA) was present in the remaining ~40% of aligned reads (Figure 1B). This mixture of *hsdM3* alleles is supported by the RNA-seq reads. The 5bp (~60%) and 10bp (~40%) components were both predicted to produce a truncated protein of 16 and 24 residues, respectively (Figure 1C). Given that isolates were subjected to a

genetic bottleneck prior to sequencing, these mixtures suggest that the *hsdM3* SSR undergoes a rapid mutation rate, with mutations potentially occurring during laboratory passage.

Liquid Chocolate Medium for Reproducible NTHi Growth. As NTHi has an absolute growth requirement for X and V blood factors (Holt, 1962), commonly used culture media for this fastidious bacterium typically includes lysed blood in a nutrient-rich agar base (e.g. CBA) (Artman et al., 1983), or BHI base supplemented with HTM (Coleman et al., 2003). However, we found uniform and reproducible growth of NTHi in HTM-supplemented BHI problematic; variable growth rates were observed both among isolates and between technical replicates, indicating inconsistent bacterial growth. Additionally, the microbial load was insufficient for RNA-seq, even after 24h incubation. We also observed inconsistent OD readings between replicates and a tendency towards flocculation in certain strains. Similar issues were observed with HTM-supplemented artificial sputum medium, which is designed to mimic cystic fibrosis sputum (Fung et al., 2010) (Figure S2A).

To overcome these issues, we developed liquid chocolate medium (LCM), a medium that is straightforward to formulate, inexpensive and produces high-quantity NTHi cellular density for downstream analysis. LCM yielded NTHi cultures with reproducible growth, consistent growth rates, little to no flocculation, and sufficient microbial load to support sequencing of late-log phase RNA. LCM was thus used to determine the growth curves of four isolates to identify an appropriate culture duration for RNA extraction. A 7.5-hour post-inoculation time point was found to represent late-log phase growth in all four cultures tested (Figure S2B). This time point, which was chosen to reflect non-logarithmic bacterial growth rates *in vivo* (Yang et al., 2008; Kasi et al., 2017), was subsequently used for RNA extraction of all isolates.

Within-patient DE and Comparison with Genetic Mutations. Culture and RNA extraction of 24 isolates was performed in duplicate. On average, 89.2% (87.1-92.0%) of sequence reads aligned uniquely to the 86-028NP reference genome, and of these, an average of 70% (68.2-74.8%) were assigned to known genomic features (Table S2). Due to this high mapping percentage, within-patient DE analysis was performed using 86-028NP as the reference genome. This approach also greatly simplified comparisons among patients. DE was observed between eight NP-BAL pairs, ranging from 2 to 58 DE genes per pair. Three pairs (60051, 60068, and 60295) had >45 DE genes, five pairs (60362, 60370, 60373_P1, 60373_P2, and 65290) had 15 or fewer DE genes (Dataset S1), and the remaining four pairs (60069, 60294, 60316, and 65001) did not exhibit any DE.

In total, 189 DE genes, representing a wide functional spectrum, were detected across the eight NP-BAL pairs. Eight genes were DE in three pairs (*comE*, *comM*, *glgC*, *ligA*, *pilB*, *pilC*, *smf* and *tnaA*), 39 were DE in two pairs (*arcC*, *argF*, *asnA*, *comABCDF*, *dmsC*, *glgABPX*, *glpK*, *hitA*, *hsdMS3*, *licBC*, *mao2*, *nagAB*, *nanAEK*, *NTHI0229*, *NTHI0232*, *NTHI0235*, *NTHI0646*, *NTHI0647*, *NTHI1183*, *NTHI1243*, *nrfCD*, *pckA*, *pilA*, *sdaA*, *siaT*, and *tnaB*), and the remaining 87 were DE in single NP-BAL pairs (Figure 2; Dataset S1). However, only ten genes exhibited concordant downregulation (*mao2*, *NTHI0646*, *NTHI0647*, *pckA*, *sdaA*) or upregulation (*asnA*, *hitA*, *hsdMS3*, *hsdS3*, *tnaB*) in the BAL isolates. In all other DE genes, discordance in the directionality of expression was observed, including the eight shared DE genes in three NP-BAL pairs (Figure 2). Additionally, genes in the 60295 pair were predominantly

251 upregulated, whereas genes were predominantly downregulated in pairs 60051 and 60068.
252 Overall, there was little overlap amongst patients.

253 There was also minimal correlation between the number of genetic mutations and the
254 number of DE genes detected between NP-BAL pairs (Table 1). For example, the 60295 NP-
255 BAL pair exhibited 46 DE genes, but only a single non-synonymous SNP ($HgpB_{Arg407Lys}$) was
256 observed in the BAL isolate. In contrast, no DE was detected between the 65001 NP-BAL pair
257 (Dataset S1), despite three indels being identified in the BAL isolate, including a deleterious
258 frameshift in the phase-variable sialyltransferase gene, *lic3A*, which truncates the Lic3A
259 protein from 320 to just 36 residues (Table 2). Two NP-BAL pairs (60294 and 60316)
260 contained no mutations and no DE genes. Conversely, two NP-BAL pairs (60051 and
261 60373_P1) contained both genetic and transcriptional alterations affecting a single phase-
262 variable gene, *hsdM3* (Table 2; Dataset S1). For both pairs, *hsdM3* and *hsdS3* were
263 upregulated in the BAL isolates, with an average $\log_2$ fold change of 2.5 and 4.6,
264 respectively. Additionally, *hsdR3* was upregulated by 2.2-fold in 60373_P1. However, both
265 BAL isolates from 60051 and 60373_P1 encode truncated HsdM proteins of 13 and 16-24
266 residues, respectively (Figure 1C), indicating that *hsdM3* upregulation in these isolates is
267 unlikely to have a functional consequence. Notably, the two isolates with mutations in *hgpB*,
268 60068 BAL Hi1 and 60295 BAL Hi1, both had a relatively high amount of DE, with 55 and 46
269 DE genes, respectively.

270 **DE Convergence and Functional Enrichment Analysis of NP-BAL Isolates.** We compared BAL
271 with NP isolates from all patients to identify genes with convergent gene expression.
272 However, no single convergent genetic mutations or DE genes were identified, suggesting
273 more complex pathways involved in lung adaptation. Therefore, we conducted a functional
274 enrichment analysis of the 134 non-redundant DE genes using COG assignments to identify
275 enriched categories. Statistical analysis identified 4/21 COG categories that were
276 significantly over-represented in the functional enrichment analysis (*Carbohydrate transport*
277 *and metabolism*, *Energy production and conversion*, *Intracellular trafficking and secretion*,
278 and *Cell motility and secretion*), and 3/21 categories (*Translation, ribosomal structure and*
279 *biogenesis*, *Coenzyme metabolism*, and *Function unknown*) that were under-represented
280 ($p \leq 0.05$, Figure 3). Of note, the *Function unknown* category encompasses 20.2% genes in the
281 86-028NP genome, yet DE was only observed in 3.5% of these genes. The basis for this
282 under-representation may partly reflect the artificially high number of gene annotations in
283 the NTHi genome. In support of this notion, 47/397 (11.8%) genes assigned to the *Function*
284 *unknown* category exhibited zero reads across our 48 transcriptomes (Dataset S2, grey
285 shaded rows), representing 37.9% of all genes with zero reads. Approximately 51.8% of the
286 other genes with zero reads corresponded with paralogous ribosomal and transfer RNA
287 species (Dataset S2).

288 The most significant over-represented COGs ($p < 0.001$) were *Carbohydrate transport and*
289 *metabolism*, which is represented by 4.9% of 86-028NP genes, yet 17.3% of DE genes
290 belonged to this category, and *Cell motility and secretion*, with only 0.6% genes in 86-028NP
291 but with 5.8% of DE genes belonging to this COG. The most significant under-represented
292 COGs were *Translation, ribosomal structure and biogenesis*, comprising 8.0% of 86-028NP
293 genes but only 0.6% of the DE genes, and *Function unknown*, which comprises 20.2% of 86-
294 028NP genes yet only 6.2% of DE genes belonged to this category (Figure 3; Dataset S1).
295 Other significant COG categories ($p < 0.01$) were *Energy production and conversion* (5.0% of

296 86-028NP genes but 12.0% DE genes) and *Coenzyme metabolism* (4.4% 86-028NP genes but
297 0% DE genes).

298 **Discussion**

299 NTHi is a pathogen of emerging public health importance (Erwin and Smith, 2007; Van
300 Eldere et al., 2014). Conventionally considered a commensal of the upper airways, it is now
301 recognised that NTHi is associated with increased severity and progression of multiple
302 polymicrobial diseases of the lower airways such as COPD, bronchiectasis, and CSLD
303 (Murphy, 2003; Purcell et al., 2014). In this study, we aimed to identify signals of NTHi
304 pathoadaptation to the lower airways using 12 paired isogenic strains retrieved from the
305 upper (NP) and the lower (BAL fluid) airways of 11 paediatric patients with CLSD or
306 bronchiectasis. Similar studies have employed WGS to document the genome-wide changes
307 leading to chronic adaptation and persistence of NTHi retrieved from COPD sputum in adults
308 (Moleres et al., 2018; Pettigrew et al., 2018). The current work expands on these prior
309 studies by examining both the genomic and transcriptomic profiles of NTHi adaptation in
310 children's airways. To ensure isolates were retrieved from the lower airways, we obtained
311 NTHi isolates from the lower airways (BAL fluid) rather than sputum, the latter of which is
312 fraught with upper respiratory tract contamination issues (Grønseth et al., 2017).

313 As part of our study criteria, only isogenic strains were selected to simplify the identification
314 of convergent pathoadaptative mechanisms. As expected, comparative genomic analyses
315 found minimal genetic differences between all isogenic NP-BAL pairs, with only nine total
316 variants in our dataset, ranging from zero to three variants per pair. Five pairs were
317 genetically identical, with the remaining seven encoding a total of two SNPs and seven
318 indels. Notably, four of these indels occurred in tandem repeat regions, or SSR tracts, of
319 phase-variable genes, which are abundant in the NTHi genome (Gilsdorf et al., 2004;
320 Pettigrew et al., 2018). SSRs provide a rapid mechanism for gene expression modulation and
321 adaptation (Vasu and Nagaraja, 2013; De Ste Croix et al., 2017). Two SSR indels occurred in
322 the hypervariable Type I restriction-modification (RM) system methyltransferase gene,
323 *hsdM3*. The remaining SSR indels occurred in the haemoglobin-haptoglobin binding protein
324 B-encoding gene *hgpB*, which is involved in NP colonisation and iron acquisition (Poole et
325 al.), and *lic3A*, which encodes a lipooligosaccharide (LOS) sialyltransferase that facilitates
326 NTHi colonisation in different anatomical sites (Hosking et al., 1999; Hood et al., 2001).

327 Our genetic findings concur with the recent studies, which found that phase variation in
328 *lic3A*, *hsdM3* and *hgpB* are common in NTHi isolates retrieved from COPD airways (Moleres
329 et al., 2018; Pettigrew et al., 2018). In addition to the *hgpB* SSR variant in 60068 BAL Hi1
330 that truncates the protein from 992 to 12 residues, a non-synonymous SNP ($HgpB_{Arg407Lys}$)
331 was seen in 60295 BAL Hi1. Likewise, the SSR region of *hsdM3* was deleteriously mutated in
332 the 60051 and 60373_P1 BAL isolates, yielding truncated proteins of ≤24 residues (Figure
333 1C). The *hsdM3* gene is part of a Type I RM system that acts as a defence system against
334 foreign DNA invasion by, e.g. bacteriophages (Murray, 2000), fine-tuning of competence
335 through variable methylation patterns, and in the methylation of self-DNA that may alter
336 gene expression (Vasu and Nagaraja, 2013). While the *hdsM3* mutations in the paediatric
337 BAL isolates would render their corresponding Type I RM systems non-functional, the phase-
338 variable nature of *hsdM3* enables rapid reverse switching back to an active state. Others
339 have shown that phase-variable RM systems allow NTHi to rapidly generate genomic and
340 phenotypic diversity, which may enhance survival and persistence, especially in new
341 environments (Zaleski et al., 2005; Atack et al., 2015). Taken together, the commonality of
342 SSR tract mutants affecting *hgpB*, *hsdM3* and *lic3A* in airway-derived isolates demonstrates

343 that phase variation of these loci is driven by strong diversifying selection, with this variation
344 being key in the long-term persistence of NTHi within the hostile lung environment.

345 Outer membrane porin (OmpP1 and OmpP2) mutations are also common in COPD-derived
346 NTHi isolates. Previous studies have identified deleterious *ompP1* mutations in ~33% of
347 COPD isolates, and in-frame *ompP2* mutations (i.e. missense SNPs and indels) in ~20% of
348 COPD isolates (Moleres et al., 2018; Pettigrew et al., 2018). Similarly, we identified an in-
349 frame *ompP2* mutation in the 65001 BAL Hi1 isolate that occurred in the extracellular loop
350 six motif (Sikkema and Murphy, 1992) and increased OmpP2 length by three amino acids.
351 However, we did not observe any genomic or transcriptomic *ompP1* variation between our
352 12 NP and BAL pairs. Although our sample size is modest, this result suggests that *ompP1*
353 variants may be much less common in paediatric NTHi lung-adapted strains than COPD lung-
354 adapted strains. Biochemical profiles in COPD airways (e.g. fatty acids with bactericidal
355 activity against NTHi wild-type strains) (Moleres et al., 2018) are potentially different to
356 those present in paediatric bronchiectasis or CSLD airways, which may explain the greater
357 selective pressure for OmpP1 inactivation in COPD airways. Further work is needed to
358 document the prevalence of *ompP1* mutations in a larger paediatric lung isolate cohort to
359 examine whether diseased paediatric airways do in fact impart different OmpP1 pressures
360 on NTHi compared with isolates from COPD airways.

361 Across the 12 within-patient DE analyses, a total of 134 non-redundant genes (range: 0 to
362 58) were DE between NP-BAL pairs, with no correlation between the number of mutations
363 and the number of DE genes. Indeed, NP-BAL pairs 60362, 60370 and 60373_P2 were
364 devoid of mutations, yet DE was observed in 15, 2, and 4 genes, respectively. Likewise,
365 65001 BAL Hi1 encoded mutations in *lic3A*, *ompP2* and a Type I RM system specificity gene,
366 *hsdS2*, yet no DE was detected in this strain. This lack of correlation suggests that DE
367 between isolate pairs is influenced by epigenetic factors (e.g. altered DNA methylation (Fox
368 et al., 2007)), or by unidentified genetic mutations due to inherent limitations with short-
369 read sequencing approaches (e.g. insertion sequence-mediated rearrangements or variants
370 in paralogues). Future studies employing methylation signature detection and long-read
371 sequencing will enable a deeper understanding of the basis for DE in these strains.

372 We did not identify any DE gene(s) common to all isolates, indicating no universal
373 expression profile associated with lung adaptation (i.e. the BAL isolates). However, some
374 degree of gene convergence was observed, with eight DE genes common to three isolate
375 pairs, and 39 common to two pairs (Dataset S1). Of these, the *com* and *pil* operons, required
376 for the biogenesis and function of the type IV pilus, were DE in three pairs. The type IV pilus
377 is involved in multiple biological roles including adherence to epithelial cells, competence,
378 twitching motility, biofilm formation and long-term NP colonisation (Bakaletz et al., 2005;
379 Jurcisek et al., 2007). The pathway responsible for uptake, transport and incorporation of N-
380 Acetylneuraminic acid (Neu5Ac) into LOS (Apicella, 2012; Wong and Akerley, 2012), was DE
381 in two pairs. Incorporation of Neu5Ac into the LOS enhances immune evasion and increases
382 resistance to killing by human serum (Mandrell et al., 1992). Most DE genes (*n*=87) occurred
383 in single pairs, suggesting multiple, parallel strategies towards lower airway adaptation. In
384 addition, the directionality of DE was inconsistent, with gene upregulation in some pairs and
385 downregulation in others (Figure 2). Lack of a convergent transcriptional signal across all
386 strains may be due to sampling difficulties, for example, the inclusion of BAL isolates that
387 have yet to adapt to the lower airways or re-seeding of the NP environment with lung-

388 adapted strains. Greater breadth and depth of strains is needed to determine whether a
389 convergent signal of lung adaptation can be identified in the NTHi transcriptome.

390 To identify parallel signatures of adaptation to the lung environment on a broader scale, we
391 categorised the functions of within-patient DE genes based on COG categories. Overall,
392 strains isolated from the lower airways showed significant enrichment of DE genes in 7/21
393 COG categories. Four categories were over-represented in the DE gene dataset when
394 compared with their prevalence in the 86-028NP genome, with *Carbohydrate transport and*
395 *metabolism*, *Cell motility and secretion*, *Intracellular trafficking and secretion,* and *Energy*
396 *production and conversion* being significant (p <0.05 or p <0.001). In comparison, the COG
397 categories *Translation, ribosomal structure and biogenesis*, *Function unknown* and
398 *Coenzyme metabolism* were significantly under-represented (p <0.01 or p <0.001) in the DE
399 gene dataset, suggesting that altered expression of these gene categories is negatively
400 associated with lung adaptation. However, analysis of the 48 transcriptomes revealed that
401 11.8% genes in the *Function unknown* category had zero reads mapping to the 86-028NP
402 genome (Dataset S2, grey-shaded rows), suggesting that many of these so-called genes
403 likely do not produce messenger RNA or proteins under any growth conditions, and may
404 represent incorrect gene annotations. Further RNA-seq studies of NTHi cultures grown
405 under different conditions will help to resolve these potential annotation issues. Another
406 noteworthy observation was that *Carbohydrate transport and metabolism* and *Energy*
407 *production and conversion* collectively contained ~35% of all DE genes and were primarily
408 downregulated. This observation is consistent with Baddal and colleagues, who observed
409 the downregulation of genes involved in NTHi central metabolism in response to
410 environmental stimuli (Baddal et al., 2015). *Intracellular trafficking and secretion* and *Cell*
411 *motility and secretion* were also over-represented in the BAL isolates and predominately
412 downregulated (Figure 3; Dataset S1). Taken together, these results suggest that NTHi alters
413 certain functional pathways in response to the lower airway environment, suggesting a
414 degree of predictability in adapting to this new niche. Although further work is needed to
415 consolidate these findings, our work sets the stage for identifying key functional pathways
416 that may be exploited for targeted treatment and eradication of this pathogen.

417 Our study chose not to assess the wider polymicrobial community of the lower airways, as
418 accurate transcriptional characterisation of entire bacterial communities remains
419 bioinformatically challenging (Lim et al., 2013). We instead chose to focus on an in-depth
420 genomic and transcriptional analysis of NTHi, a species known to play a crucial role in the
421 progression and severity of lung disease (Erwin and Smith, 2007; Van Eldere et al., 2014;
422 Slack, 2017). Genetically diverse NTHi isolates from different patients were selected to
423 enable identification of convergent adaptation strategies to the lung environment of
424 paediatric patients. Due to the necessary passaging of strains to ensure purity and to obtain
425 sufficient RNA material for sequencing, we cannot discount that these procedures may have
426 influenced the NTHi expression profiles so that they no longer represent their *in vivo*
427 counterparts. However, as previously demonstrated with other bacterial species, isolates
428 appear to maintain their expression profiles even after microbiological handling and
429 culturing *in vitro* (Viberg et al., 2017). For future studies, we recommend maximising the
430 number of strains isolated from individual patients together with longitudinal sampling to
431 better understand pathogen adaptation over time.

432 In conclusion, our study provides new insights into NTHi adaptation to the lower airways in
433 paediatric chronic lung diseases. Through genomic and transcriptomic characterisation of 12

434 paired NTHi isolates from the NP and the lung, we found that NTHi employs several avenues
435 of pathoadaptation that enables this pathogen to persist in the lower airways. Although we
436 did not identify evidence of convergent pathoadaptive mechanisms at the single-gene level,
437 our study identified parallel DE at the functional level, suggesting that NTHi adaptation to
438 the paediatric airways is a complex but ultimately predictable process. The next steps
439 require characterising larger isolate panels using genomic and transcriptomic methods to
440 better understand the pathoadaptive mechanisms that enable NTHi to persist and cause
441 disease. Such findings will be crucial for the informed development of effective therapeutic
442 interventions to prevent NTHi-driven chronic lung diseases.

443 **Conflict of Interest**
444 The authors declare that the research was conducted in the absence of any commercial or
445 financial relationships that could be construed as a potential conflict of interest.
446
447 **Author Contributions**
448 AC conducted patient recruitment and specimen collection. EN, JB conducted specimen
449 processing, genotyping, and DNA extractions. AA, JB, HSV, EPP, TMH conducted isolate
450 selection. AA conducted culturing, RNA extraction, and bioinformatics analysis with
451 supervision and assistance from DSS, EPP, TMH. AA wrote the initial manuscript draft, and
452 DSS, EPP and TMH critically reviewed and edited the manuscript. DSS, HSV, AC, and EPP
453 conceived of the study and DSS, HSV, and EPP obtained funding. All authors reviewed and
454 approved the final manuscript.
455
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465
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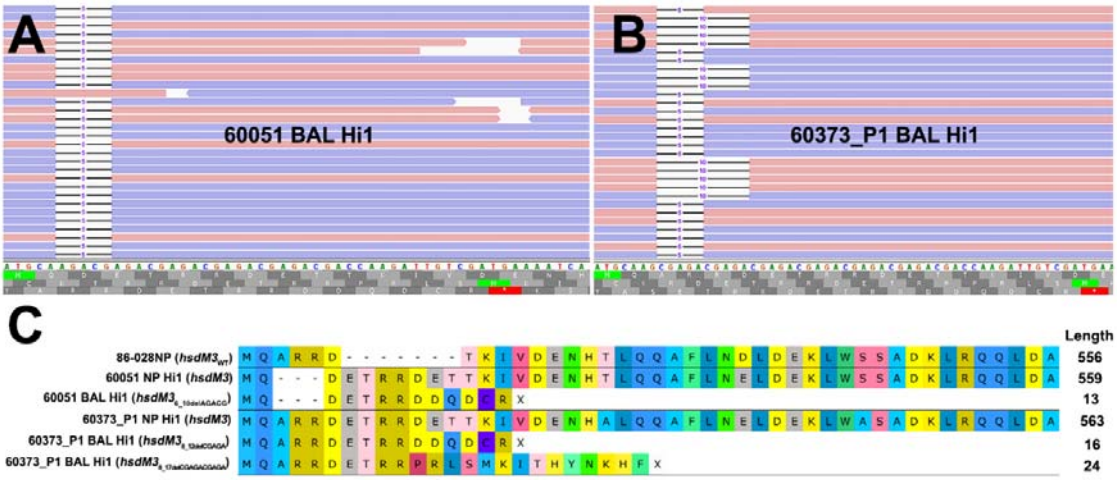
470 **Figures**



471

472 **Figure 1. Read mapping analysis of the highly mutable *hsdM3* pentanucleotide short**
473 **sequence repeat (SSR) tract in 60051 and 60373_P1 NP-BAL pairs.** (A) Graphical
474 representation of 60051 BAL Hi1 Illumina reads aligned against the *hdsM3* region of the
475 60051 NP Hi1 reference assembly. One allele consisting of a 5bp deletion (AGACG) was
476 observed. (B) Graphical representation of 60373_P1 BAL Hi1 Illumina reads aligned against
477 the 60373_P1 NP Hi1 reference assembly. Two different alleles consisting of 5bp (AGACG)
478 and 10bp (AGACGx2) deletions were observed in the BAL isolate. (C) Amino acid alignment
479 of *hsdM3* against reference strain 86-028NP (wild-type). NP isolates from 60051 and
480 60373_P1 encode the full-length protein, with minor differences resulting from the variable
481 SSR track. Indels in the same SSR track of both BAL isolates, including the two alleles
482 observed in 60373_P1, result in a deleterious frameshift that is predicted to yield a
483 truncated protein of 13-24aa.

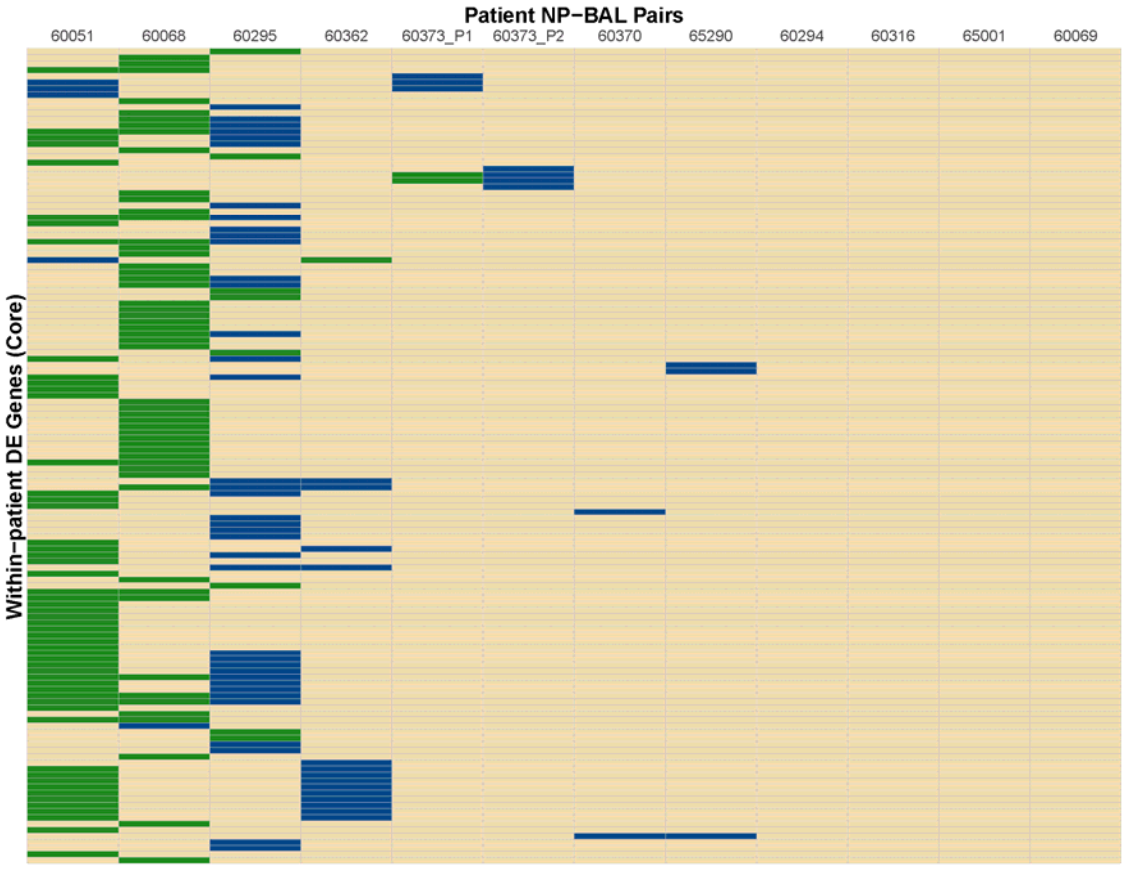
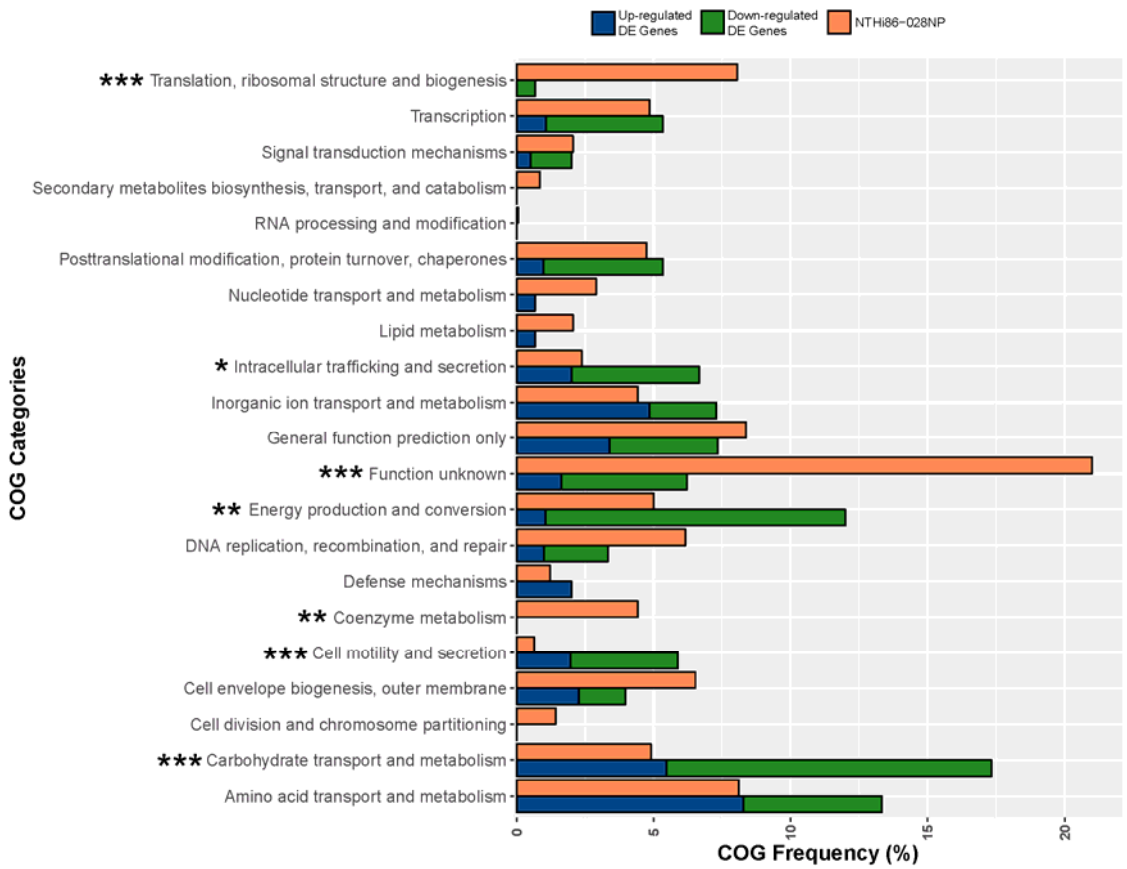


Figure 2. Heat map of differentially expressed (DE) genes identified between the NP-BAL pairs from 11 paediatric chronic lung disease patients. In total 134 non-redundant DE genes were detected across eight NP-BAL pairs. DE was observed in eight NP-BAL pairs; the remaining four pairs contained no DE. Genes were predominantly upregulated in the 60295 NP-BAL pair but predominately downregulated in the 60051 and 60068 NP-BAL pairs. The direction of $\log_2$ fold change of DE genes in BAL is colour-coded as follows: upregulation (blue), downregulation (green), no DE (tan).



492

493 **Figure 3. Clusters of Orthologous Group (COG) analysis comparing the frequency of**
494 **categories in the 86-028NP genome with all DE genes in the 12 NP-BAL pairs.** Seven COG
495 categories were significantly enriched in DE genes from BAL-derived isolates, comprising
496 four over-represented categories (*Carbohydrate transport and metabolism, Cell motility and*
497 *secretion, Energy production and conversion* and *Intracellular trafficking and secretion*) that
498 were majority downregulated and three under-represented categories (*Coenzyme*
499 *metabolism, Function unknown,* and *Translation, ribosomal structure and biogenesis*). *** p
500 < 0.001, ** $p < 0.01$, * $p < 0.05$.

501 **Tables**

502 **Table 1.** Summary of genomic and transcriptomic differences between paired, isogenic NTHi
503 isolates obtained from the nasopharynx (NP) and lower airways (bronchoalveolar lavage;
504 BAL) of 11 Australian children with chronic lung disease.

Patient ID	Site of isolation	Isolate no.	No. SNPs	No. indels	No. differentially expressed genes (up/down)	Genes with transcriptional and genetic alterations
60051	NP	1	0	1	58 (4/54)	*hsdM3*
	BAL	1				
60068	NP	3	0	1	54 (1/53)	---
	BAL	1				
60069	NP	2	0	1	0	---
	BAL	1				
60294	NP	1	0	0	0	---
	BAL	1				
60295	NP	1	1	0	46 (38/8)	---
	BAL	1				
60316	NP	1	0	0	0	---
	BAL	1				
60362	NP	1	0	0	15 (14/1)	---
	BAL	2				
60370	NP	2	0	0	2 (2/0)	---
	BAL	2				
60373_P1	NP	1	0	1*	5 (3/2)	*hsdM3*
	BAL	1				
60373_P2	NP	3	0	0	4 (4/0)	---
	BAL	4				
65001	NP	2	0	3	0	---
	BAL	1				
65290	NP	3	1	0	4 (4/0)	---
	BAL	4				

505 Abbreviations: SNP, single-nucleotide polymorphism; indel, insertion-deletion.
506 *The BAL strain from this patient contains a mixture of two deleterious indel mutations in *hsdM3* at a 40:60 ratio (See Figure 1).

507 **Table 2.** Summary of genetic variants identified between 12 paired, isogenic non-typeable *Haemophilus influenzae* isolates retrieved from the
508 nasopharynx vs. lower airways (bronchoalveolar lavage; BAL).

Patient ID[a]	86-028NP Locus Tag	Gene	NP to BAL Genotype		Amino Acid Change (WT Length)	WT Isolate[b]	Mutation Effect in BAL isolate	Affected Protein	Protein Function	COG category
60051	*NTHI1838*	*hsdM3**	AAGACG	A	Glu9fs (556)	NP	Deleterious frameshift; premature stop yields 13aa product	Type I restriction enzyme HindVIIP M protein	DNA binding; site-specific DNA-methyltransferase (adenine-specific) activity	Defense mechanisms
60068	*NTHI0782*	*hgpB**	GCCAACCAA	G	Thr35fs (992)	NP	Deleterious frameshift; premature stop yields 12aa product	Hemoglobin-haptoglobin binding protein B	Receptor and transporter activity	Inorganic ion transport and metabolism
60069	Intergenic	---	GATTATT	G	---	NP	Intergenic (62bp upstream of NTHI0369 [*hxuC*])	---	---	---
60295	*NTHI0782*	*hgpB*	C	T	Arg407Lys (992)	NP	Non-synonymous	Hemoglobin-haptoglobin binding protein B	Receptor and transporter activity	Inorganic ion transport and metabolism
60373_P1†	*NTHI1838*	*hsdM3**	CGAGA (60%); CGAGACGAGA (40%)	A	Glu12fs (556)	NP	Deleterious frameshift; premature stop yields 16aa or 24aa product	Type I restriction enzyme HindVIIP M protein	DNA binding; site-specific DNA-methyltransferase (adenine-specific) activity	Defense mechanisms
65001	*NTHI0472*	*lic3A**	TTTGA	T	Ser18fs (320)	NP	Deleterious frameshift; premature stop yields 36aa product	CMP-Neu5Ac--lipooligosaccharide alpha 2-3 sialyltransferase	Transferase activity; transfer of glycosyl groups	Cell envelope biogenesis
	NTHI0225	*ompP2*	C	CAGCAGTAGG	Ala269_Gly270insValGlyAla (365)	NP	In frame insertion of ValGlyAla; 368aa product	Outer membrane protein P2	Porin activity	Cell envelope biogenesis
	NTHI0315	*hsdS2*	ACTTACCAGCGAG	A	Thr173_Leu176del (390)	NP	In-frame deletion of 4aa	Type I restriction-modification system specificity protein	DNA binding and modification	Defense mechanisms
65290	*NTHI1084*	*bamA*	T	C	Glu508Gly (800)	NP	Non-synonymous	Protective surface antigen D15	Cell outer membrane assembly; protein insertion into membrane	Cell envelope biogenesis

[a]No genetic variants were found between the 60294, 60316, 60362, 60370 and 60373_P2 pairs; only pairs with genetic differences are shown

[b]Wild-type isolate is defined as the isolate which produces full length protein of similar aa length to 86-028NP reference

*Mutation found in a phase-variable region

[†]The BAL strain from this patient contains a mixture of two deleterious indel mutations in *hsdM3* at a 40:60 ratio (See Figure 1).

Abbrevations: aa, amino acid; BAL, bronchoalveolar lavage; COG, cluster of orthologous groups; NP; nasopharynx; WT, wild type

509 **Supplemental material legends**

510 **Text S1.** Supplementary methods.

511 **Figure S1.** Maximum parsimony phylogenomic analysis of non-typeable *Haemophilus*
512 *influenzae* isolates from paediatric lung disease against a global strain dataset.

513 **Figure S2.** Non-typeable *Haemophilus influenzae* growth kinetics in different growth media.

514 **Table S1.** Updated Cluster of Orthologous Group terms for non-typeable *Haemophilus*
515 *influenzae* strain 86-028NP. Figshare link: https://doi.org/10.6084/m9.figshare.7956191.v1

516 **Table S2.** Whole-genome sequencing and RNA sequencing summary information. Figshare
517 link: https://doi.org/10.6084/m9.figshare.7956194.v5

518 **Dataset S1.** Within-patient Non-typeable *Haemophilus influenzae* differential expression
519 analysis comparing nasopharynx against bronchoalveolar isolates. Figshare link:
520 https://doi.org/10.6084/m9.figshare.7956158.v1

521 **Dataset S2**. RNA sequencing raw read counts against Non-typeable *Haemophilus influenzae*
522 strain 86-028NP. Figshare link: https://doi.org/10.6084/m9.figshare.7956161.v1

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