Genome-scale approaches to identify genes essential for *Haemophilus influenzae* pathogenesis

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Haemophilus influenzae is a Gram-negative bacterium that has no identified natural niche outside of the human host. It primarily colonizes the nasopharyngeal mucosa in an asymptomatic mode, but has the ability to disseminate to other anatomical sites to cause otitis media, upper, and lower respiratory tract infections, septicemia, and meningitis. To persist in diverse environments the bacterium must exploit and utilize the nutrients and other resources available in these sites for optimal growth/survival. Recent evidence suggests that regulatory factors that direct such adaptations also control virulence determinants required to resist and evade immune clearance mechanisms. In this review, we describe the recent application of whole-genome approaches that together provide insight into distinct survival mechanisms of *H. influenzae* in the context of different sites of pathogenesis.

Keywords: *Haemophilus influenzae*, lung infection, bacteremia, ArcA, FNR, lipopolysaccharide, genomics

INTRODUCTION

Haemophilus influenzae's highly specialized host tropism presents a challenge in that many aspects of pathogenesis cannot be experimentally addressed in humans, yet there is no animal model that recapitulates all aspects of human infection. To address this gap in knowledge, a range of *in vivo* and *in vitro* model systems have been devised to mimic *H. influenzae*'s interaction with host components under varied conditions. Animal models are used to reconstruct interactions within the complex physiological and immunological environments occurring in different stages of natural infections including nasopharyngeal colonization (Weiser et al., 1990, 1998), invasion into the blood (Hood et al., 1996; Herbert et al., 2002; Wong et al., 2011), lung infection (Pang et al., 2008; Gawronski et al., 2009), or spread to the middle ear (Mason et al., 2003; Figueira et al., 2007). Targeted *in vitro* models can then be used to address specific molecular interactions in more precisely defined contexts. Several studies have applied whole-genome analytical approaches toward this undertaking (Hood et al., 1996; Herbert et al., 2002; Qu et al., 2010; Nakamura et al., 2011). Recently, application of a high-resolution technology for comprehensive analysis of fitness of bacterial mutants during infection has generated results that can be integrated with information from other genome-scale studies to derive a better understanding of *H. influenzae*'s adaptations relevant to its survival within the host (Gawronski et al., 2009). In this article, we focus on common themes that have emerged relating to *H. influenzae*'s lifestyle in different sites of pathogenesis. The results highlight roles during infection of bacterial nutrient acquisition, stress defense, cell surface structures mediating immune evasion, and regulatory systems controlling these adaptations.

SURVIVAL AND PERSISTENCE OF *H. INFLUENZAE* IN THE LUNG

Haemophilus influenzae is a prevalent cause of lung infections, particularly in exacerbation of chronic obstructive pulmonary disease (COPD; Sethi and Murphy, 2001; Vila-Corcoles et al., 2009; Doring et al., 2011). It is also a leading cause of community-acquired pneumonia (De Schutter et al., 2011). In populations receiving the effective vaccine against strains that produce the serotype b capsule, the majority of lung infections are caused by non-encapsulated, non-typeable *H. influenzae* strains (NTHi).

Several genes of *H. influenzae* have been implicated in infection in a murine lung model (Lorenz et al., 2005; Pang et al., 2008). Until recently, the relative importance of the majority of this bacterium's genes in this setting was unknown. A means of addressing this question was provided by the development of a methodology termed high-throughput insertion tracking by deep sequencing (HITS) that utilizes a whole-genome *mariner* transposon mutant bank in combination with deep sequencing to analyze genes involved in bacterial pathogenesis (Gawronski et al., 2009). This approach exploits a negative selection strategy to identify the genes essential for growth or survival under a condition of interest *in vitro* or during infection in a model host. Identification of these genes is based on measuring the relative decrease in abundance of transposon insertion mutants deficient in specific genes within a mutant library subjected to selection in the host in comparison to pre-selective growth conditions (i.e., *in vitro*). Results from HITS technology provide rapid and comprehensive information about the selection conditions that the bacterium must negotiate in order to survive within that site. Application of the HITS procedure with an *H. influenzae* library comprised of ~75,000 mutants to analyze genes required by *H. influenzae* to resist clearance in a mouse model of pulmonary infection over a 24 h infection period identified 135 genes required for optimal growth/survival in the lungs (Gawronski et al., 2009). This analysis utilized an *H. influenzae* Rd strain with a high level of genetic competence for DNA uptake to allow high-density transposon mutagenesis. Although Rd lacks some of the virulence factors present in NTHi isolates, it exhibits infection properties in animal models similar to those of clinical isolates and serves as a useful model for many aspect of *H.*

influenzae biology and pathogenesis (Weiser et al., 1995; Daines et al., 2003; Wong et al., 2007; Rosadini et al., 2008). With refinements in understanding natural transformation mechanisms in *H. influenzae* or by scaling up mutagenesis procedures, it is likely that large mutant libraries suitable for the HITS procedure could be generated in NTHi isolates. Importantly, all of the genes identified via HITS are conserved in the majority of clinical isolates that have been characterized. Moreover, independent validation of HITS results by evaluating defined NTHi mutants in the lung model has been fully concordant with these data (Gawronski et al., 2009; Rosadini et al., 2011).

An overall theme from the results of this experiment is that during infection of the lung *H. influenzae* encounters an oxidatively stressful environment in which certain nutrients are limiting. Under aerobic growth conditions bacteria need to cope with oxygen toxicity that results from the formation of reactive intermediates such as hydrogen peroxide, superoxide, and hydroxyl radicals, all by-products of aerobic metabolism which can damage proteins, nucleic acids, and cell membranes (Storz and Imlay, 1999; Imlay, 2003). In addition to coping with natural by-products of aerobic metabolism, pathogenic bacteria also face additional challenges from exogenous oxidants produced by phagocytes as part of the host's innate immune response. From the HITS data, DNA recombination and repair genes including *recC*, *recR*, *xerD*, *xerC*, *ruvB*, *ruvA*, and *ruvC* were needed for survival in the lung (**Table 1**), of which several have been shown to be protective specifically against oxidative stress caused by hydrogen peroxide in another mucosal pathogen of humans, *Neisseria gonorrhoeae* (Stohl and Seifert, 2006). Also implicated in bacterial survival in the lung was the *pgdX* gene encoding a hybrid peroxiredoxin/thioredoxin glutathione-dependent peroxidase (Pauwels et al., 2003). Peroxiredoxin and thioredoxin are ubiquitous anti-oxidant enzymes present in all known organisms and have been implicated in defense against reactive oxygen species in bacteria (Zeller and Klug, 2006) and in mammalian airways (Rahman et al., 2006; Lehtonen et al., 2008).

Bacterial nutrient scavenging was implicated during lung infection as genes in several amino acid biosynthetic pathways were needed such as those for phenylalanine (*pheA*), tryptophan (located in probable operons *trpEGDC*, *trpBA*), tyrosine (*tyrA*), methionine (*metB*, *metC*, *metE*, *metX*), serine (*serA*, *serB*), asparagine (*asnA*), aspartate (*aspC*), and branched amino acids (*ilvE*). The lung environment appears to be limiting for purines and pyrimidines as genes in these respective biosynthetic pathways were also needed (e.g., *purA*, *purB*, *purE*, *pyrG*) as well as those for purine salvage (*deoD*, *apaH*). In addition, essential trace minerals may be sequestered from bacteria in the lung. Transport genes for iron (*yfeB*) and zinc (*znuC*, *znuB*, *znuA*) were needed as well as two hypothetical transport genes, HI1249 and HI1248. Targeted studies of the latter two genes, now designated *zevA* and *zevB*, in a clinical NTHi isolate indicated that they encode components of a high-affinity zinc binding system required for growth under severe zinc restriction, and NTHi deletion mutants lacking either *zevA* or *zevB* were verified to be attenuated in the mouse lung model (Rosadini et al., 2011).

Some of the physiological adaptations required by *H. influenzae* in the lung appear to be analogously required by other respiratory pathogens. For example, three large-scale studies utilizing a hybridization-based method termed signature tagged mutagenesis (STM; Hensel et al., 1995) identified genes needed for lung colonization by *Streptococcus pneumoniae*, a Gram-positive bacterial pathogen that inhabits a niche similar to that of *H. influenzae* in the human respiratory tract (Polissi et al., 1998; Lau et al., 2001; Hava and Camilli, 2002). Together these experiments identified several hundred candidate virulence genes of *S. pneumoniae*, however the sets identified exhibited limited overlap between studies, probably because the mutant libraries used were not large enough to fully represent all of the genes in the genome. Of note, many of the genes detected encode components of pathways similar to those needed for survival of *H. influenzae* in the lungs, including purine metabolism, metal ion transport (zinc and iron), and amino acid synthesis (e.g., tryptophan, branched chain, and aromatic amino acids). Although *H. influenzae* and *S. pneumoniae* differ extensively at the genetic and phenotypic level and utilize numerous species-specific virulence factors, it appears that they exhibit certain core survival requirements in this niche. Overall, however, the genetic and phenotypic diversity in virulence factors used by the two pathogens in the lung appears to be very high, and a direct comparison is challenging in this context, compounded by the differences in HITS versus STM methodologies. Recently, a method similar to HITS, termed Tn-Seq (van Opijnen et al., 2009), was employed to study the fitness of mutants of *S. pneumoniae* libraries *in vitro*, and application of such an approach to *S. pneumoniae* in the lung infection model would greatly facilitate a comprehensive comparison to the *H. influenzae* HITS data. Moreover, as the STM studies revealed similarities and differences in the genes required in *S. pneumoniae* lung infection versus a model of bloodstream infection, it would be of particular interest to compare its genetic requirements for pathogenesis to those of *H. influenzae* in both sites.

The HITS data also indicated that perturbation of outer membrane lipid asymmetry was damaging for *H. influenzae* in the lung. Genes in this category that were required in the lung were associated with trafficking of phospholipids between the leaflets of the cell envelope such as *vacJ* and a set of genes that are putative orthologs of an ABC transport system of *Escherichia coli* encoded by the *mlaA* and *mlaBCDEF* loci (currently termed *yrb* loci in *H. influenzae*; Malinverni and Silhavy, 2009). The *vacJ* and *yrb* genes were also implicated in *Shigella flexneri* pathogenesis (Suzuki et al., 1994; Hong et al., 1998). More recently, Nakamura et al. (2011) screened a transposon bank of 6912 mutants in the NTHi R2866 strain background for sensitivity to killing by human serum. The *yrb/mla* genes were identified in this screen, and appear to influence the spatial arrangement of the cell surface lipooligosaccharide (LOS) leading to increased antibody binding to this structure (Nakamura et al., 2011). The potential role of complement in defense against *H. influenzae* in the lung is not fully understood, and it will be of interest to determine whether the attenuation of these mutants in the lung model reflects their complement sensitivity, greater recognition by antibodies, or other virulence defects associated with perturbation of the cell surface.

Lipooligosaccharide glycoforms are well established virulence factors of *H. influenzae* (Moxon and Maskell, 1992). The *H. influenzae* LOS glycolipid is similar to LPS of other Gram-negative

Table 1 | *Haemophilus influenzae* genes involved in pathogenesis.

Homologous locus in Rd	Locus	Function	Attenuated *in vivo*/serum	Gene/protein induced *in vivo*/sputum
AMINO ACID METABOLISM				
HI1145	*pheA*	Chorismate mutase/prephenate dehydratase	a	
HI1387	*trpE*	Anthranilate synthase component I	a	
HI1388	*trpG*	Anthranilate synthase component II	a	
HI1389	*trpD*	Anthranilate phosphoribosyltransferase	a	
HI1389.1	*trpC*	Bifunctional indole-3-glycerol phosphate synthase/ phosphoribosylanthranilate isomerase	a	
HI1431	*trpB*	Tryptophan synthase subunit beta	a	
HI1432	*trpA*	Tryptophan synthase subunit alpha	a	
HI1290	*tyrA*	Bifunctional chorismate mutase/prephenate dehydrogenase	a	
HI0086	*metB*	Cystathionine gamma-synthase	a, d	
HI0122	*metC*	Cystathionine beta-lyase	a	
HI1702	*metE*	5-Methyltetrahydropteroyltriglutamate – homocysteine methyltransferase	a	
HI1263	*metX*	Homoserine *O*-acetyltransferase	a	
HI0465	*serA*	D-3-phosphoglycerate dehydrogenase	a	
HI1033	*serB*	Phosphoserine phosphatase	a	
HI0564	*asnA*	Asparagine synthetase	a	
HI1617	*aspC*	Aromatic amino acid aminotransferase	a	
HI1193	*ilvE*	Branched chain amino acid aminotransferase	a	
HI0596	*argF*	Ornithine carbamoyltransferase		e
LOS BIOSYNTHESIS				
HI1114	*rfaD*	ADP-l-glycero-d-mannoheptose-6-epimerase	a	
HI1181	*gmhA*	Phosphoheptose isomerase	a	
HI0621.1	*gmhB*	D,D-heptose 1,7-bisphosphate phosphatase	a	
HI0261	*opsX*	Heptosyltransferase I	a, c	
HI0351	*galE*	UDP-glucose 4-epimerase	a, b	
HI0523	*orfH*	Heptosyltransferase-like protein	a, b, c	
HI0653	*lgtF*	Beta-1,4-glucosyltransferase	a, b	
HI0765	*lpsA*	Glycosyl transferase	a, b, c	
HI0812	*galU*	Glucosephosphate uridylyltransferase	a, b, c	
HI1105	*rfaF*	ADP-heptose-LPS heptosyltransferase II	a, c	
HI0740	*pgmB*	Phosphomannomutase	a, b, c	
HI0550	*lic2A*	Glycosyl transferase	b	
HI0258	*lgtC*	Glycosyl transferase	b, c	
HI0872	*rfbP*	Undecaprenyl-phosphate galactosephosphotransferase	c	
HI0873	*rfbB*	dTDP-glucose 4,6-dehydratase	c	
Not present in Rd	*lex2B*	Beta-1,4-glucosyltransferase	b	
HI1537	*licA*	Choline kinase	d	
HI1540	*licD*	Choline phosphotransferase		f
HI0260.1	*orfZ*	3-Deoxy-d-manno-octulosonic acid kinase	c	
CELL SURFACE				
HI0718	*vacJ*	Lipoprotein	a, b	
HI1083	*yrbB*	Putative NTP binding protein, contains STAS domain	b	
HI1084	*yrbC*	ABC transporter	a	
HI1085	*yrbD*	ABC transporter periplasmic protein	a, b	
HI1086	*yrbE*	ABC transporter permease	a, b	
HI1087	*yrbF*	ABC transporter ATPase	a	
HI1732	*hia*	Adhesin	d	
ANTI-OXIDANT/STRESS DEFENSE				
HI0572	*pdgX*	Hybrid peroxiredoxin/thioredoxin glutathione-dependent peroxidase	a	f

(Continued)

Table 1 | Continued

Homologous locus in Rd	Locus	Function	Attenuated *in vivo*/serum	Gene/protein induced *in vivo*/sputum
HI1434.1	*cspD*	Cold shock-like protein		e
HI0713	*tig*	Trigger factor (peptidyl-prolyl isomerase), ribosome-associated chaperone	a	
HI0084	*trxA*	Thioredoxin		f
HI1349	*dps*	DNA-binding ferritin-like protein		f
HI1426	*uspE*	Universal stress protein		f
HI1238	*dnaJ*	Molecular chaperone		f
HI0543	*groEL*	Chaperonin		f
HI0542	*groES*	Co-chaperonin		f
DNA RECOMBINATION/REPAIR				
HI0309	xerD	Site-specific tyrosine recombinase	a	
HI0312	ruvB	Holliday junction DNA helicase B	a	
HI0313	ruvA	Holliday junction DNA helicase motor protein	a	
HI0314	ruvC	Holliday junction resolvase	a	
HI0676	xerC	Site-specific tyrosine recombinase	a	
HI0942	recC	Exodeoxyribonuclease V gamma chain	a	
HI0443	recR	Recombination protein	a	
UREA METABOLISM				
HI0535	*ureH*	Urease accessory protein	a	e
NUCLEOTIDE METABOLISM				
HI1633	*purA*	Adenylosuccinate synthetase	a	
HI0639	*purB*	Adenylosuccinate lyase	a	
HI1615	*purE*	Phosphoribosylaminoimidazole carboxylase catalytic subunit	a	e
HI1077	*pyrG*	CTP synthetase	a	
HI0518	*deoD*	Purine nucleoside phosphorylase	a	
HI0551	*apaH*	Diadenosine tetraphosphatase	a	
COFACTOR/VITAMIN SYNTHESIS				
HI0764	*ribB*	3,4-Dihydroxy-2-butanone 4-phosphate synthase		e
HI1647	*pdxS*	Pyridoxine biosynthesis protein	a	e
NUTRIENT ACQUISITION ZINC				
HI0119	*znuA*	High-affinity zinc transporter periplasmic component	a	f
HI0407	*znuB*	Zinc transport system permease protein	a	
HI0408	*znuC*	Zinc transport system ATP-binding protein	a	
HI1249	*zevA*	ABC transporter periplasmic component (zinc binding system)	a	
HI1248	*zevB*	ABC transporter permease (zinc binding system)	a	
IRON				
HI0362	*yfeA*	Iron-chelated ABC transporter periplasmic-binding protein		f
HI0361	*yfeB*	Iron (chelated) transporter ATP-binding protein	a	
HI0097	*hitA*	ABC transporter, iron-utilization periplasmic protein		f
HI0129	*afuB*	Ferric transport system permease-like protein	d	
HEME				
HI0263	*hxuB*	Heme/hemopexin-binding protein B		f
RIBOSE				
HI0503	*ribsC*	Ribose ABC transporter permease		e
TRANSPORTERS				
HI0561	–	Putative oligopeptide transporter (OPT) family	d	
HI1218	*lctP*	l-lactate permease	d	
HI0898	*emrA*	Multidrug resistance protein A	d	
METABOLIC PROCESSES/ELECTRON TRANSPORT				
HI1245	–	Bifunctional malic enzyme oxidoreductase/phosphotransacetylase	d	
HI1170	–	Similar to para-aminobenzoate synthetase required for folate biosynthesis	d	
HI0406	*accA*	Acetyl-CoA carboxylase carboxyltransferase subunit alpha	d	

(Continued)

Table 1 | Continued

Homologous locus in Rd	Locus	Function	Attenuated *in vivo*/serum	Gene/protein induced *in vivo*/sputum
HI0432	*nudC*	NADH pyrophosphatase	d	
HI0527	*fdx-2*	Ferredoxin	d	
HI0936	–	Cytochrome C-type biogenesis	d	
TRANSCRIPTION/DNA PROCESSING				
HI0700	–	Hypothetical protein	d	
HI0814	*alaS*	Alanyl-tRNA synthetase	d	
HI1056	–	Type III restriction-modification system methyltransferase-like protein	d	
HI1459	–	RNA polymerase sigma-70 factor	d	
HI1528	*parE*	DNA topoisomerase IV subunit B	d	
PROTEIN MODIFICATION				
HI0428	*dsbB*	Disulfide bond formation protein B	a, d	e
HI1152	*pmbA*	Peptidase protein	d	
HI1368	–	Putative zinc protease	d	
HYPOTHETICAL PROTEIN/OTHER				
HI0364	*pbpG*	Penicillin-binding protein 7	d	
HI1018		Insertion sequence IS1016 group transposase	d	
HI1598	–	Hypothetical protein	d	
HI0877	*obgE*	GTPase	d	
HI0094	–	Hypothetical protein HI0094		e

a, H. influenzae Rd genes required in mouse lung (Gawronski et al., 2009).

b, NTHi strain R2866 genes needed for resistance to normal human serum (Nakamura et al., 2011).

c, H. influenzae type b (Eagan) genes needed for survival in infant rat blood (Hood et al., 1996).

d, Rd (b+) (strain containing type b capsule locus) genes needed for survival in infant rat blood (Herbert et al., 2002).

e, NTHi strain 86-028NP genes induced in chinchilla middle ear effusions (Mason et al., 2003).

f, NTHi strain 11P6H proteins induced in human sputum (Qu et al., 2010).

bacteria except that it lacks extended O antigen structures, but consist of Lipid A, an inner core comprised of several sugars including a single 3-deoxyc-D-*manno*-octulosonic acid linked to three conserved heptose residues, and a variable outer core containing a heteropolymer of glucose and galactose modified with various substituents such as sialic acid, *N*-acetylgalactosamine, and phosphorylcholine (Hood et al., 1999; Risberg et al., 1999; **Figure 1**). The HITS data indicate the importance of the LOS structure for survival in the lung, as numerous LOS biosynthesis genes were required. The genes needed in the steps to generate the nucleotide-activated heptose precursor of the inner core (*rfaD*, *gmhA*, *gmhB*) and for extension from the heptose residues of the inner core (*opsX*, *rfaF*, and *orfH*) were most important *in vivo* in the lung with estimated degrees of attenuation ranging from ∼80- to 300-fold. Also critical are genes required for precursor production for LOS outer core hexose extensions (*galU*, *pgmB*, and *galE*) which were ∼90-, ∼70-, and 40-fold attenuated *in vivo*, respectively. GalU converts glucose-1-phosphate to uridine diphosphate glucose (UDP-glucose), the substrate for glucose residue addition to the LOS. Based on mass spectrometry data, a *galU* mutant of *H. influenzae* does not contain hexose in the outer core consistent with studies of mutants deficient in this enzyme in other species (Choudhury et al., 2005; Wong and Akerley unpublished data). The *pgmB* (or *yhxB*) encoded enzyme is a phosphohexose mutase that putatively functions in the conversion of glucose-6-phosphate to glucose-1-phosphate, a metabolic substrate for GalU.

GalE converts UDP-glucose to UDP-galactose, and therefore the LOS of *galE* mutants is predicted to contain decreased levels of galactose containing structures (**Figure 1**; Maskell et al., 1992).

Genes in which mutations result in less dramatic truncations of the LOS outer core were less important for survival as determined via HITS. For example, *lgtF*, which mediates hexose extension from the first heptose, and *lpsA*, which is required for hexose extension from the terminal heptose (Hood et al., 2001, 2004) were ∼7- and 4-fold attenuated *in vivo*, respectively. Single strain infections with a defined *lpsA* mutant of non-typeable *H. influenzae* verified that this gene was non-essential for infection in the lung, as the mutant only exhibited an ∼1.5-fold trend of attenuation that did not attain statistical significance. Distal extensions and modifications of the LOS outer core mediated by genes *lic2A*, *lgtC*, *lgtD*, *lic3A*, and genes of the *lic1* locus, respectively (**Figure 1**) appeared to be non-essential in the lungs at 24 h. Of note, a *lic1D* gene encoding the transferase required for phosphorylcholine addition to the LOS was previously examined in the murine lung model and exhibited only a modest, strain specific attenuation at 24 h with more consistent attenuation at 48 h (Pang et al., 2008), suggesting that distal modifications of the LOS may become more critical at a later stage of infection when immune responses are likely to be more fully engaged. Taken together, the results indicate that truncation of the LOS inner core or inability to extend hexose residues from the inner core results in varying degrees of enhanced clearance of *H. influenzae* from the lung.
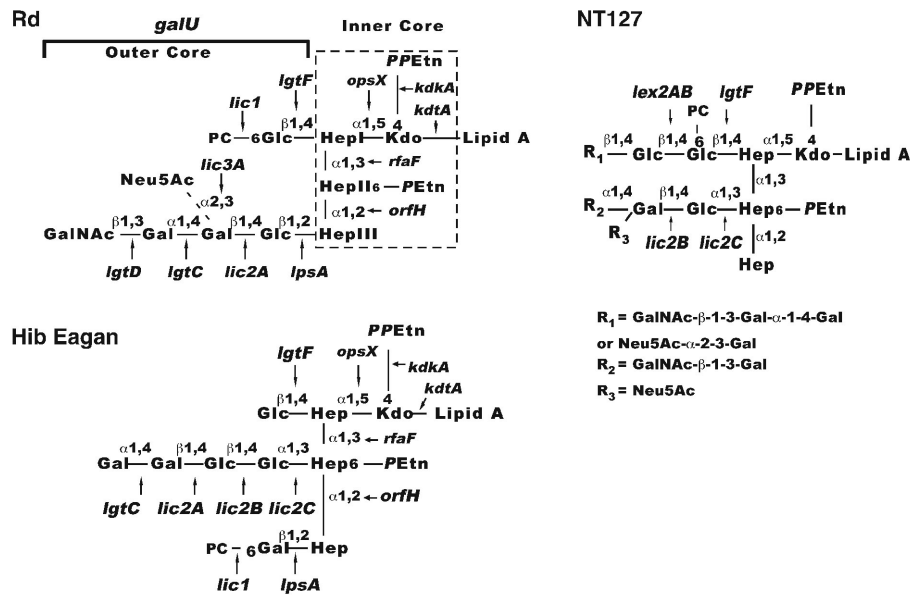
FIGURE 1 | Schematic diagram of *H. influenzae* LOS. Composite models based on structural studies are shown for *H. influenzae* Rd (Risberg et al., 1999; Hood et al., 2001), Hib (Masoud et al., 1997, 2003; Schweda et al., 2000; Griffin et al., 2003; Hood et al., 2004; Twelkmeyer et al., 2011), and non-typeable strain NT127 (Wong et al., 2011). The conserved inner core (boxed with dashed lines on the Rd diagram) is present in most strains as three heptose sugars designated HepI (proximal to Kdo), HepII (center), HepIII (the terminal inner core structure). Extending from these heptose residues are the outer core structures (in brackets) dependent on *galU* for LOS hexose precursor production. Arrows indicate linkages added to the structure by the designated genes. Abbreviations: Glc, glucose; Gal, galactose; Hep, heptose; Kdo, 2-keto-3-deoxyoctulosonic acid; *PP*Etn, pyrophosphoethanolamine; *P*Etn, phosphoethanolamine; GalNac, *N*-acetylgalactosamine; Neu5Ac, sialic acid; PC, phosphorylcholine.

INVASIVE INFECTION AND BACTEREMIA

Encapsulated strains, and most frequently *H. influenzae* type b (Hib) expressing the type b capsule, cause invasive infections including meningitis, pneumonia, and septicemia. Despite the implementation of an effective vaccine against Hib, which has dramatically reduced the incidence of type b disease in developed countries, Hib remains prevalent in the developing world (Peltola, 2000; Agrawal and Murphy, 2011). In populations receiving the Hib vaccine, NTHi strains have replaced type b as the most common bloodstream isolate (Agrawal and Murphy, 2011). NTHi predominantly cause respiratory tract infections and otitis media, but in rare cases can invade the bloodstream leading to meningitis. This disease profile raises the possibility that genes promoting intravascular invasion could be present among NTHi strains. The molecular basis for the invasive properties of *H. influenzae* that promote transmission from the nasopharynx to the bloodstream or middle ear are not fully understood. NTHi lack capsular polysaccharide, a major virulence factor of Hib promoting survival in the bloodstream, however they share a number of virulence determinants that are also required for Hib bacteremia. Moreover, it is possible that factors involved in adaptation of *H. influenzae* to interstitial invasion in the nasopharynx, a property that may enhance persistent asymptomatic or subclinical persistence, also promote bacteremia. Therefore, comparisons between Hib and non-encapsulated strains in models of bloodstream infection will likely provide insight into both persistence and disease.

In one of the earliest demonstrations of the utility of complete genomic DNA sequencing information, Hood et al. (1996) utilized the sequence of *H. influenzae* Rd to identify putative LOS biosynthetic genes for virulence screening in mice. Mutants of the Hib strain deficient in these candidate LOS genes were examined in an infant rat model of infection to identify LOS structures required for intravascular dissemination. The Hib LOS genes reported to be required for bacteremia in the rat model are *opsX*, *rfaF*, *pgmB*, *galU*, *rfbB*, *orfZ*, *orfH*, *lgtC*, *lpsA*, and *rfbP* (**Figure 1**; **Table 1**). Similarly, *galU* and *lpsA* mutants of the non-encapsulated *H. influenzae* Rd strain were evaluated in a mouse model of bacteremia and also exhibit persistence defects (Wong et al., 2007). Of note, the Hib mutants that produced the most severely truncated LOS structures (*opsX*, *rfaF*, *pgmB*, *galU*, *orfH*) and were the most attenuated in the rat bacteremia model (Hood et al., 1996) were also markedly attenuated for survival in the lung as indicated by HITS data (Gawronski et al., 2009). In contrast to their more severe effects on bloodstream infection, mutations in LOS genes of the outer core that produced less dramatic truncations conferred either partial attenuation (*lgtF*, *lpsA*) or no appreciable effect on survival (*lic2A*, *lgtC*) in the lung (Gawronski et al., 2009). In summary, LOS structures are critical for adaptation to both the lung and bloodstream environments, yet the bacterium's requirement for specific structures varies between these sites.

Also using an infant rat model of systemic infection, Herbert et al. (2002) screened a random transposon insertion library of 1632 *H. influenzae* mutants using the STM method (Hensel et al., 1995) to identify 25 genes essential for bacteremia. This experiment utilized an Rd-b+ strain in which the locus encoding the capsule of the type b strain was introduced into strain Rd,

thereby conferring enhanced virulence and permitting efficient intravascular infection. Eleven of the genes detected were involved in metabolic processes and in nutrient/cofactor acquisition, five were involved in transcription or DNA processing, three are involved in protein modification, four have putative roles in well established virulence phenotypes, and two encode hypothetical proteins (**Table 1**). Mutants containing deletions of *afuB* (ferric transport system permease), *accA* (fatty acid metabolism), *nudC* (NADH pyrophosphatase), *lctP* (L-lactate permease), HI1245 (malic enzyme or malate dehydrogenase), and HI0700 (hypothetical protein) were used to verify the requirement for these genes in bacterial survival in the infant rat bloodstream. Thus the results confirm the requirement for genes mediating nutrient utilization and metabolism by *H. influenzae* for survival in the blood. The requirement for L-lactate permease is of interest as the presence of lactate *in vitro* induces resistance to complement-mediated bacterial killing, potentially involving increased production of LOS (Kuratana and Anderson, 1991). Interestingly, acquisition of iron and lactate is also important for bloodstream colonization by another respiratory tract pathogen that can become invasive, *Neisseria meningitidis*, as a transferrin-binding deficient mutant (TbpB) and a *lctP* mutant in this organism were more deficient for survival *ex vivo* in whole human blood (Echenique-Rivera et al., 2011).

The majority of genes detected by STM in the bacteremia model were not required according to HITS in the lung. This observation may relate to differences in Rd versus the Rd-b+ strain, but also likely reflects that very different survival strategies are required at these sites. Both experiments detected a requirement for methionine synthesis (*metB*) suggesting that this amino acid is unavailable at multiple sites in the host. Methionine is an essential dietary amino acid not synthesized by humans, and is also an important host-signaling molecule, and therefore its abundance in the extracellular milieu is likely tightly controlled. Preliminary analysis of HITS data recently obtained with a bank of ∼34,000 mutants of *H. influenzae* Rd recovered from the bacteremia model (Wong and Akerley, unpublished) has yielded concordant results with all of the genes implicated as most critical for bacteremia by Hood et al. (1996) and several of the genes identified via STM by Herbert et al. (2002; e.g., *licA, lctP, dsbD, afuB, HI0936*). We anticipate that re-evaluating *H. influenzae* growth and survival in the context of bacteremia with a more comprehensive library of mutants via the HITS methodology will provide additional insight into the physiological and immunological selection pressures *H. influenzae* experiences in different sites of infection.

CHINCHILLA MODEL OF OTITIS MEDIA

Non-typeable *H. influenzae* is a predominant bacterial isolate in chronic otitis media with effusion (Klein, 1997). To identify candidate genes that may be important in causing otitis media, Mason et al. (2003) used a green fluorescent protein (GFP) promoter trap system with a differential fluorescence induction strategy combined with fluorescence-activated cell sorting to identify *H. influenzae* genes potentially induced in the chinchilla middle ear relative to *in vitro* growth on rich medium. NTHi strain 86-028NP containing a GFP promoter trap library was inoculated into the middle ear of chinchillas and clones containing promoter

elements induced *in vivo* were enriched following recovery from middle ear effusions. The screen revealed 52 genes of which 26 were confirmed by RT-qPCR conducted on RNA isolated from NTHi recovered from the chinchilla middle ear. Five genes expressed at ≥5-fold levels higher in the middle ear are involved with membrane transport (the ribose transporter *rbsC*), purine synthesis (*purE*), biosynthetic/metabolic functions (*ribB*, which functions in riboflavin synthesis and *argF* which functions in arginine degradation), and a hypothetical gene, HI0094. The authors suggest that induction of these genes implies the middle ear contains ribose, whereas the levels of purines and riboflavin may be low in this environment and therefore synthesis would be required. Five genes of the 26 on this list were also implicated in survival in the mouse lung model, *uxuA, dsbB, ureH, purE*, and *pdxS* (**Table 1**; Gawronski et al., 2009). While expression analysis and studies with specific mutants have provided insight into adaptations at this site, the complete set of genes involved in middle ear infection has not been identified. Application of the HITS methodology would therefore represent an attractive opportunity to address this important question at a genomic scale in animal models of otitis media.

EXPRESSION OF NTHi GENES DURING GROWTH IN HUMAN SPUTUM

Proteomic expression studies of NTHi grown in human sputum by Qu et al. (2010) can complement studies of genes that are required in infection models. This type of approach can identify potentially "redundant" factors as well as minor contributors that, when combined, mediate enhanced virulence. In addition, it can help describe physiological responses and identify genes that are essential for growth *in vitro* that may need to be upregulated *in vivo*. This study aimed to simulate conditions in the lower respiratory tract that occur during COPD, which predisposes individuals to exacerbations involving lung colonization by NTHi.

The expression profile of NTHi 11P6H strain grown in human sputum from COPD patients identified 1402 unique proteins from 1759 predicted coding regions. Thirty-one proteins were present in greater abundance by more than 1.5-fold during growth in sputum versus in chemically defined media. These include proteins involved in anti-oxidant/stress response such as PdgX, thioredoxin (TrxA), the DNA-binding ferritin-like protein Dps, universal stress protein UspE, and molecular chaperones DnaJ, GroEL, and GroES. Other proteins induced in the sputum include those required for uptake of minerals such as iron (HitA, YfeA) and zinc (ZnuA; **Table 1**).

This protein expression profile is consistent with the view that the human respiratory tract presents NTHi with an oxidatively stressful environment that is relatively deficient of nutrients and trace metals such as zinc, similar to the profile of genes implicated in survival in the mouse lung model (Gawronski et al., 2009). Both PdgX and ZnuA were implicated in survival in the mouse lungs with mutants exhibiting ∼13- and 10-fold attenuation, respectively, as estimated by HITS. Despite potential zinc limitation in sputum, the ZevA/ZevB zinc uptake system, which is required in the mouse lung, was not detected as being more highly expressed in sputum *in vitro*. This result may reflect that expression of *zevAB* does not respond to zinc levels but rather is activated under aerobic conditions and repressed in low oxygen

by the anaerobic regulator, FNR (Rosadini et al., 2011). Therefore differential expression of ZevAB *in vivo* likely involves transitions between aerobic and anaerobic environments, in contrast to the comparison between two different aerobic conditions in the study by Qu et al. (2010). Overall, expression analysis in this *in vitro* model shows many correlations to the genetic requirements for growth in the murine lung model, and can provide insight into the nutrients and other host factors influencing *H. influenzae's* adaptation to life at the mucosal surface.

ENVIRONMENTAL SENSING AND SIGNAL TRANSDUCTION IN ADAPTATION TO SITES OF INFECTION

The ability to sense and adapt to environmental conditions is an important survival strategy for many bacterial pathogens, however the role of signaling by *H. influenzae* during infection is not well understood. Comparison of the genetic requirements for *H. influenzae* at different infection sites, combined with expression studies under conditions reflective of these environments, can provide insight into the ways that this bacterium may sense and respond to its environment *in vivo*, and ultimately how such responses may influence pathogenesis.

Accumulating evidence indicates that modulation of gene expression in response to varying oxygen levels in diverse environments is needed by *H. influenzae* to evade and resist host defenses for efficient colonization and pathogenesis. *H. influenzae* contains two global transcriptional regulatory systems, ArcAB and FNR, which are similar to their respective homologs in *E. coli* (Shaw et al., 1983; Lynch and Lin, 1996b), and modulate the expression of the genes needed for adaptation to changes in oxygen availability. The *H. influenzae* ArcB can phosphorylate *E. coli* ArcA *in vitro*, and complementation experiments have demonstrated that the *H. influenzae* ArcB and ArcA can interact with the appropriate *E. coli* Arc components *in vivo* to control *E. coli* ArcAB regulated genes with a similar response to varied reduction/oxidation (redox) conditions of growth (Manukhov et al., 2000; Georgellis et al., 2001b). Under low oxygen conditions, the transmembrane sensor component ArcB responds to the redox status of the quinone pool and autophosphorylates, leading to activation of the ArcA response regulator by phosphoryl transfer (Georgellis et al., 2001a; Malpica et al., 2004; Bekker et al., 2010). Phosphorylated ArcA transcriptionally activates or represses diverse target genes including genes of the tricarboxylic acid cycle and genes involved in other aspects of respiratory or fermentative metabolism (Lynch and Lin, 1996a,b; De Souza-Hart et al., 2003; Wong et al., 2007). Under high oxygen conditions ArcB activity is abrogated by interaction with oxidized quinone (Malpica et al., 2004).

Unlike the ArcAB system, which indirectly senses oxygen availability via the redox state of the quinone electron carrier, FNR of *E. coli* is considered to be a direct oxygen sensor by virtue of its iron–sulfur center, which is required for maintaining an active conformation that promotes dimerization and DNA-binding (Lazazzera et al., 1996). Upon oxidation, the iron–sulfur center undergoes a transition leading to conversion of FNR to its inactive monomeric form (Crack et al., 2004; Dibden and Green, 2005). The biochemical properties of FNR in *H. influenzae* have not been studied, however its predicted amino acid sequence is 79% identical to that of *E. coli* and it regulates target genes containing a

similar FNR binding motif in their promoter regions under low oxygen conditions consistent with a similar function (Stewart and Bledsoe, 2005; Harrington et al., 2009). Despite functional similarities of these regulators to those of *E. coli*, there are important differences in the sets of genes these two factors control in *H. influenzae*, and these differences have been linked to mechanisms of pathogenesis (Wong et al., 2007, 2011; Harrington et al., 2009).

In *H. influenzae*, ArcAB appears to protect the bacterium from oxidative stress in a pre-emptive manner during transition from low to high oxygen conditions (Wong et al., 2007), a condition that likely exists in the bloodstream upon sudden exposure to the oxidative defenses of phagocytic cells. This anti-oxidant effect involves ArcA mediated activation of expression of the ferritin-like protein Dps, which in *E. coli* has been shown to sequester iron in the presence of hydrogen peroxide by converting Fe(II) to a ferric oxide mineral core within the Dps complex, preventing Fenton reactions that would create toxic hydroxide radicals. Regulation of Dps by ArcA has not been demonstrated in *E. coli* or other species, and therefore this pre-emptive anti-oxidant defense may relate to unique adaptations of *H. influenzae* to its niche within the human host.

FNR is needed by *H. influenzae* for defense against nitrosative stress under anaerobic conditions (Harrington et al., 2009). During immune responses to bacteria, host cells produce the free radical gas, nitric oxide (NO) via metabolism of L-arginine by inducible nitric oxide synthase (iNOS or NOS2; for reviews see MacMicking et al., 1997; Bogdan et al., 2000). Additional reactions with NO generate other reactive nitrogen species (RNS) such as S-nitrosothiols, which are thought to exceed the cytotoxicity of NO, leading to diverse antimicrobial effects (Brunelli et al., 1995; Fang, 2004). Production of nitric oxide by macrophages and epithelial cells is significant in human sinuses and resistance to RNS is also likely to be an important adaptation for *H. influenzae* in specific locations within the nasopharynx (Lundberg et al., 1995; Lundberg, 2008). Alcohol dehydrogenase (*adhC*) has been implicated in defense of *H. influenzae* against one type of RNS, *S*-nitrosoglutathione, under normoxic conditions (Kidd et al., 2007). Under low oxygen conditions and positively controlled by FNR, resistance to RNS requires the *ytfE* gene, predicted to encode a di-iron protein similar to a family of iron–sulfur repair proteins involved in RNS and ROS resistance in *E. coli* and a diverse group of additional bacterial species (Overton et al., 2008). Genes similar to *ytfE* in other species are controlled by other regulatory factors or, in the case of *E. coli*, negatively controlled by FNR (Justino et al., 2006). Therefore, analogous to *H. influenzae* specific aspects of the Arc regulon, FNR mediated activation of *ytfE* appears to be a distinctive property of *H. influenzae*.

In some cases, it is possible to relate the regulatory profiles of specific virulence factors to the requirements for these factors in sites of infection. For example, FNR was shown to negatively regulate expression of the *zevA* promoter of the *zevAB* zinc binding system, which was required for NTHi survival in the lung (Rosadini et al., 2011). The promoter activity of *zevA* is therefore higher in aerobic versus anaerobic growth, consistent with the requirement for *zevAB* in the lung. *H. influenzae* may encounter zinc limitation in the respiratory tract as genes of the *znu* locus encoding a high-affinity zinc transport system were required in the lung

as indicated by HITS, and ZnuA expression was induced during growth in human sputum as determined by proteomic expression analysis (Qu et al., 2010). It's not clear why *zevAB* is controlled by aerobiosis rather than zinc levels. It is possible that high levels of oxygen may be utilized by *H. influenzae* to sense that it is growing at the mucosal surface, and initiate a program of gene expression that includes increased scavenging of zinc along with other oxygen regulated adaptations.

Data from HITS analysis of mutants in the lung model and NTHi protein expression in human sputum support the concept that *H. influenzae* encounters an aerobically stressful environment in the lung. It is not surprising then that the anaerobic regulators ArcA and FNR were not implicated in survival in the lung, yet a role for the oxidative stress responsive regulator OxyR was detected (Gawronski et al., 2009). The *H. influenzae* OxyR regulon includes genes that are upregulated in response to hydrogen peroxide exposure (Harrison et al., 2007) and have known or probable roles in detoxifying reactive oxygen species such as the peroxidase gene, *pgdX* (Pauwels et al., 2003).

In contrast to the aerobic environment in the lung, results from animal models of bacteremia support the view that bloodstream colonization by *H. influenzae* requires microaerobic adaptations. While ArcA was not required in the mouse lung, it was required in mouse models of bacteremia (De Souza-Hart et al., 2003; Wong et al., 2007). Moreover, the OxyR regulated *pgdX* gene in *H. influenzae* was not required in an infant rat model of bacteremia (Vergauwen et al., 2006). Similarly, *H. influenzae* *sodA* encoding superoxide dismutase participates in oxidative stress defense *in vitro* and was implicated in colonization of the nasopharynx, but not in the bloodstream in infant rats (D'Mello et al., 1997). These observations highlight the concept that survival of *H. influenzae* at mucosal surfaces such as lung or nasopharynx versus the bloodstream requires different stress defense and nutrient acquisition strategies in each environment, and that responses to oxygen levels play an important role in controlling expression of these adaptations.

Haemophilus influenzae not only responds to aeration conditions in defense against oxidative stress, but also to control genes involved in immune evasion during infection such as the LOS structure. Modification of the *H. influenzae* LOS with phosphorylcholine was found to decrease under high oxygen and increase in microaerobic conditions (Wong and Akerley, 2005). Microarray analysis suggested a model in which this regulatory profile was mediated by changes in expression of UDP-glucose pyrophosphorylase encoded by *galU*. Moreover, expression of *galU* from a constitutive promoter resulted in equal levels of phosphorylcholine display during growth in both high and low oxygen as determined via western immunoblotting with anti-phosphorylcholine monoclonal antibody, TEPC 15 (Wong and Akerley, unpublished). These results suggest that aeration conditions influence the structure of the entire outer core, which is dependent on UDP-glucose for hexose addition to the LOS. Consistent with this concept, a LOS epitope in NTHi was reported to show reduced expression during aerobic growth on an agar plate and higher levels during biofilm growth in broth medium, a condition that is likely to restrict oxygen availability (Murphy and Kirkham, 2002). More recently, the *lic2B* glycosyltransferase was demonstrated to be activated by ArcA under low oxygen conditions. The *lic2B* gene is required for addition of a galactose residue to the LOS outer core (**Figure 1**), and is required for both survival in the bloodstream and resistance to serum complement in NTHi (Wong et al., 2011). *H. influenzae* LOS is essential for pathogenesis in animal models of middle ear and bloodstream infection by contributing to evasion of complement and antimicrobial peptides (Lysenko et al., 2000; Figueira et al., 2007; Ho et al., 2007). However, LOS structures are also targets for components of innate and acquired immunity (Leon and Young, 1971; Weiser et al., 1998; Shaw et al., 2000), and downregulation of these structures may be beneficial in sites of infection in which they are not essential for bacterial survival.

The differential requirement for specific LOS biosynthesis genes in models of lung infection versus bacteremia is consistent with the hypothesis that they are differentially regulated in response to environmental signals at these locations. While some LOS structures are required in both models, distal modifications of the LOS outer core mediated by genes such as *lic3A*, which adds sialic acid, or the *licABCD* locus responsible for phosphorylcholine display, appeared to be non-essential in the lung by HITS, yet are required for bloodstream colonization (Weiser et al., 1990; Hood et al., 1996; Herbert et al., 2002). Taken together, the expression profile of LOS modifications in response to aeration conditions (Wong and Akerley, 2005) seems consistent with the *in vivo* importance of the genes responsible for generating those LOS structures during infection in niches predicted to differ in oxygen levels. This provides insight into the factors influencing *H. influenzae's* transition between diverse infection sites in pathogenesis while it evades the host immune system.

CONCLUDING REMARKS

Integration of genome-scale data from expression profiling studies and mutagenesis experiments is providing a much more comprehensive view of the factors that are likely to be critical for human infection and disease. An emerging theme is that *H. influenzae* expresses adaptations that allow it to survive in different environmental niches as it transits between the relatively high oxygen levels of the airway surface to sites that may be lower in oxygen as it disseminates to the middle ear, or enter the bloodstream. Understanding differential requirements for specific virulence factors in these sites is likely to provide new candidate bacterial targets for development of strategies to specifically block disease progression. Overall, we are only in the early stages of exploiting highly effective, "next generation" genome-scale approaches to understanding *H. influenzae*, and more extensive application of these technologies promises to provide unprecedented insight into this bacterium's mechanisms of pathogenesis and strategies for persistent colonization.

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