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# Interspecies Transfer of the Penicillin-Binding Protein 3-Encoding Gene *ftsI* between *Haemophilus influenzae* and *Haemophilus haemolyticus* Can Confer Reduced Susceptibility to β-Lactam Antimicrobial Agents

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**Mutations in *ftsI*, encoding penicillin-binding protein 3, can cause decreased β-lactam susceptibility in *Haemophilus influenzae*. Sequencing of *ftsI* from clinical strains has indicated interspecies recombination of *ftsI* between *H. influenzae* and *Haemophilus haemolyticus*. This study documented apparently unrestricted homologous recombination of *ftsI* between *H. influenzae* and *H. haemolyticus in vitro*. Transfer of *ftsI* from resistant isolates conferred similar but not identical increases in the MICs of susceptible strains of *H. influenzae* and *H. haemolyticus*.**

*Haemophilus influenzae* is the major human pathogen of the genus *Haemophilus* (1, 2), and infections are usually treated with β-lactam antimicrobial agents. Amino acid substitutions in penicillin-binding protein 3 (PBP3), encoded by the *ftsI* gene, can confer decreased susceptibility to β-lactams in strains lacking β-lactamase genes (3, 4). Genetically defined β-lactamase-negative ampicillin-resistant (gBLNAR) isolates carry either the N526K (Ubukata group II) or the R517H substitution in PBP3, while the additional substitutions S385T and/or L389F (Ubukata group III) further reduce susceptibility (3, 5–7). A range of other amino acid substitutions has been identified, but their significance is unclear (3–5, 8, 9).

Whether the worldwide spread of gBLNAR isolates is caused by clonal dissemination or horizontal gene transfer is controversial (8, 10, 11). *H. influenzae* and *Haemophilus haemolyticus* are close relatives, (1) and putative transfer of *ftsI* between these two species is indicated by mosaic structures of *ftsI* from clinical and nasopharyngeal carriage isolates (12, 13).

(Part of these data was presented on a poster at the 2014 International Pasteurellaceae Conference, Prato, Italy, 15 May 2014.)

The present study was undertaken to examine putative species barriers and delineate transformation events after *ftsI* transfer under standardized *in vitro* conditions. *ftsI* genes (1,833 bp) plus flanking regions from two gBLNAR *H. influenzae* strains and two gBLNAR *H. haemolyticus* strains were amplified by PCR (primers used for amplification and sequencing are listed in Table S1 in the supplemental material) and used for electroporetic transformation of susceptible strains of *H. influenzae* and *H. haemolyticus* (one representative each) as previously described (3). Characteristics of the donor and recipient strains are listed in Table 1, and overall similarity of *ftsI* and PBP3 are given in Table S2 in the supplemental material. Transformants were selected on chocolate agar (Columbia agar [Oxoid] supplemented with 5% horse blood and Vitox [Oxoid]) containing 0.5 μg/ml ampicillin (AMP) and screened for the presence of the N526K substitution using real-time PCR as previously described (14). We observed intraspecies *ftsI* transformation frequencies between $4.2 \times 10^{-7}$ and $6.7 \times 10^{-7}$ with *H. influenzae* strain Rd as the recipient and between $7 \times 10^{-5}$ and $1 \times 10^{-4}$ with *H. haemolyticus* strain ATCC 33390$^T$ as the recipient; we observed interspecies *ftsI* transformation frequencies between $3.3 \times 10^{-7}$ and $1.7 \times 10^{-6}$ with *H. influenzae* strain Rd as the recipient and between $1.6 \times 10^{-5}$ and $2.4 \times 10^{-5}$ with *H. haemolyticus* strain ATCC 33390$^T$ as the recipient. Thus, we observed no obvious difference in transformation frequencies of *ftsI* between strains of the same species and strains of separate species, although transformation occurred more frequently in *H. haemolyticus* recipient strain ATCC 33390$^T$ than in *H. influenzae* recipient strain Rd.

Recombination events were delineated by sequencing the *ftsI* gene in five transformants of each recipient-donor combination. Mosaic structures of *ftsI* (Fig. 1) with sequence identities of >99.6% to the donor DNA in these segments documented homologous recombination in all 40 transformants. Recombination sites varied between recombinants of the same experiment and between different combinations of donor and recipient cells. Recombined fragments tended to be larger in

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TABLE 1 Bacterial strains used in this study

| Strain | Species | Group[a] | Amino acid at position[b]: | | | | | | | | | | | | | | | | MIC range[c] (μg/ml) of: | | Reference or source |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 273 | 274 | 344 | 350 | 352 | 355 | 356 | 357 | 377 | **385** | **389** | 449 | 502 | **526** | 547 | 554 | 561 | 562 | 569 | AMP | CTX | |
| Recipient | | | | | | | | | | | | | | | | | | | | | | | | |
| Rd (KW20) | *H. influenzae* | | S | E | K | D | T | K | V | S | M | S | L | I | A | N | V | A | A | V | N | 0.125–0.19 | 0.008–0.016 | 18 |
| ATCC 33390$^T$ | *H. haemolyticus* | | | D | R | N | G | T | V | | I | | | | | | I | | | | S | 0.19–0.25 | 0.012–0.016 | 19 |
| Donor | | | | | | | | | | | | | | | | | | | | | | | | |
| ATCC 49247 | *H. influenzae* | II | A | | | | | | | | | | | | | **K** | I | T | E | | S | 3 | 0.19–0.25 | 19 |
| UTAS252 | *H. influenzae* | III | | | | N | | | | N | I | T | **F** | | | **K** | I | | | L | S | 0.5–1 | 0.5–1 | This study |
| L23 | *H. haemolyticus* | II | | | | N | | | | | I | | | | V | **K** | I | | | | S | 1.5–2 | 0.094–0.125 | 20 |
| L48 | *H. haemolyticus* | II | | D | R | N | G | T | V | | I | | | V | | **K** | I | | | | S | 1–1.5 | 0.012–0.023 | 20 |

[a] *ftsI* mutation classification according to Ubukata et al. (3).
[b] Amino acid (aa) positions of substitutions in the transpeptidase region (aa 265 to 580 of PBP3) relative to *H. influenzae* strain Rd (3). The critical mutations for group classification are in bold.
[c] MIC range from two to four independent measurements.

intraspecies recombinations. When *H. influenzae* strain Rd was transformed with *ftsI* from *H. influenzae* strain ATCC 49247, all sequenced recombinants harbored almost the entire *ftsI* open reading frame (ORF) (1,833 nucleotides [nt]) of strain ATCC 49247 (Fig. 1A), while the recombined fragment varied from <600 to >1,500 nt when *H. haemolyticus* strain ATCC 33390$^T$ was transformed with the same DNA (Fig. 1B); however, even the smallest of these fragments encoded five (N526K, V547I, A554T, A561E, and N569S) of the six amino acid substitutions in the transpeptidase domain of PBP3 (see Fig. S1B in the supplemental material). The difference in size of the recombined fragments between intraspecies and interspecies recombinations was less prominent for other donor-recipient combinations (Fig. 1C to F). β-Lactams target the transpeptidase region of PBP3 that is encoded by nt 796 to 1,741 of *ftsI* (3). The entire transpeptidase region of the donor strain was present in almost all of the recombinants (Fig. 1).

A previous comparison of *ftsI* sequences from clinical and surveillance strains of *H. influenzae* and *H. haemolyticus* clustered mosaic fragments of the gene into distinct groups of recombination profiles, indicating a preference for specific recombination events (13). In that analysis, there was no indication of horizontal transfer of the entire ORF of *ftsI* despite *H. influenzae* being capable of specific uptake and homologous recombination of segments in excess of 10 kb (15). In this *in vitro* study, we observed a wide variation in the size of recombined fragments, and for 8 of 40 recombinants, the entire ORF of *ftsI* was replaced. Moreover, the size and position of inserted fragments did not cluster into distinct groups of recombination profiles. The reason for these differences is not clear. We used electroporation to introduce DNA into recipient cells; hence, the recombined fragments did not depend on the specific uptake of genomic DNA fragments carrying the DNA uptake signal sequences that facilitates transformation *in vivo* (16). Also, we have no indication of the fitness of our recombinants when exposed to the selective forces of a commensal lifestyle. On the other hand, the apparent high frequency of interspecies recombination of *ftsI in vivo* (13) indicates events that may occur on multiple occasions, thereby blurring the origin of separate regions of the gene.

Ampicillin and cefotaxime (CTX) MICs were assessed using Etest (bioMérieux) on Mueller-Hinton agar (Oxoid) with 5% horse blood and HTM supplement (Oxoid). The results showed that transfer of segments of *ftsI* from gBLNAR strains conferred a rise in the AMP and CTX MICs for all recombinants (Table 2; see also Table S3A and B in the supplemental material). The increase in the AMP MIC was modest (3-fold to 6-fold increase) except when *H. haemolyticus* strain ATCC 33390$^T$ was transformed with the Ubukata group III *ftsI* gene from *H. influenzae* UTAS252; this transfer increased the AMP MIC of *H. haemolyticus* ATCC 33390$^T$ recombinants profoundly (13-fold), even surpassing the AMP MIC of the donor strain. The largest change in CTX susceptibility was also observed after transfer of the *ftsI* gene from strain UTAS252, which increased the CTX MICs of *H. influenzae* and *H. haemolyticus* recombinants more than 50 times (Table 2). Transfer of the Ubukata group II *ftsI* gene from *H. influenzae* strain ATCC 49247 *ftsI* altered the susceptibility of *H. haemolyticus* recombinants to a larger extent than that of the *H. influenzae* recombinants (6-fold and 9-fold versus 3-fold and 5-fold, respectively). Thus, *ftsI* from the same donor may alter the antimicrobial susceptibilities of the two species differently.

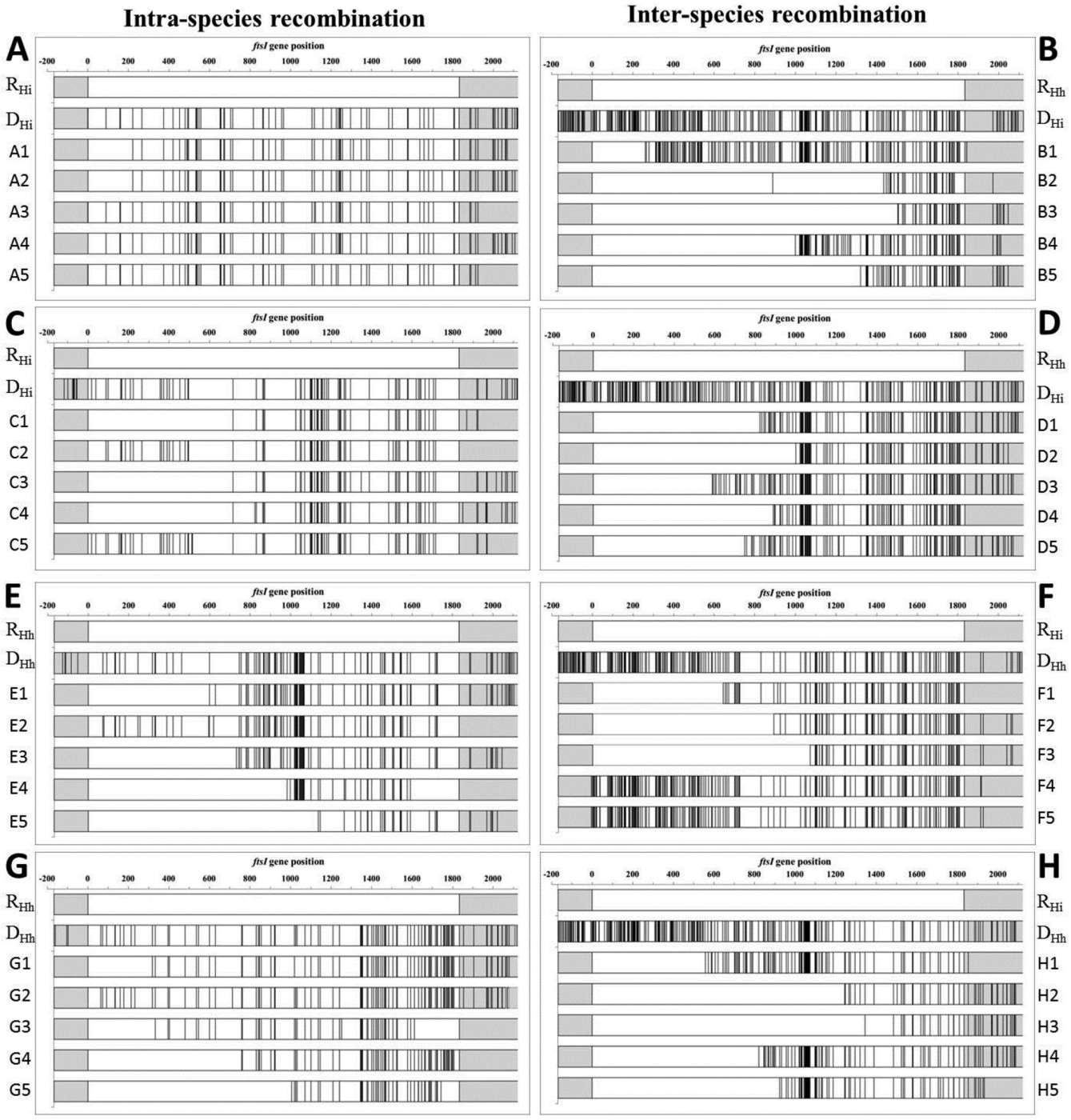


**FIG 1** Schematic representation of single nucleotide polymorphisms (vertical lines) in donor *H. influenzae* ($D_{Hi}$) or donor *H. haemolyticus* ($D_{Hh}$) and recombinant strains relative to recipient strains ($R_{Hi}$ or $R_{Hh}$). Left lane (A, C, E, G), intraspecies gene transfer; right lane (B, D, F, H), interspecies gene transfer. (A and B) *H. influenzae* strain ATCC 49247 donor; (C and D) *H. influenzae* strain UTAS252 donor; (E and F) *H. haemolyticus* strain L23 donor; (G and H) *H. haemolyticus* strain L48 donor. (A, C, F, and H) *H. influenzae* strain Rd recipient; (B, D, E, and G) *H. haemolyticus* strain ATCC 33390$^T$ recipient. White region, *ftsI* ORF; gray region, flanking regions. Numbers are relative to the first nucleotide of the *ftsI* ORF.

The exceptionally high AMP MIC of the *H. influenzae* BLNAR reference strain ATCC 49247 was only partially transferred by horizontal transfer of *ftsI* (Table 2). The additional, non-PBP3 and non-β-lactamase resistance mechanisms have not been clearly identified (4, 17).

The present study unambiguously documents intraspecies and interspecies recombination of *ftsI* in *H. influenzae* and *H. haemolyticus in vitro*, resulting in mosaic structures of the gene. Unexpectedly, the interspecies recombination of *ftsI* appeared relatively unaffected by the sequence divergence between the two species.

**TABLE 2** MICs of AMP and CTX for *ftsI* recombinants

| Group[a] | Recombinant | | AMP MIC (μg/ml) | | | CTX MIC (μg/ml) | | |
|---|---|---|---|---|---|---|---|---|
| | Donor | Recipient | GM[b] | Range | Fold change | GM | Range | Fold change |
| A | ATCC 49247 | *H. influenzae* strain Rd | 0.47 | 0.25–0.75 | 3 | 0.056 | 0.032–0.094 | 5 |
| B | ATCC 49247 | *H. haemolyticus* strain ATCC 33390$^T$ | 1.35 | 1–2 | 6 | 0.118 | 0.064–0.19 | 9 |
| C | UTAS252 | *H. influenzae* strain Rd | 0.66 | 0.5–0.75 | 4 | 0.596 | 0.38–0.75 | 53 |
| D | UTAS252 | *H. haemolyticus* strain ATCC 33390$^T$ | 2.86 | 1.5–4 | 13 | 0.772 | 0.75–1 | 56 |
| E | L23 | *H. haemolyticus* strain ATCC 33390$^T$ | 1.11 | 0.5–2 | 5 | 0.047 | 0.012–0.094 | 3 |
| F | L23 | *H. influenzae* strain Rd | 0.53 | 0.25–1 | 3 | 0.034 | 0.012–0.064 | 3 |
| G | L48 | *H. haemolyticus* strain ATCC 33390$^T$ | 0.89 | 0.38–1.5 | 4 | 0.023 | 0.012–0.032 | 2 |
| H | L48 | *H. influenzae* strain Rd | 0.49 | 0.25–0.75 | 3 | 0.023 | 0.008–0.047 | 2 |

[a] Letters relate to recombinant groups depicted in Fig. 1.
[b] GM, geometric mean of five sequenced transformants, each determined twice in separate experiments.

However, phenotypic expression of the same *ftsI* gene may differ between *H. haemolyticus* and *H. influenzae*.

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## REFERENCES

1. **Norskov-Lauritsen N.** 2014. Classification, identification, and clinical significance of *Haemophilus* and *Aggregatibacter* species with host specificity for humans. Clin Microbiol Rev **27:**214–240. http://dx.doi.org/10.1128/CMR.00103-13.
2. **Turk DC.** 1984. The pathogenicity of *Haemophilus influenzae*. J Med Microbiol **18:**1–16. http://dx.doi.org/10.1099/00222615-18-1-1.
3. **Ubukata K, Shibasaki Y, Yamamoto K, Chiba N, Hasegawa K, Takeuchi Y, Sunakawa K, Inoue M, Konno M.** 2001. Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Antimicrob Agents Chemother **45:**1693–1699. http://dx.doi.org/10.1128/AAC.45.6.1693-1699.2001.
4. **Tristram S, Jacobs MR, Appelbaum PC.** 2007. Antimicrobial resistance in *Haemophilus influenzae*. Clin Microbiol Rev **20:**368–389. http://dx.doi.org/10.1128/CMR.00040-06.
5. **Dabernat H, Delmas C, Seguy M, Pelissier R, Faucon G, Bennamani S, Pasquier C.** 2002. Diversity of beta-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. Antimicrob Agents Chemother **46:**2208–2218. http://dx.doi.org/10.1128/AAC.46.7.2208-2218.2002.
6. **Osaki Y, Sanbongi Y, Ishikawa M, Kataoka H, Suzuki T, Maeda K, Ida T.** 2005. Genetic approach to study the relationship between penicillin-binding protein 3 mutations and *Haemophilus influenzae* beta-lactam resistance by using site-directed mutagenesis and gene recombinants. Antimicrob Agents Chemother **49:**2834–2839. http://dx.doi.org/10.1128/AAC.49.7.2834-2839.2005.
7. **Garcia-Cobos S, Campos J, Lazaro E, Roman F, Cercenado E, Garcia-Rey C, Perez-Vazquez M, Oteo J, de Abajo F.** 2007. Ampicillin-resistant non-beta-lactamase-producing *Haemophilus influenzae* in Spain: recent emergence of clonal isolates with increased resistance to cefotaxime and cefixime. Antimicrob Agents Chemother **51:**2564–2573. http://dx.doi.org/10.1128/AAC.00354-07.
8. **Skaare D, Anthonisen IL, Caugant DA, Jenkins A, Steinbakk M, Strand L, Sundsfjord A, Tveten Y, Kristiansen BE.** 2014. Multilocus sequence typing and *ftsI* sequencing: a powerful tool for surveillance of penicillin-binding protein 3-mediated beta-lactam resistance in nontypeable *Haemophilus influenzae*. BMC Microbiol **14:**131. http://dx.doi.org/10.1186/1471-2180-14-131.
9. **Barbosa AR, Giufre M, Cerquetti M, Bajanca-Lavado MP.** 2011. Polymorphism in *ftsI* gene and beta-lactam susceptibility in Portuguese *Haemophilus influenzae* strains: clonal dissemination of beta-lactamase-positive isolates with decreased susceptibility to amoxicillin/clavulanic acid. J Antimicrob Chemother **66:**788–796. http://dx.doi.org/10.1093/jac/dkq533.
10. **Kishii K, Chiba N, Morozumi M, Hamano-Hasegawa K, Kurokawa I, Masaki J, Ubukata K.** 2010. Diverse mutations in the *ftsI* gene in ampicillin-resistant *Haemophilus influenzae* isolates from pediatric patients with acute otitis media. J Infect Chemother **16:**87–93. http://dx.doi.org/10.1007/s10156-009-0011-6.
11. **Park C, Kim KH, Shin NY, Byun JH, Kwon EY, Lee JW, Kwon HJ, Choi EY, Lee DG, Sohn WY, Kang JH.** 2013. Genetic diversity of the *ftsI* gene in beta-lactamase-nonproducing ampicillin-resistant and beta-lactamase-producing amoxicillin-/clavulanic acid-resistant nasopharyngeal *Haemophilus influenzae* strains isolated from children in South Korea. Microb Drug Resist **19:**224–230. http://dx.doi.org/10.1089/mdr.2012.0116.
12. **Takahata S, Ida T, Senju N, Sanbongi Y, Miyata A, Maebashi K, Hoshiko S.** 2007. Horizontal gene transfer of *ftsI*, encoding penicillin-binding protein 3, in *Haemophilus influenzae*. Antimicrob Agents Chemother **51:**1589–1595. http://dx.doi.org/10.1128/AAC.01545-06.
13. **Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A.** 2014. Role of inter-species recombination of the *ftsI* gene in the dissemination of altered penicillin-binding-protein-3-mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. J Antimicrob Chemother **69:**1501–1509. http://dx.doi.org/10.1093/jac/dku022.
14. **Witherden EA, Kunde D, Tristram SG.** 2013. PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. J Antimicrob Chemother **68:**2255–2258. http://dx.doi.org/10.1093/jac/dkt189.
15. **Mell JC, Shumilina S, Hall IM, Redfield RJ.** 2011. Transformation of natural genetic variation into *Haemophilus influenzae* genomes. PLoS Pathog **7:**e1002151. http://dx.doi.org/10.1371/journal.ppat.1002151.
16. **Redfield RJ, Findlay WA, Bosse J, Kroll JS, Cameron AD, Nash JH.** 2006. Evolution of competence and DNA uptake specificity in the *Pasteurellaceae*. BMC Evol Biol **6:**82. http://dx.doi.org/10.1186/1471-2148-6-82.
17. **Kaczmarek FS, Gootz TD, Dib-Hajj F, Shang W, Hallowell S, Cronan M.** 2004. Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* with unusually high resistance to ampicillin. Antimicrob Agents Chemother **48:**1630–1639. http://dx.doi.org/10.1128/AAC.48.5.1630-1639.2004.
18. **Fleischmann RD, Adams MD, White O, Clayton RA, Kirkness EF, Kerlavage AR, Bult CJ, Tomb JF, Dougherty BA, Merrick JM.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512. http://dx.doi.org/10.1126/science.7542800.
19. **ATCC.** 2015. ATCC: the global bioresource center. ATCC, Manassas, VA. http://www.lgcstandards-atcc.org/. Accessed 7 April 2015.
20. **Witherden EA, Tristram SG.** 2013. Prevalence and mechanisms of beta-lactam resistance in *Haemophilus haemolyticus*. J Antimicrob Chemother **68:**1049–1053. http://dx.doi.org/10.1093/jac/dks532.