Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung

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Rapid genome-wide identification of genes required for infection would expedite studies of bacterial pathogens. We developed genome-scale “negative selection” technology that combines high-density transposon mutagenesis and massively parallel sequencing of transposon/chromosome junctions in a mutant library to identify mutants lost from the library after exposure to a selective condition of interest. This approach was applied to comprehensively identify Haemophilus influenzae genes required to delay bacterial clearance in a murine pulmonary model. Mutations in 136 genes resulted in defects in vivo, and quantitative estimates of fitness generated by this technique were in agreement with independent validation experiments using individual mutant strains. Genes required in the lung included those with characterized functions in other models of H. influenzae pathogenesis and genes not previously implicated in infection. Genes implicated in vivo have reported or potential roles in survival during nutrient limitation, oxidative stress, and exposure to antimicrobial membrane perturbations, suggesting that these conditions are encountered by H. influenzae during pulmonary infection. The results demonstrate an efficient means to identify genes required for bacterial survival in experimental models of pathogenesis, and this approach should function similarly well in selections conducted in vitro and in vivo with any organism amenable to insertional mutagenesis.

In this study we report a technique termed “high-throughput insertion tracking by deep sequencing” (HITS) that uses a whole-genome transposon mutant bank in combination with massively parallel sequencing to efficiently analyze bacterial genes involved in pathogenesis. HITS allowed analysis of genes required by Haemophilus influenzae to resist clearance from the lung, a site colonized during pneumonia and chronic obstructive pulmonary disease (5, 6). Because deep sequencing is used for detection, background signal is easily identified and removed during data analysis, and the dynamic range of detection is limited only by the number of sequencing reads, which can be readily increased. The results highlight the utility of HITS in systematic discovery and analysis of virulence genes required in environments encountered by bacteria during pathogenesis.

**Results**

**Overview of the HITS Technique.** HITS is outlined schematically as two steps in Fig. 1A and B. The first step involves fragmentation and ligation of adapters to sheared genomic DNA prepared from a high-density mutant bank carrying random transposon insertion mutations. In this study, mutagenesis was performed with a minitransposon derived from the Himar1-mariner transposon, which inserts efficiently in the genomes of H. influenzae and other bacteria, with only the dinucleotide TA as the apparent insertion site specificity (7–9). Selective amplification of transposon/chromosome junction regions is performed by PCR, and the resulting amplicons are purified by affinity capture. Sequencing is performed en masse on the Illumina next-generation sequencing platform. The second step identifies the genomic location of each transposon insertion site within the bank by mapping chromosomal sequences adjacent to inverted terminal repeats of the transposon to the reference genome. The fitness of insertion mutants containing disruptions in a given gene is reflected in both the relative number of insertion sites detected within the gene and the number of times each site is detected by the sequence analysis.

**Generation of a Mutant Bank Selected for Growth or Survival in Vivo in the Murine Lung Model.** The mechanisms that allow H. influenzae to persist in the lung are not well understood. Mouse pulmonary infection provides a well-established model for investigation of mechanisms used by H. influenzae and other

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**Author contributions:** J.D.G., S.M.S.W., and B.J.A. designed research; J.D.G., S.M.S.W., and G.G. performed research; J.D.G., G.G., and D.V.W. contributed new reagents/analytic tools; J.D.G., S.M.S.W., and B.J.A. analyzed data; and J.D.G., S.M.S.W., and B.J.A. wrote the paper. The authors declare no conflict of interest.

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Data deposition: Draft genome sequences for H. influenzae strains have been submitted to the National Center for Biotechnology Information: Rd BA042 (RdAW) (accession no. AC5N00100000) and NT127 (accession no. ACSL000000000).

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**Tracking insertion mutants within libraries by deep sequencing and a genome-wide screen for Haemophilus genes required in the lung**

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bacteria to persist and resist host defenses during lung pathogenesis (10–12), yet there have been no comprehensive studies to identify H. influenzae genes needed at this site. To evaluate the utility of HITS for virulence gene identification using the mouse lung model of infection, we inoculated 5 mice with 10^7 cfu of a ∼75,000 member insertion mutant library of H. influenzae generated with a Himar1 mariner–derived minitransposon. At 24 h after inoculation an average of 9.2 × 10^2 cfu were recovered from the lungs of each mouse. Chromosomal DNA was isolated for analysis from both the inoculum and from the ex vivo bacterial populations. The numbers of cfu in the inoculum and recovered from mice suggested that the mutant library was likely to be sufficiently represented in both populations and that mutants had been subjected to in vivo selection.

Analysis of Genomic Mutant Banks by HITS and Application to Genome-Wide Identification of H. influenzae Genes Required in the Lung. We conducted the HITS procedure (Fig. 1) on the input library and mapped the insertions to their chromosomal positions (Fig. S1). Insertions were evenly distributed around the chromosome, and ∼44% of the 131,960 total possible chromosome TA target sites for mariner were found to have sustained insertions in this library. Before passage in vivo, a total of 534,567 sequencing reads mapped to nonrepetitive chromosomal regions immediately flanking 55,935 unique sites, with 44,270 in predicted protein coding genes and 11,665 in intergenic regions or structural RNAs. Of 1,657 annotated genes, no insertions were detected within 268 genes and 90 sustained insertions in <5% of their possible TA insertion sites, implicating at least 358 genes as essential for growth or viability on laboratory medium in vitro. Twenty-five genes with <8 possible TA insertion sites were excluded from analysis on the basis of an estimated probability of 0.05 that they could fail to sustain insertions owing to chance at this level of transposon insertion density in the library. Thirty-five genes could not be analyzed because they either contained extensive repetitive sequences or were duplicated in the genome. After subtraction of essential genes, genes containing repetitive sequence, or genes with very few possible insertion sites, there were 1,239 genes that could be analyzed.

For fitness analysis of mutants after in vivo selection, we considered the number of transposon insertions detected in the first 5–80% of each gene, the region in which insertions are expected to abrogate gene function. To exclude genes in which insertion mutations led to potential in vitro growth defects, we set a threshold requiring that candidate virulence genes sustain insertions in at least 40% of their possible mariner transposon target sites before in vivo selection, and 201 genes sustained densities of insertions below this threshold (Fig. 2 and Table S1). In the 1,038 annotated protein-coding genes that were dispensable in vitro, an average of 287 sequencing reads detecting insertions in the 5’ 5–80% region of each gene was observed.
HITS implicated 8.1% of the 1,657 annotated genes in the lung. Comparisons between wild-type and mutants were statistically significant via one-way ANOVA with Tukey's multiple comparison test (p < 0.001). LLD, lower limit of detection. Fold differences in mean cfu recovered for NT127 wild-type strain vs. the galU or orfH mutants in individual mutant infections (brackets) are compared with HITS results (below the chart). HITS survival indices were 0.011 for galU and 0.012 for orfH, corresponding to in vivo attenuations (calculated as the reciprocal of the s.i.) of 89-fold and 82-fold, respectively. In A–C, genome coordinates of transposon insertion sites detected via HITS analysis were reoriented with respect to the chromosomal primer positions used in footprinting. The y axis was modeled to the migration of the molecular weight (MW) standard of footprinting gels using nonlinear regression, and the x axis represents the number of sequencing reads mapped to insertion sites. The scale of the MW standards on the right of each panel applies to both the genetic footprints and the HITS analysis plots. White, nonessential genes; gray, genes required for growth or survival in the lung.

(Fig. S2). HITS analysis of these genes was quite reproducible. When two preparations of genomic DNA from the transposon mutant bank were independently analyzed, the number of insertions detected in each gene was similar, with the majority (82%) of genes having <20% variation in insertion density between samples (Fig. S2). Therefore, both the complexity of the transposon bank and the detection of mutations by sequencing seemed to be sufficiently saturating for reproducible analysis of the relative abundance of mutants in the library.

To identify genes required during infection, we analyzed the relative number of insertions in each gene in the output library obtained after lung infection vs. insertions in the library before in vivo selection. The results are shown graphically in Fig. 2, and the complete data are listed in Table S1. A total of 903 genes had similar numbers of insertions before and after selection in the lung, indicating that they were not required in this model. This large number of genes with insertion patterns in the output library that were similar to those in the input library indicated that significant stochastic loss of mutations had not occurred in the infection model. The 135 genes that sustained insertions in >40% of their possible TA insertion sites and in which the number of insertions decreased by at least 3.3-fold after selection in the lung were considered candidate virulence genes (Table S2). Representative insertion patterns for genes detected as being required during infection (galU and orfH) vs. those that are not required in vivo (xylA) are shown in Fig. S3. In summary, HITS implicated 8.1% of the 1,657 annotated genes in the genome in survival or growth of H. influenzae in the lung.

Genetic footprinting provides a means for analyzing insertions in discrete genes to verify results obtained with HITS. Genetic footprinting uses PCR with a specific chromosomal primer paired with a transposon primer for physical mapping of insertions to the chromosome in a bank of mutants (13). For a given gene, PCR results in a set of products varying in size that correspond to the distance between the chromosome-specific primer and each transposon mutation within that gene. Specificity is further assured by conducting the procedure with a primer 5′ of the gene and independently with a primer 3′ of the gene. For this validation we chose genes of LPS biosynthesis (opsX, rfaF, orfH, and galU) in which mutations resulted in pronounced attenuation relative to wild-type according to HITS data (Table S2). H. influenzae produces a short chain carbohydrate on its LPS (also called lipooligosaccharide, LOS) and lacks the repeating O-antigen carbohydrate typical of some bacterial LPS. The LOS of H. influenzae consists of a conserved “inner core” usually composed of 3 heptose residues and an “outer core” composed of variable-length oligosaccharide extensions from the heptose residues. The opsX, rfaF, and orfH genes encode heptosyltransferases I, II, and III, respectively, and generate the chain of 3 heptose residues initiating at a single 3-deoxy-D-manno-octulosonic acid, which is attached to lipid A (14–16). The galU gene encodes a UDP-glucose pyrophosphorylase that catalyzes the UTP-dependent conversion of D-glucose-1-phosphate into UDP-glucose, the activated form of the sugar required for biosynthesis of various carbohydrates, and galU is required for addition of glucose and galactose residues to the LPS of diverse pathogenic bacteria (17–19).

Representative genetic footprinting results are shown for galU and orfH and compared with insertion patterns detected by HITS in Fig. 3 A and B. The decrease in insertion mutations detected in these genes after in vivo passage of the bank provided verification of selection against mutants with disruptions in these genes, and band intensities on genetic footprinting gels were in good agreement with the abundance of insertions at each site as detected by HITS. Genetic footprinting also detected in vivo attenuation of mutants with insertions in opsX, rfaF, and galE, and similar results were obtained in reactions with primers positioned either 5′ or 3′ of each gene (Fig. S4). In contrast, xylA, a gene of D-xylene metabolism that is not required for bacteria (20), exhibited similar mutational profiles in both the input and output banks (Fig. 3C), indicating that it is dispensable in the lung model. Therefore, these results provided a verification of HITS results by an independent method, identifying virulence factors previously implicated in bacteremia.

To assess whether genes identified by HITS as being required in vivo are also required in single-strain infections, we generated nonpolar mutations removing the complete coding sequences of galU or orfH, genes implicated as being required in the lung model by HITS. To address mutant phenotypes with a recent clinical isolate, mutations were constructed in the nontypeable
*H. influenzae* strain, NT127 (21). In agreement with HITS and genetic footprinting results, both mutants were attenuated for survival in the lung. Moreover, the degree of attenuation calculated by HITS falls within the variation in fold difference observed between single-strain infections of individual mice. (Fig. 3D). The gallU and orfH genes were previously shown to be essential for survival of *H. influenzae* in bloodstream models of infection (22, 23). A requirement for these genes in the lung supports the view that *H. influenzae* utilizes structures of the LPS inner and outer core in virulence strategies to combat clearance mechanisms of the host found in both of these environments.

**Discussion**

The genes implicated in bacterial growth or survival in the lung were functionally diverse, although several general categories were notable (Table S3). On the basis of Clusters of Orthologous Groups (COG) classifications, categories that were overrepresented in the attenuated gene set relative to their representation in the genome overall were “cell wall/membrane/envelope biogenesis,” “amino acid transport and metabolism,” and “nucleotide transport and metabolism” (Tables S2 and S3). The genes identified provided insight into the selection conditions encountered by *H. influenzae* in the lung model.

Components of the bacterial cell surface are frequently the most direct participants in host–pathogen interactions. A major class of genes related to the cell envelope that was identified as markedly attenuated in vivo consisted of genes of LPS synthesis. LPS is essential in models of *H. influenzae* pathogenesis in the middle ear and blood and contributes to numerous aspects of NTHI infection, including evasion of complement and antimicrobial peptides (24–26). Genes needed for extension of the LPS inner-core structures (opsX, rfaF, and orfH) were required in vivo in the lung [survival index (s.i.) ≤0.012] (Table S2), in agreement with the requirement for these genes for bacteremia (22, 23). Genes required for precursor production for LPS carbohydrate outer-core hekex extensions (gaiU and gaiE) were also required (s.i. ≤ 0.025), suggesting that unmodified inner-core LPS results in enhanced clearance of *H. influenzae* from the lung. Genes required for hekex extensions from the first hektope, lgsF (s.i. = 0.152), or the terminal hektope of the inner core, *lipA* (s.i. = 0.258), were partially required (16, 27), and a trend of moderate attenuation (∼1.5-fold) was also observed in single-strain infections with an *lpsA* mutant (Fig. S5). Distal modifications of the LPS outer-core structure mediated by genes such as *lic1A*, which adds acidic or the *lic1* locus responsible for addition of phosphorylcholine seemed to be nonessential in vivo in these experiments. The *lic1D* gene was previously implicated in the lung model at a late time during infection, but not at 24 h (28), and it is possible that other distal modifications also are more important at later times.

Numerous genes involved in transport of proteins or other substrates were implicated in the lung model, including the complete twin-arginine translocation system (*tatA*, *tatB*, and *tatC*), which translocates folded proteins that lack Sec-dependent signal sequences across the plasma membrane and contributes to virulence in multiple pathogens (29). An intriguing set of genes with recently predicted functions in maintenance of outer-membrane lipid asymmetry (30) was implicated in this pathogenesis in the lung. These genes included *vacJ* and a set of 5 genes annotated as “hypothetical genes” that are putative orthologs of an ABC transport system of *Escherichia coli* encoded by the *mlaA* and *mlaBCDEFG* loci (for clarity, *E. coli* names of these genes are noted in Table S2). Orthologs of these genes were implicated in virulence of enteroinvasive *E. coli*, *Shigella flexneri*, and *Burkholderia pseudomallei* (31–33). The *mla* gene orthologs were required late during the intracellular life cycle for escape from the phagocytic vacuole (31). A role for the *mla* genes in both intracellular pathogens and *H. influenzae* suggests that *H. influenzae* may encounter membrane-membrane-host defenses, such as cationic peptides or stress conditions in the lung, that are similar to those found in the phagocytic vacuole.

Additional overrepresented COG groups included genes involved in nutrient acquisition and interrelated adaptations to physiologic stress. Pathways of amino acid metabolism were required in the lung and included enzymes for synthesis or interconversion of methionine, asparagine, aspartate, serine, tryptophan, and branched-chain amino acids. Consistent with amino acid limitation, genes predicted to encode regulators involved in the stringent response were implicated in vivo, including RelA, which synthesizes (pppGpp in response to amino acid starvation (34), DksA, which modulates rRNA expression in response to (pppGpp (35), and Lon protease, which is activated by phosphorylation generated from (pppGpp (36) and has been implicated in proteolytic control of virulence factors (reviewed in ref. 37). Genes of nucleotide uptake and metabolism included those required for synthesis of purines and pyrimidines, in addition to genes involved in NAD uptake, *nadN* and *hel*. These genes mediate sequential conversion of NAD to NMN and nicotinamide riboside for uptake of this nucleotide that *H. influenzae* is unable to synthesize de novo (38). The complete set of genes for phosphate uptake (psaA, *psaB*, *psaC*, and *psbC*) was implicated in pathogenesis, as was the gene predicted to encode PhoB, a conserved response regulator protein that becomes active under low-phosphate conditions and controls diverse virulence functions in bacterial pathogens (reviewed in ref. 39). PhoR, a sensor kinase that activates PhoB, was not implicated in the lung. In other species, PhoB can be activated by “cross-talk” with other signaling systems independently of PhoR (40), and therefore *H. influenzae* PhoB may be required for responses to alternative signals. Resistance to oxidative stress is important for many pathogens. Genes involved in adaptations to oxidative stress conditions were identified, including *pgdX*, encoding a glutathione-dependent peroxidase (41), *oxyR*, which regulates genes critical for oxidative stress resistance, including *pgdX* (42), and genes of recombination pathways (ruvA, *ruvB*, *ruvC*, *recR*, *recC*, *recX*, and *xerD*) required to repair DNA damaged by oxidative stress (43). Several genes implicated in the lung model (*nadN*, *hel*, and *pgdX*) are dispensable for bloodstream colonization by *H. influenzae* type b (41, 44). It is possible that *H. influenzae* strains differ in their requirements for these genes in vivo, or that these genes are specifically needed in the lung, where nucleotide sources and levels of oxidative stress may differ from those in the blood.

**Conclusion**

HITS provides a massively parallel system to simultaneously monitor the relative fitness of thousands of individual mutants undergoing a selection condition of interest. In this report, a large library of ∼75,000 *H. influenzae* mutants was subjected to selection in a murine pulmonary model of pathogenesis to identify genes required for prolonging survival of *H. influenzae* in the lung. Analysis of the mutant library by HITS was easily performed, highly reproducible, and remarkably comprehensive. Sequencing of transposon/chromosome junctions revealed independent insertions in nearly 56,000 genomic sites. More than 96% of *H. influenzae* protein coding genes were analyzed using a conservative cutoff that excluded 35 genes that were duplicated or contained repetitive sequences and 25 genes that had <8 TA dinucleotides available for *mariner* insertion. It is anticipated that with improvements in high-throughput sequencing technology, the depth of sequencing coverage will substantially increase to allow an even greater level of resolution and dynamic range. The results provide a genome-wide assessment of the genetic requirements of this bacterium for growth or survival in the lung, and also represent the most comprehensive fitness analysis that has been applied to *H. influenzae* mutants in any animal model.
The profile of genes required in this environment provides a view of the host–pathogen interactions occurring during pulmonary pathogenesis and will provide insight into potential strategies for the design of vaccines or therapeutics to specifically target *H. influenzae* in this site of disease.

The HITS procedure was demonstrated using a mariner transposon bank in *H. influenzae*; however, none of the procedures are organism specific, and the approach should be applicable to any organism amenable to mutational analysis with transposons. A major advantage of the approach we present in this report is that it can be applied to existing mutant libraries and does not require use of a specifically engineered transposon. In fact, the procedure should be readily adaptable to libraries generated with any insertion mutation capable of providing a primer-binding site. Although a complete genome sequence is useful for HITS, mapping insertions to annotated contigs of draft genome sequences should yield much of the same information. Although HITS was used in this report to obtain a genome-wide assessment of the requirements for lung pathogenesis, the procedure should be equally effective for analysis of requirements for growth or survival under any selective condition that can be applied to large populations of mutants *en masse*. Because of the speed and resolution of HITS, it will be possible to efficiently conduct fitness analyses in diverse contexts of host–microbe interactions. Application of this approach is expected to generate multifaceted views of the genetic requirements of pathogens in the environments they encounter in diverse stages of pathogenesis.

**Materials and Methods**

**High-Density Mutagenesis of *H. influenzae* by In Vitro Transposition.** *H. influenzae* Rd strain BA042 and clinical isolate nontypeable strain NT127 (21) were grown in brain heart infusion broth (BHI) supplemented with 10 μg/ml hemin and 10 μg/ml NAD (sBHI) or on sBHI agar plates at 35 °C. Media contained kanamycin sulfate at 20 μg/ml (sBHI-Kan) where indicated. The mini-mariner transposon mntTrck (carried on plasmid pENTrck) was derived from magel-1 (8) by replacement of the endogenous promoter for the kanamycin resistance gene, aph, with the trc promoter. Transposition reactions were performed in vitro as described in ref. 45. Transposition products were transformed into *H. influenzae* as described previously (8, 46). After selection on sBHI-Kan plates, the insertion library (~75,000 colonies) was harvested in BHI with 20% glycerol and stored at ~80 °C.

**Selection of Transposon Insertion Mutant Library in the Lung Model.** The *H. influenzae* insertional mutation library (0.1 × 10^10 cfu) was inoculated in 50 ml sBHI and grown with shaking at 225 rpm to a final OD_{600} of 0.45. For representation of the input library, cells from 35 ml of culture were collected by centrifugation and stored at ~80 °C. Inoculum for murine lung infection was prepared by pelleting 5 ml of the culture, washing in 1× Hank’s buffered salt solution, and dilution to concentrations of 2.5 × 10^5 cfu/ml. Forty microliters (10^7 cfu) was inoculated into the naries of 5 female C57BL/6 mice (7 to 8 weeks old) anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) by i.p. injection. At 24 h of infection, lungs were harvested and homogenized using a Fisher Tissueemome. Dilutions of homogenates were plated on sBHI to enumerate total cfu per lung. To recover the output library, homogenates from each mouse lung were plated on 12 sBHI agar plates, and resulting colonies were collected for chromosomal DNA isolation using phenol chloroform extraction (8). All experiments with mice were conducted with prior approval of the University of Massachusetts Institutional Animal Care and Use Committee (IACUC).

**Genetic Footprinting.** Genetic footprinting was conducted on *H. influenzae* genomic DNA from input and output libraries as described elsewhere (45) with transposon-specific primer, marout, and gene-specific primers that bind 3′ or 5′ of each gene. Primer design, PCR conditions, and image analysis are described in *SI Methods*, and footprinting primers are listed in Table S4.

**Illumina Sequencing of Transposon–Chromosome Junctions from Mutant Libraries.** Genomic DNA from mutant libraries prepared before and after in vivo selection was sequenced using a Covaris S2 device. Pair-ended Illumina libraries were created by ligation of adapters to sheared DNA as described by Bentley et al. (47) and size selected between 200 and 400 bp. Enrichment of transposon-chromosome junction reads was performed by PCR amplification with a 5′ biotinylated transposon enrichment primer, PE1MAR, and adapter-specific PCR PE2.0 enrichment primer (Table S4). Thermocycler settings were as follows: 30 s, 98 °C; 18 cycles of 10 s, 98 °C, 30 s, 65 °C, 30 s, 72 °C; 5 min, 72 °C. Fragments between 250 and 300 bp were gel purified and added to Dyal MyOne C1 beads (Invitrogen) to capture biotinylated templates containing transposon insertions. The beads were washed according to the manufacturer's instructions, and the resulting eluted strand was eluted with 125 μM sodium hydroxide (NaOH). Supernatants were recovered from beads, neutralized, and templates purified with MinElute PCR purification columns (Qiagen). The resulting transposon libraries were quantified on an Agilent Bioanalyzer 2100 RNA Pico6000 chip (Agilent Technologies). Single-stranded templates were cluster amplified and sequenced on an illumina GAII, as described in ref. 47.

**Analysis and Mapping of Illumina Sequencing Data.** The illumina sequencing reads that contained the Himar1 inverted terminal repeat (ITR) sequence and the adjacent TA insertion site were identified in the raw fasta files and trimmed of the ITR sequence. The processed sequencing reads are provided as multifasta files for Input Library Sample1 (Dataset S1), Input Library Sample2 (Dataset S2), and Lung Output Library (Dataset S3). Processed reads typically 53 bp in length, were aligned to the *H. influenzae* Rd KW20 genome sequence (48) (GenBank accession no. L42023) using SOAPv1.11 alignment software using default settings (2 mismatches allowed per read) (49). A custom PERL script was used to parse insertion site coordinates from the SOAP output file to report the number of reads mapped per site and strand orientations of aligned reads (*SI Computer Script*). The data were imported into Microsoft Excel, and insertion site coordinates were mapped to positions within protein coding genes annotated in protein table RefSeq file NC_000907.ppt (from the National Center for Biotechnology Information: ftp://ftp.ncbi.nih.gov/). For each gene, the number of insertion sites identified and the total number of sequencing reads in the internal 50–80% of the gene were determined using Excel functions. Additional details are provided in *SI Methods*. A draft version of *H. influenzae* strain RdAW (also referred to as BA042) genome sequence was generated and is at least 99.8% identical to strain Rd KW20 (48).

**Single-Strain Infections in the Pulmonary Clearance Model.** Nonpolar mutations deleting the galU and orfH genes were introduced into nontypeable *H. influenzae* strain, NT127 (*SI Methods*). Each *H. influenzae* strain was used to inoculate C57BL/6 mice by the intranasal route as described above. At 24 h of infection, mice were killed and pulmonary cfu in the lungs were enumerated as described above. The number of cfu recovered from the lungs of each mouse was compared by one-way ANOVA with Tukey’s multiple comparison test. Blood samples obtained immediately before killing revealed no detectable cfu. Procedures were approved by the University of Massachusetts IACUC.

**Note Added in Proof.** During review of this manuscript we learned of an independent report submitted to *Nature Methods* by Tim van Opijnen, Kip L. Bodi, and Andrew Camilli, in which transposon junction sequencing was successfully applied to study genetic networks (personal communication).

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Table S2. Genes required for growth or survival in the lung model detected by HITS

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<th>Hit Index</th>
<th>COG</th>
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<tr>
<td>HI0812</td>
<td>68.4</td>
<td>0.011</td>
<td>M</td>
<td>galU  UDP-glucose pyrophosphorylase</td>
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<td>0.012</td>
<td>M</td>
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</tr>
<tr>
<td>HI0428</td>
<td>46.4</td>
<td>0.012</td>
<td>O</td>
<td>dsbB  disulfide bond formation protein B</td>
</tr>
<tr>
<td>HI0942</td>
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<td>0.014</td>
<td>L</td>
<td>recC  exodeoxyribonuclease V gamma chain</td>
</tr>
<tr>
<td>HI0351</td>
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<td>0.014</td>
<td>T</td>
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<tr>
<td>HI0740</td>
<td>51.7</td>
<td>0.015</td>
<td>G</td>
<td>yhbX  phosphomannomutase (pgm)</td>
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<tr>
<td>HI0465</td>
<td>67.2</td>
<td>0.017</td>
<td>H</td>
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<td>HI0846</td>
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<td>O</td>
<td>por   periplasmic oxidoreductase DsbA</td>
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<tr>
<td>HI1193</td>
<td>42.6</td>
<td>0.021</td>
<td>E</td>
<td>ilvE  branched-chain amino acid aminotransferase</td>
</tr>
<tr>
<td>HI0221</td>
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<tr>
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<td>nagB  glucosamine-6-phosphate deaminase</td>
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<td>ahaT  aminotransferase AlaT</td>
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<td>P</td>
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<td>aspC  aromatic amino acid aminotransferase</td>
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<td>amiB  N-acetylMuramoyl-L-alanine amidase</td>
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<td></td>
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<tr>
<td>HI1290</td>
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<td>50.0</td>
<td>S, hypothetical protein HI0371</td>
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<tr>
<td>HI0669</td>
<td>40.0</td>
<td>C, mioC, flavodoxin</td>
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<td>trpC, bifunctional indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase</td>
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<td>43.9</td>
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<tr>
<td>HI1388</td>
<td>46.2</td>
<td>trpG, anthranilate synthase component II</td>
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<td>65.8</td>
<td>E, metC, cystathionine beta-lyase</td>
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<td>HI0055</td>
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<td>G, uxxA, mannionate dehydratase</td>
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</table>
The set of *H. influenzae* genes listed in the table were determined by two criteria as depicted in Fig. 2. Genes were considered to be specifically required in vivo if they sustained insertions in at least 40% of the possible insertion sites in the internal 5-80% of the gene in the in vitro grown input library and the total number of sequencing reads mapping to insertion sites in each gene decreased at least 3.3-fold after in vivo passage relative to the input library.

- Percentage of TA dinucleotides with detected insertions in the 5’ 5 to 80% of each gene.
- Survival index calculated as the fraction of total sequencing reads mapped to insertions in the output library relative to the input library.
- Identifier for the Cluster of Orthologous Groups functional classification.
- For HI1380, the gene *pstB* has been annotated as containing an “artificial frameshift” and was not listed in the *H. influenzae* Rd KW20 (NC_000907.ppt) protein table.
Table S3. Distribution of genes required \textit{in vivo} organized by functional categories

<table>
<thead>
<tr>
<th>Category(^a)</th>
<th>Genes required \textit{in vivo}</th>
<th>Total \textit{H. influenzae} genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid transport and metabolism (E)</td>
<td>23(^b) 16.9%</td>
<td>148 8.9%</td>
</tr>
<tr>
<td>Cell wall/membrane/envelope biogenesis (M)</td>
<td>18(^b) 13.2%</td>
<td>118 7.1%</td>
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<tr>
<td>Nucleotide transport and metabolism (F)</td>
<td>13(^b) 9.6%</td>
<td>56 3.4%</td>
</tr>
<tr>
<td>DNA Replication, recombination and repair (L)</td>
<td>10 7.4%</td>
<td>108 6.5%</td>
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<tr>
<td>Inorganic ion transport and metabolism (P)</td>
<td>10 7.4%</td>
<td>85 5.1%</td>
</tr>
<tr>
<td>Posttranslational modification, chaperones (O)</td>
<td>8 5.9%</td>
<td>85 5.1%</td>
</tr>
<tr>
<td>Carbohydrate transport and metabolism (G)</td>
<td>7 5.1%</td>
<td>104 6.3%</td>
</tr>
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<td>Cell division and chromosome partitioning (D)</td>
<td>6(^b) 4.4%</td>
<td>23 1.4%</td>
</tr>
<tr>
<td>Signal transduction mechanisms (T)</td>
<td>5 3.7%</td>
<td>31 1.9%</td>
</tr>
<tr>
<td>Coenzyme transport and metabolism (H)</td>
<td>4 2.9%</td>
<td>72 4.3%</td>
</tr>
<tr>
<td>(^2) metabolites biosynthesis and transport (Q)</td>
<td>4(^b) 2.9%</td>
<td>14 0.8%</td>
</tr>
<tr>
<td>Intracellular trafficking and secretion (U)</td>
<td>3 2.2%</td>
<td>26 1.6%</td>
</tr>
<tr>
<td>Translation, ribosomal structure (J)</td>
<td>3 2.2%</td>
<td>148 8.9%</td>
</tr>
<tr>
<td>Energy production and conversion (C)</td>
<td>3 2.2%</td>
<td>94 5.7%</td>
</tr>
<tr>
<td>Transcription (K)</td>
<td>2 1.5%</td>
<td>73 4.4%</td>
</tr>
<tr>
<td>Defense mechanisms (V)</td>
<td>2 1.5%</td>
<td>17 1.0%</td>
</tr>
<tr>
<td>Unknown(^c)</td>
<td>14 10.3%</td>
<td>404 24.4%</td>
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<tr>
<td>Total</td>
<td>135</td>
<td>1657</td>
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</tbody>
</table>

“Percentage in category” refers to the fraction of genes within an individual functional class relative to the total. Letters in parenthesis denote the COG classification identifiers.

\(^a\) Categories in which no genes were identified for growth or survival \textit{in vivo} are not shown

\(^b\) Increases in the number of genes in category identified by HITS relative to the entire genome are significant (p < 0.05) by chi squared analysis.

\(^c\) Genes with COG designations of Function Unknown (S), General Function Prediction Only (R) and those without an assigned designation.
### Table S4. Oligonucleotides used in this study

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<tbody>
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<td><strong>HITS enrichment primers</strong></td>
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<tr>
<td>PE1MAR&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5'-biotinTEG-AAATGATACGCCAGACCCAGATCTACACTCTTTTCTCCTACACGCCGCTCTTCCGATCTCCGGGAGTATTCGATCCTGGAAGATATCGTCTTCCGATCT</td>
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<tr>
<td>PCR PE2.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>CAAGCAGAAAGACGGCATACGATGCTCCTCGGACTTACCTGCTGAACCAGCTCTTCCGATCT</td>
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<td><strong>Genetic Footprinting</strong></td>
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<td>marout</td>
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<tr>
<td>rfaF_F</td>
<td>TCATCGTGCCTGTCATCGAATTTAAGTAGG</td>
</tr>
<tr>
<td>rfaF_R</td>
<td>GCTAGGGGATTTGGACCTGTAAGTGTG</td>
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<td>GGTAGCGGCTAACGATCGACAGC</td>
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<td>orfH_R</td>
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<tr>
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<tr>
<td>galE_F</td>
<td>ATCACAACACTATGGACATAGCCT</td>
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<tr>
<td>galE_R</td>
<td>GATGTTATTGCTTAGCCAAAACAGGCA</td>
</tr>
<tr>
<td>xylA_F</td>
<td>CAGTTAATGCCACAAACGAGGTAAGTAAAGCTGAT</td>
</tr>
<tr>
<td>xylA_R</td>
<td>CATGTTTTCGTAACCAACTCCATC ACT</td>
</tr>
</tbody>
</table>

**Primers used in mutant construction.** Single-strain infections in the pulmonary clearance model

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>HI0524-int5'</td>
<td>CAAAAAGTTTTGTCTAGTCTAAAG</td>
</tr>
<tr>
<td>HI0523-out</td>
<td>TTGAAATAGCGTCTGTATTTTATGT</td>
</tr>
<tr>
<td>15kan+HI0523</td>
<td>GATGAGTTTTCTTAAAGAAAGAGCTCATAATGTTAAG</td>
</tr>
<tr>
<td>HI0521-int3'</td>
<td>TAAATCGCTAGCCTATCATATAATC</td>
</tr>
<tr>
<td>HI0523-15</td>
<td>CGACCGCACTTTAAAATGAGCCCATATTAACGCCGGAACG</td>
</tr>
<tr>
<td>kan3&lt;sup&gt;b&lt;/sup&gt;+TAA</td>
<td>TTAGAAAACATCATGAGCATAACA</td>
</tr>
<tr>
<td>812at1945for</td>
<td>GCCGATAAGTTCCGTATTTTAC</td>
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<tr>
<td>811interg3&lt;sup&gt;c&lt;/sup&gt;2</td>
<td>GTAAAATCAATGTAGCTCTTAAG</td>
</tr>
<tr>
<td>764-5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ATGAATCGTCAATTTTATC</td>
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<tr>
<td>767-5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>ATGAAAAATACAAATACCG</td>
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</table>

<sup>a</sup> The Illumina primer PCR PE1.0 (underlined) was adapted for enrichment of transposon/chromosomal junctions

<sup>b</sup> Oligonucleotide sequences © 2006 Illumina, Inc. All rights reserved.
Supporting Information

Gawronski et al. 10.1073/pnas.0906627106

SI Text

Analysis and Mapping of Illumina Sequencing Data. The last 20 nucleotides of the PE1MAR enrichment primer are complementary to the inverted terminal repeats of the hinmar-derived minitransposon, mmrTrkKan, and allowed selective enrichment of transposon/chromosomal junctions. The Illumina sequencing primer site was introduced into enriched fragments via PE1MAR immediately 5’ of the transposon-specific sequence. As a result, the sequencing read of an amplified transposon/chromosomal junction fragment started with the sequence “ggaggatctaggcaacCTGta,” with the following regions denoted: transposon-specific 3’ end of the PE1MAR primer (lowercase), the remainder of the ITR of the hinmar transposon (uppercase), and the chromosomal TA insertion site (italics), which is duplicated upon transposon integration. Sequencing reads containing the exact string (above) were trimmed of the ITR-derived sequence to leave the putative TA insertion site at the 5’ end of the sequence. The sequences were aligned to the H. influenzae Rd KW20 genome reference genome (1) using SOAPv1.11 alignment software (2), which allowed for 2 mismatches per read. In the Input Library Sample1 (Dataset S1), 564,484 of the 708,731 trimmed reads (80%) were mapped to the reference genome. In Input Library Sample2 (Dataset S2), 559,459 of the 736,631 reads (76%) and in the Lung Output Library (Dataset S3) 263,237 of the 447,370 (59%) were aligned to the reference genome.

A custom PERL script was used to extract the TA dinucleotide insertion site coordinates from the SOAP output file (SI Computer Script). For reads aligning to the plus strand of the genome, the genome coordinate at position 1 of the trimmed read was determined. For reads aligning to the minus strand, the genome coordinate at position 2 of the read was calculated to represent the TA coordinate position with respect to the plus strand. For each TA insertion site detected by alignment, the total number of reads and the strand orientation was determined.

To adjust for sequencing coverage among samples for calculation of survival indices, the number of sequencing reads obtained for each insertion site in the input library was multiplied by a normalization factor of 0.63 defined as (Ro/Ri)/(So/Si), in which the variables represent the total number of sequencing reads (R) and insertion sites (S) detected in the input (i) and output (o) libraries, respectively.

Genetic Footprinting of Input and Lung Selected Libraries. Chromosomal footprinting primers were designed by FastPCR software (www.biocenter.helsinki.fi/bi/programs/fastpcr.htm). The primers were analyzed using standalone MEGABlast (3) against H. influenzae Rd KW20 genome (1) (NC_000907.fna) with the options “-W 8 -F” to search of potential of nonspecific amplification. At secondary sites, the identity at the 3’ end of the primer was <14 bp. PCR reactions consisted of 200 ng library genomic DNA, 1 µM marout primer, 1 µM gene-specific primer, 250 µM dNTP, 2.1 U Taq polymerase, and 0.17 U DeepVent DNA polymerase in 1X Thermopol buffer (NEB) in a 40 µL reaction. The PCR settings were as follows: 95 °C for 2 min; 30 cycles of 94 °C for 30 s, 68 °C for 3.5 min + 10 s per cycle; hold at 8 °C. Primers are listed in Table S4. PCR reactions were examined by agarose gel electrophoresis using 0.9% agarose gels after visualization with ethidium bromide. Gels were imaged using the Kodak Gel Logic 200 imaging system. In the genetic footprinting data, gene positions were determined using the method of Schaffer and Sederoff (4). Migration distances of the 1-kb plus ladder (Invitrogen) were fit using nonlinear least-squares regression by Microsoft Excel and the Solver Add-in.

Single-Strain Infections in the Pulmonary Clearance Model: Strain Construction. The NT127 orfH mutant was generated by double-crossover homologous recombination using a kanamycin resistance-marked gene replacement construct. The replacement cassette was assembled from the following 3 fragments: a 1,065-bp PCR product containing the 5’ flanking region of orfH was amplified from H. influenzae Rd BA042 with primers H10524-int5’ and H10523out; a 1,140-bp PCR product containing the 3’ flanking region of orfH was amplified from Rd with primers 15kan+HI0523 and HI0521-int3’; and an 815-bp fragment containing the kanamycin resistance gene, aphl, from Tn903 was amplified with primers HI0523–15 and kan3’+TA (5). Primer sequences are provided in Table S4. Overlap extension PCR of the 3 purified fragments using primers H10524-int5’ and HI0521-int3’ yielded a final 3.0-kb “stitched” product. The synthetic construct was used to transform H. influenzae NT127. Transformants were selected on sBHI containing 20 µg/mL kanamycin and verified by PCR analysis.

The galU mutant was generated by transformation of H. influenzae NT127 with a PCR product amplified from strain RdgalU (6) using primers 812at1945for and 811inter3’ (Table S4). The ~3.3-kb PCR product contains a nonpolar galU mutation, in which the galU ORF was replaced with that of aphl kanamycin resistance gene. Transformants were selected on sBHI agar containing 20 µg/mL kanamycin and verified by PCR analysis.

The lpsA mutant strain ΔrepRcp5 was generated by transformation of H. influenzae Rd Δrep (7) with a PCR product amplified from H. influenzae Rd strain Rcp5 (5) using primers 764–5’ and 767–5’ (Table S4). The strain Δrep contains a deletion of 16 tandem CAAT repeats in lcsA and stabilizes the ORF to prevent stochastic phase variation (5). The ~4-kb PCR product from Rcp5 contains a transposon insertion mutation in lpsA at position 635 of the protein coding sequence (7). Transformants were selected on sBHI agar containing 20 µg/mL kanamycin and verified by PCR analysis.

The N127 mutant strain orfHΔrepRcp5 was generated by transformation of H. influenzae Rd Δrep (7) with a PCR product amplified from H. influenzae Rd strain Rcp5 (5) using primers 764–5’ and 767–5’ (Table S4). The strain Δrep contains a deletion of 16 tandem CAAT repeats in lcsA and stabilizes the ORF to prevent stochastic phase variation (5). The ~4-kb PCR product from Rcp5 contains a transposon insertion mutation in lpsA at position 635 of the protein coding sequence (7). Transformants were selected on sBHI agar containing 20 µg/mL kanamycin and verified by PCR analysis. For each strain, 40 µL (10^7 cfu) was inoculated into the nases of 7 C57BL/6 mice (5–8 weeks old) anesthetized with ketamine (50 mg/kg) and xylazine (5 mg/kg) by i.p. injection. At 24 h of infection, lungs were harvested and homogenized using a Fisher Tissuemiser. Dilutions of homogenates were plated on sBHI to enumerate total cfu per lung. Procedures were approved by the University of Massachusetts IACUC.

Fig. S1. Genomic distribution of transposon insertions sites in input and output libraries. The percentage of TA dinucleotide sites sustaining insertions identified by HITS analysis are displayed in 1-kb increments along the H. influenzae Rd KW20 genome. Light gray bars, input library; dark gray bars, lung-selected output library. Arrows denote the location of several genes discussed in text. Genes located in regions A and B are involved in essential cell functions. Region A: transcription, translation, and protein secretion (pos 846,000–853,000 bp; rplE, rpsN, rpsH, rplF, rplR, rpsE, rpmD, rplO, secY, rpmI, rpsM, rps11, rpsD, and rpoA). Region B: cell division (pos 1,198,000–1,215,000 bp; ftsI, murE, murF, mraY, murD, ftsW, murG, murC, ddl, ftsQ, ftsA, ftsZ, and lpxC).
Replicate analysis of the input library by HITS. Two chromosomal preparations of the input library were analyzed by HITS. Axes represent the total number of sequencing reads mapped to insertion sites in all genes (5' 5–80% of the coding sequence) for each sample. Dotted lines indicate the upper and lower 20% deviation in x- and y-values from the best fit line (y = 0.9889x) determined by linear regression analysis. Of 1,038 genes containing insertions in >40% of possible sites, 850 (82%) fell within these boundaries.
Fig. S3. Insertion patterns in representative genes before and after in vivo selection. Comparison of the number of HITS reads mapped to insertion sites in the input and output pools for galU (A) orfH (B), and xylA (C). The positions of the sites sustaining insertions in the entire coding sequence of the genes are denoted sequentially on the x axis, and the numbers of reads mapped to insertions are shown on the y axis. Open bars, input library; solid bars, output library. Gene lengths: galU, 888 bp; orfH, 1,041 bp; xylA, 1,320 bp.
Fig. S4. Genetic footprinting of *H. influenzae* whole-genome transposon insertion library. In each panel A–F, the left image shows the genetic footprint analyzed in the forward direction and the right image from the reverse direction. PCR was conducted on genomic DNA from each input and output library using transposon-specific primer, marout, and a chromosomal-specific primer to examine insertions either the 5′ or 3′ direction. I, input library; O, output library. Gene and molecular weight standards positions are displayed to the right of the gel images for each genetic footprint. White, nonessential genes; gray, genes required for growth or survival in the lung; black, genes implicated as essential for growth or survival in vitro. (A) Insertion profiles were examined in *opsX*, encoding heptosyltransferase I, using (Left) primer *opsX*F (positioned 259 bp 5′ of *opsX*) and (Right) primer *opsX*R (positioned 454 bp 3′ of *opsX*). Similarly, insertions were examined in the following genes using the 5′ primer (Left) and the 3′ primer (Right): (B) rfaF, heptosyltransferase II, rfaF F (355 bp) and rfaF R (259 bp); (C) orfH, heptosyltransferase III, orfH F (202 bp) and orfH R (125 bp); (D) galE, UDP-glucose 4-epimerase, galE F (162 bp) and galE R (271 bp); (E) galU, UDP-glucose pyrophosphorylase, galU F (278 bp) and galU R (170 bp); and (F) xylA, xylose isomerase, xylA F (279 bp) and xylA R (153 bp). The primer sequences are provided in Table S4.
Fig. S5. Effect of lpsA mutation in single-strain infections compared with HITS. *H. influenzae* Rd parental strain and the lpsA insertion mutant were recovered from lungs of C57BL/6 mice 24 h after intranasal inoculation with each strain. Bars represent the mean cfu per lung (parent, 6.04 × 10⁵ cfu per lung; lpsA, 3.98 × 10⁵ cfu per lung). The fold decrease in mean cfu recovered from the lpsA mutant vs. Rd parental strain is indicated (bracket). HITS attenuation for lpsA was calculated as the reciprocal of the HITS s.i. value of 0.258 and represents the number of reads mapping to lpsA in the input library relative to the lung-passaged output library.

Other Supporting Information Files

Table S1
Table S2
Table S3
Table S4
Dataset S1
Dataset S2
Dataset S3
SI Computer Script