Full-length Paper

Carbapenem-non-Susceptible *Haemophilus influenzae* with Penicillin-Binding Protein 3 containing Amino Acid Insertion

Running title: Inset in PBP3 of Carbapenem-non-susceptible BLNAR

Kazuki Kitaoka[a], Kouji Kimura[a]#, Hiromitsu Kitanaka[a], Hirotsugu Banno[a], Wanchun Jin[a], Jun-ichi Wachino[a], and Yoshichika Arakawa[a]

[a]Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

#Address correspondence to Kouji Kimura, koujikim@med.nagoya-u.ac.jp

Abstract

Prevalence of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae* (BLNAR) has become a clinical concern. In BLNAR, amino acid substitutions in penicillin-binding protein 3 (PBP3) are relevant to the β-lactam resistance. Carbapenem-non-susceptible *H. influenzae* isolates have been rarely reported. Through antimicrobial susceptibility testing and nucleotide sequence analysis of *ftsI*, encoding PBP3, and utilizing a collection of *H. influenzae* clinical isolates in our laboratory, we obtained a carbapenem-non-susceptible clinical isolate (NUBL1772) that possessed an altered PBP3 containing V525_N526insM. The aim of this study was to reveal the effect of altered PBP3 containing V525_N526insM on reduced carbapenem susceptibility. After generating recombinant strains with altered *ftsI*, we performed antimicrobial susceptibility testing and competitive binding assays with fluorescent penicillin (Bocillin FL) and carbapenems. Elevated carbapenem MICs were found for the recombinant strain harboring the entire *ftsI* gene of NUBL1772. The recombinant PBP3 of NUBL1772 also exhibited reduced binding to carbapenem. These results demonstrate that altered PBP3 containing V525_N526insM influences reduced carbapenem susceptibility. The revertant mutant lacking the V525_N526insM exhibited lower MICs of carbapenem than NUBL1772, suggesting that this insertion affects reduced carbapenem susceptibility. MICs of β-lactam for NUBL1772 was higher than those for the recombinant possessing *ftsI* of NUBL1772. NUBL1772 harbored AcrR with early termination, resulting in low-level transcription of *acrB* and high efflux pump activity. These findings suggest that the disruption of AcrR also contributes to the reduced carbapenem susceptibility found in NUBL1772. Our results provide the first evidence that the altered PBP3 containing V525_N526insM is responsible for reduced susceptibility to carbapenem in *H. influenzae*.

Introduction

Although the incidence of invasive diseases caused by *H. influenzae* type b (Hib) has decreased in many countries as a result of the universal Hib vaccination, *H. influenzae* still remains as a major pathogen involved in respiratory tract infections; these conditions are usually caused by nontypeable strains (1). In general, β-lactams have been used to treat *H. influenzae* infections. Two mechanisms of β-lactam resistance in *H. influenzae* have been reported, including β-lactamase-producing ampicillin-resistant (BLPAR) and β-lactamase-negative ampicillin resistant (BLNAR) (2, 3), which can be attributed to reduced binding affinity of β-lactam for penicillin-binding-protein 3 (PBP3) (4, 5). In Japan, the prevalence of BLNAR *H. influenzae* has become a clinical concern. In a nationwide survey of pediatric patients in Japan, BLNAR isolates occurred at the high frequency of 46.7 % in 2012 (6).

Carbapenem-non-susceptible *H. influenzae* isolates have been rarely reported. Although the number of reports concerning such strains is limited, all were reported to harbor altered PBP3 (7-9). In addition, slowed drug influx or direct efflux regulation appeared to be involved in reduced carbapenem susceptibility (10). Altered PBP3 in carbapenem-non-susceptible *H. influenzae* isolates has usually included various amino acid substitutions. To the best of our knowledge, only one report has mentioned an altered PBP3 containing the amino acid insertion, V525_N526insM (11). However, no comprehensive antimicrobial susceptibility testing, including that using various carbapenems, for the isolate harboring this altered PBP3 containing V525_N526insM was performed. Moreover, the involvement of the altered PBP3 with β-lactam resistance, based on recombination studies and/or PBP3 affinity studies, has not been documented.

We investigated antimicrobial susceptibility and the nucleotide sequence of *ftsI*, encoding PBP3, in our collection of *H. influenzae* clinical isolates. Through this analysis, a carbapenem-non-susceptible clinical isolate harboring the altered PBP3 containing

V525_N526insM was obtained.

Although there have been many reports of carbapenem-resistant gram-negative pathogens, carbapenems are prescribed as the last line in many bacterial infections (12, 13). Carbapenem-non-susceptible *H. influenzae* might be selected, and this may well cause treatment failure with carbapenems.

Thus, the aim of the study was to reveal the effect of altered PBP3 containing V525_N526insM on reduced carbapenem susceptibility.

Results and Discussion

Characteristics of clinical isolates with reduced carbapenem susceptibility

Among 157 clinical isolates of *H. influenzae*, we obtained seven isolates with reduced carbapenem susceptibility (imipenem MIC, ≥ 4 μg/ml or biapenem MIC, ≥ 8 μg/ml). The detailed information of these isolates with reduced carbapenem susceptibility is summarized in Table 1. All isolates were derived from the pediatric department and were recovered from the nose, except one isolate (vagina). All isolates were nontypeable and classified as biotype II (5/7) or V (2/7). All isolates were β-lactamase negative and non-susceptible to ampicillin; five belonged to BLNAR (ampicillin MIC, ≥ 4 μg/ml). According to the Clinical and Laboratory Standards Institute (CLSI) breakpoints, only the NUBL1772 isolate showed non-susceptibility to meropenem, imipenem, and doripenem, although the CLSI does not define the "resistant" category of carbapenems (14). Table 2 shows MIC values for NUBL1772. NUBL1772 showed high-level resistance to ampicillin-sulbactam and amoxicillin-clavulanic acid and intermediate resistance to clarithromycin.

According to the *ftsI* grouping proposed by Skaare et al., four isolates belonged to group III+, two isolates belonged to group II, and NUBL1772 was not classified because it was found to harbor

97 the V525_N526insM in PBP3 (9). The deduced amino acid sequence of PBP3 of NUBL1772 is
98 aligned in Fig. 1.

99 **Transformation, antimicrobial susceptibility and PBP3 competitive binding assay**

100 Considerably elevated MICs of ampicillin and carbapenems were found for the recombinant
101 strains harboring the entire *ftsI* gene of NUBL1772 compared to those for Rd (Table 3). Based on
102 competitive binding assay to the PBP3 proteins using Bocillin FL and ampicillin or carbapenems,
103 the 50 % inhibitory concentration (IC_{50}) values of ampicillin and carbapenems for NUBL1772
104 PBP3 were significantly higher than those for Rd PBP3, as summarized in Table 4. These
105 findings suggest that altered PBP3 containing V525_N526insM influences reduced carbapenem
106 susceptibility.

107 The nucleotide sequence of *ftsI* and deduced amino acid sequences of PBP3 in NUBL1772
108 were identical to those of *H. influenzae* isolates 92 and 118 from Wajima's report (11). NUBL1772
109 showed antimicrobial susceptibilities that were similar to those of *H. influenzae* isolates 92 and
110 118 reported by Wajima et al. within their description.

111 The reduced carbapenem susceptibility caused by the altered PBP3 containing
112 V525_N526insM in *H. influenzae* appears to be attributed to the insertion. The revertant isolate
113 possessing NUBL1772 PBP3 without the insertion showed lower MICs to carbapenem than
114 NUBL1772 (Table 3). Epidemiologically, Sanbongi et al. proposed that the amino acid
115 substitution at position 526 is important for the response to carbapenem (15). The introduction of
116 an insertion instead of an amino acid substitution at position 526 might reduce susceptibility to
117 carbapenems.

118 In terms of computer modeling, the effect of the altered PBP3 containing V525_N526insM
119 on reduced susceptibility to carbapenem was investigated (Fig. S1). The PBP3 protein of
120 *Escherichia coli* (PDB: 4BJP) was used as a template. N526 was located on the loop between β3

and β4. In NUBL1772, the loop was increased in length and shifted toward the active site, although three conserved motifs (-STVK-, -SSN-, and –KTG-) constituting the active site were unchanged. Regarding the PBPs 1a and 2b of *Streptococcus pneumoniae*, amino acid substitutions from some β-lactam-resistant isolates were found to be positioned on the loop between β3 and β4, and this resulted in increased flexibility of the loop, which was found to important for the generation of high-level β-lactam resistance (16-18). Similarly, the insertion in NUBL1772 might impart flexibility to the loop between β3 and β4, leading to reduced β-lactam susceptibility. However, this explanation has a limitation because no crystal structure of *H. influenzae* PBP3 has been solved yet, and actual flexibility of this region remains unclear.

Concerning other species, altered PBP3 containing a series of insertions in *E. coli* or altered PBP2 containing an insertion in *Neisseria gonorrhoeae* have been reported (19, 20). The altered PBP3 containing a series of insertions in *E. coli* was involved in the resistance to aztreonam and NUBL1772 showed elevated MIC values of aztreonam. However, the altered PBP3 containing a series of insertions in *E. coli* or the altered PBP2 containing an insertion of *N. gonorrhoeae* did not affect reduced carbapenem susceptibility. Each insertion was located on a different site (β2b-β2c in *E. coli*, β2a-β2d in *N. gonorrhoeae*, and β3-β4 in NUBL1772) and thus their susceptibilities to carbapenem appeared to be different from each other.

One limitation of this study was that a recombinant with only the insertion did not show reduced susceptibility to β-lactams (Table 3). Modeling of the PBP3 structure of Rd *ftsI* with V525_N526insM resulted in only a conformational change in the loop between β3 and β4, that the loop was slightly expanded and shifted toward the active site. The low level of loop modification compared to that of NUBL1772 might not affect β-lactam resistance. Thus, the methionine insertion results in reduced susceptibility to carbapenem only when it co-exists with other amino acid substitutions in NUBL1772 PBP3.

Effect of AcrR on reduced carbapenem susceptibility in NUBL1772

AcrR is a repressor of the AcrAB efflux pump, which might contribute to β-lactam resistance (21). The *acrR* gene of NUBL1772 was sequenced and the coding region (564 bp) was compared to that of Rd. NUBL1772 had nucleotide deletions at position 442–451. These deletions caused a frameshift after the amino acid sequence of AcrR at position 148. This change resulted in premature termination of AcrR at position 149, despite the intact length of wild-type AcrR is 187 amino acids. Thus, these results predicted a loss of function mutation in the AcrR protein of NUBL1772. Indeed, the transcription of *acrB* gene in NUBL1772 was significantly elevated, compared to that in Rd (Table 5). Efflux pumps including acrAB efficiently pump out ethidium bromide (EB) and the velocity of EB accumulation will decline in accordance with the efflux pump activity. The initial velocity of EB accumulation in NUBL1772 was significantly lower than that of Rd, as shown in Table 5. These results suggest that the disruption of AcrR affects the reduced carbapenem susceptibility of NUBL1772.

Kaczmarek et al. and Seyama et al. reported some amino acid sequences that predict the early termination of AcrR in *H. influenzae* (21, 22). Deletions found in *acrR* of NUBL1772 were different from these reports. The results for NUBL1772 were consistent with those for *H. influenzae* isolates harboring AcrR with an early termination described by Seyama et al. in terms of low-level *acrB* transcription and high efflux pump activity (22, 23). Moreover, the results for NUBL1772 were consistent with those for *H. influenzae* isolates harboring AcrR with an early termination described by Kaczmarek et al. in terms of the effect of efflux on ampicillin resistance (21). The fact that MICs of β-lactam for NUBL1772 were higher than those for the recombinant possessing *ftsI* of NUBL1772 (Table 3) and the MIC of clarithromycin for NUBL1772 was higher than that for Rd (Table 2) might be mediated by the disruption of AcrR. One limitation is that small molecules like β-lactams appear to abolish the effect of any efflux system based on the

rapid influx through porin channels (24). Many studies concerning *acrR* sequence analysis in BLNAR are required.

Dissemination of mutants with methionine insertion in PBP3

Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) using seven *H. influenzae* isolates with reduced carbapenem susceptibility demonstrated the presence of various genetic backgrounds (Table 1, Fig. S2). Strains NUBL970, NUBL1771, NUBL1775, and NUBL1777 were registered as a new sequence type (ST). Although NUBL1767 and NUBL1772 belonged to the identical sequence type (ST) and biotype, PFGE results indicated that these two isolates were not genetically related. Growth curves showed that the growth rate and maximum cell density of NUBL1772 and Rd recombinant carrying the entire *ftsI* gene of NUBL1772 were lower than those of Rd (Fig. S3), suggesting that the altered PBP3 containing V525_N526insM is associated with a growth disadvantage. These results would indicate limited spread for this variant.

Wajima et al. reported *H. influenzae* isolates that possess the same altered PBP3 as NUBL1772 (11). It is unclear whether the spread of BLNAR is dependent on clonal dissemination or horizontal gene transfer (25-31). When *H. influenzae* isolates possessing the same *ftsI* gene were isolated in different hospitals, each appeared to show different PFGE pulsotypes (31). Therefore, the fact that NUBL1772 and the isolates reported by Wajima et al. were detected in different prefectures might suggest the occurrence of genetic transfer of the altered PBP3 containing V525_N526insM. However, whether the clonal dissemination or horizontal gene transfer occurred between these isolates cannot be fully determined without performing PFGE on these isolates with one gel, MLST analysis, or whole genome analysis.

Clinical impact of mutants with methionine insertion in PBP3

NUBL1772 is a nontypeable *H. influenzae* nasopharyngeal isolate recovered from a 1-year

old child in 2012 (Table 1). Such isolates are predominantly associated with otitis media and sinusitis, which have been treated with amoxicillin, amoxicillin-clavulanic acid and ceftriaxone (32, 33). Furthermore, in Japan, treatment guidelines for otitis media and sinusitis state that the use of oral carbapenem can be considered as the last line upon clinical failure (34, 35). NUBL1772 showed non-susceptibility to these antibiotics, which might cause treatment failure, although the MIC of meropenem for NUBL1772 was very close to the clinical breakpoint.

NUBL1772 was susceptible to piperacillin-tazobactam and fluoroquinolone. Although susceptibility to piperacillin-tazobactam was not well investigated, to the best of our knowledge, no piperacillin-tazobactam resistant *H. influenzae* has been reported (6) to date. The prevalence of fluoroquinolone-resistant *H. influenzae* has been still low (6, 36, 37). Therefore, the use of piperacillin-tazobactam or fluoroquinolone might be effective for the treatment of infections with *H. influenzae* such as NUBL1772. Actually, the possibility of using piperacillin-tazobactam as an antibiotic therapy against meningitis caused by *H. influenzae* was previously examined (38). If NUBL1772 acquires resistances to other antimicrobials, drug choice would become even more difficult and limited.

Thus, because carbapenem is considered a last-resort drug, the emergence and spread of *H. influenzae* with reduced carbapenem susceptibility harboring an altered PBP3 containing V525_N526insM might pose a threat for treatment failure.

Conclusion

Our study provided the first evidence that altered PBP3 containing an insertion, namely V525_N526insM is responsible for reduced susceptibility to carbapenem in *H. influenzae*, based on recombination studies and PBP3 affinity studies. The insertion, together with other amino acid substitutions, plays a role in the reduced carbapenem susceptibility. Early termination of AcrR also partially contributes to the reduced susceptibility to carbapenem found in NUBL1772. Given that

carbapenems are reserved as the last line of treatment in many situations, it is necessary to focus on the trends in the development and spread of *H. influenzae* harboring the V525_N526insM insertion in PBP3.

Materials and methods

Clinical isolates

One hundred and fifty-seven clinical isolates of *H. influenzae* isolated at Miroku laboratory in Japan between 2011 and 2012 were sent to our laboratory. Miroku laboratory collected clinical specimens from various clinics and medical institutions in Japan, isolated *H. influenzae* from specimens in this study and performed bacteriological testing of them. According to the results of antimicrobial susceptibility testing, we selected seven isolates with reduced susceptibility to carbapenem (MIC of imipenem ≥ 4 μg/ml or MIC of biapenem ≥ 8 μg/ml) for further investigation. Capsular serotype was determined by capsular swelling with antisera (Denka Seiken, Tokyo, Japan) and PCR capsular genotyping (39). Indole, urease, and ornithine decarboxylase reactions were used to assess the biotype with the API-NH system (SYSMEX bioMérieux, Lyon, France). Production of β-lactamase was tested using Cefinase™ disks (Becton Dickinson and Company, Franklin Lakes, NJ.) and amplification of bla_{TEM-1} and bla_{ROB-1} was performed by PCR (15). *H. influenzae* Rd (ATCC 51907), *H. influenzae* ATCC 49247, and *H. influenzae* ATCC 49766 were purchased from the American Type Culture Collection (Manassas, VA).

Media and antibiotics

For broth cultures, Bacto™ Brain Heart Infusion (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 10 μg/ml β-NAD (Nacalai Tesque, Kyoto, Japan) and 10 μg/ml hemin (Nacalai Tesque, Kyoto, Japan) or *Haemophilus* Test Medium (HTM) broth consisting of BBL™ Muller-Hinton broth Cation Adjusted (Becton, Dickinson and Company, Franklin Lakes,

NJ.), 15 μg/ml β-NAD, 15 μg/ml hemin, and 5 g/l Bacto™ Yeast Extract (Becton, Dickinson and Company, Franklin Lakes, NJ) were typically used. Chocolate II agar (Becton, Dickinson and Company, Franklin Lakes, NJ) or HTM agar were used as the agar for bacterial growth. Cultures were incubated at 37 °C with 5 % CO_2 for 20–24 h.

Antimicrobial susceptibility testing

Antimicrobial susceptibility was assessed by broth microdilution testing according to CLSI guidelines (14). HTM broth was used and incubation conditions were 20 h in an ambient atmosphere. *H. influenzae* ATCC 49247 and *H. influenzae* ATCC 49766 were used for quality control. The breakpoints were interpreted according to CLSI criteria (14).

Subtyping of *ftsI*

The *ftsI* genes of seven *H. influenzae* isolates with reduced carbapenem susceptibility were amplified by PCR using the primers ftsIfrw and ftsIrev described by Cerquetti et al (7). PCR was performed using PrimeSTAR® HS DNA Polymerase (Takara Bio, Kusatsu, Japan) with a TaKaRa PCR Thermal Cycler Dice® Standard (Takara Bio, Kusatsu, Japan).

Purified PCR products were sent to Eurofins Genomics K.K (Tokyo, Japan) and sequenced using a BigDye terminator v3.1 with a 3730xl DNA Analyzer (Thermo Fisher Scientific, Waltham, MA). Although several classifications for PBP3 amino acid substitution patterns have been suggested, grouping proposed by Skaare et al. was determined to be more relevant to reduced carbapenem suscptibility (9). According to the Skaare classification, deduced PBP3 amino acid sequences from *ftsI* gene sequences were grouped as follows: Group I (R517H) and Group II (N526K) as low-rPBP3; Group III (S385T, N526K), Group III+ (S385T, L389F, N526K), Group III-like (S385T, R517H) and Group III-like+ (S385T, L389F, R517H) as high-rPBP3.

Cloning of *ftsI*

The *ftsI* genes of *H. influenzae* Rd and NUBL1772 were amplified by PCR with primers

containing restriction sites, as presented in Table S1. Each PCR product was digested with the relevant enzymes and inserted into the appropriate plasmid vector. For recombinant constructs, the upstream primer was designed to contain a NheI site. The downstream primer was designed to contain a KpnI site, starting from the 3´ end of the *ftsI*, to avoid the effect of unnecessary sequences. pBAD18-Cm was used as the vector. Furthermore, using a PrimeSTAR® Mutagenesis Basal Kit (Takara Bio, Kusatsu, Japan), pBAD 18-Cm containing Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526 del was constructed. Colonies of recombinants containing Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526 del were not able to be picked; therefore, an antibiotic cassette was added to these *ftsI* mutated genes to increase the frequency of picking a recombinant. The antibiotic cassette, *KanR2*, was excised from pBAD18-Kan by PCR, using primers containing KpnI and BamHI sites. The *murE* gene, which consists of a sequence located downstream of *ftsI* in *H. influenzae* Rd, was amplified by PCR using primers containing BamHI and SalI sites. These PCR products of *KanR2* and *murE* were inserted sequentially into the KpnI-SalI sites of pBAD18-Cm containing Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526 del.

For the expression and purification of PBP3, the primers were designed as previously described with some modifications (21). The upstream primer contained a SmaI site, and started after the region encoding a putative transmembrane domain for solubilization. The downstream primer contained a BamHI site, and started from the 3´ end of *ftsI*. pET47-b (+) (Merck, Darmstadt, Germany) carrying an N-terminal His-tag-coding sequence was used as the vector.

The recipient cell was *E. coli* DH10B for recombinant construction or *E. coli* Rosetta™ 2 (DE3) pLysS (Merck, Darmstadt, Germany) for expression and purification of PBP3.

Transformation

ftsI PCR products were introduced into *H. influenzae* Rd by electroporation (5, 40). NUBL1772 *ftsI* was amplified using the primers ftsIfrw and ftsIrev, as described by Cerquetti et al

(7), from NUBL1772 chromosomal DNA. Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526 del was amplified using *ftsI-KanR2* gene-*MurE* from plasmid DNA of the relevant vector. The conditions for electroporation were 1.25 kV/cm, 200 Ω, and 25 μF with time constants of 4.7 to 4.8 ms using the Gene Pulser Xcell (Bio-Rad Laboratories, Hercules, CA). Recombinants were selected on HTM agar containing 4 μg/ml biapenem for NUBL1772 *ftsI* or 10 μg/ml kanamycin for Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526 del. Each recombination was checked by DNA sequencing.

Expression and purification of PBP3

E. coli Rosetta™ 2 (DE3) pLysS including pET47b (+), into which the partial *ftsI* sequence of Rd or NUBL1772 was inserted, was grown overnight in medium containing 30 μg/ml kanamycin and 30 μg/ml chloramphenicol. The culture was added to 1 l of LB medium containing 100 μg/ml kanamycin and incubated at 37 °C. When the optical density reached 0.5 at an absorbance of 600 nm (OD_{A600}), production of PBP3 was induced by 1.0 mM isopropyl β-D-1-thiogalactopyranoside and cells were grown for 3 h. The cells were collected by centrifugation and resuspended in 50 mM HEPES (pH 7.5), 500 mM NaCl and 30 mM imidazole. Cell lysis was achieved by freezing and thawing twice and then sonication. The cell lysate was centrifuged at 30000 × *g* for 3 h at 4 °C and the supernatant was subjected to a HisTrap HP (GE Healthcare Japan, Tokyo, Japan). His-tagged protein was eluted with a linear increasing gradient of imidazole. The purity of eluted PBP3 proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and samples were stored at -20 °C.

PBP3 competitive binding assays

PBP3 competitive binding assays using Bocillin FL (Thermo Fisher Scientific, Waltham, MA) and carbapenems were performed as previously described with some modifications (21). Purified PBP3 of *H. influenzae* Rd or NUBL1772 was quantified by the bicinchoninate method

and was prepared to 500 ng in HEPES buffer (10 mM HEPES [pH 7.5] and 500 mM NaCl). Ampicillin, meropenem, doripenem, imipenem, or biapenem was mixed with the PBP3 at 37 °C for 10 min, which was followed by the addition of Bocillin FL for 30 min. Concerning final concentrations, ampicillin and biapenem were used at 0.06–2 μM, other carbapenems were used at 0.03–1 μM, and Bocillin FL was used at 5 μM. These reaction mixtures were boiled in SDS sample buffer to stop the reaction. Fluorescence-labeled PBP3 was quantified using an LAS 4010 (GE Healthcare Japan, Tokyo, Japan). Using Kaleida graph version 4.5 (Synergy Software, Reading, PA), the IC_{50} values of β-lactams for PBP3 were determined.

acrR sequence analysis

The *acrR* gene of *H. influenzae* Rd and NUBL1772, which encodes a regulatory component of the AcrAB efflux pump, was amplified by PCR with primer sets, as previously described (21).

qRT-PCR

H. influenzae Rd or NUBL1772 was grown to the logarithmic growth phase (0.3 at OD_{A600}). RNA was extracted using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). cDNA synthesis and PCR were performed using EXPRESS One-Step SYBR® GreenER™ Kits (Thermo Fisher Scientific, Waltham, MA) with the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA). *acrB* gene expression was calculated relative to the housekeeping gene, *gyrA* using the equation $2^{-\Delta\Delta Ct}$ (41). The *acrB* genes were amplified by PCR with a sense primer (5′-AGTGCGCCTAGTAGTTCGAC-3′) and a reverse primer (5′-GCTCCCACCTGAAGAAGAGG-3′). The *gyrA* genes were amplified by PCR using the primers gyrAfrw and gyrArev by Giufre et al (42).

Ethidium bromide efflux assays

Ethidium bromide (EB) efflux assays were performed according to the previously described procedure (43). To assess efflux pump activity, *H. influenzae* Rd and NUBL1772 were treated

with EB only. The fluorescence of accumulated EB was read using the Synergy™ H1 (BioTek, Winooski, VT). The initial velocity of EB accumulation from 5 to 15 min was calculated to quantify the efflux speed, as previously described (23).

MLST

MLST, using seven *H. influenzae* isolates with reduced carbapenem susceptibility, was performed by sequencing of seven housekeeping genes, specifically *adk, atpG, frdB, fucK, mdh, pgi,* and *recA*, as previously described (44). The ST was determined based on the *H. influenzae* MLST website (https://pubmlst.org/hinfluenzae/) at the University of Oxford (45). For cases in which a ST could not be assigned, new MLST profiles were submitted to the *H. influenzae* MLST website, which was followed by the assignment of a ST.

PFGE

PFGE using seven *H. influenzae* isolates with reduced carbapenem susceptibility was performed as described previously (46-48). For plug preparation, 1.2 % SeaKem® Gold Agarose (Lonza, Basel, Switzerland) was used. The plug was incubated with 1 mg/ml lysozyme (Merck, Darmstadt, Germany) and 0.5 mg/ml proteinase K (Wako Pure Chemical Industries, Osaka, Japan). Restriction enzyme digestion of DNA was performed using 40 U of SmaI. DNA fragments were electrophoresed using 1% pulse field certified agarose (Bio-Rad Laboratories, Hercules, CA) with a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA) for 20 h at 6 V/cm and 14 °C with a ramped pulse time of 5.3–34.9 s.

Growth kinetics

The growth kinetics of *H. influenzae* NUBL1772 and Rd recombinant carrying the whole *ftsI* gene of NUBL1772 were compared to those of *H. influenzae* Rd, as previously described with some modifications (30, 49). An overnight culture was suspended in 0.9 % saline at a McFarland standard of 1.0. The suspension was diluted 1000-fold in 5 ml HTM broth and measured every 1 h

for 24 h at OD_{A600} using an OD-Monitor C&T (Taitec, Koshigaya, Japan). Incubation was performed at 37 °C at ambient atmosphere with 180 rpm of shaking.

Statistical analysis

Each experiment was repeated three times independently. Unpaired t-tests were used for a specific contrast. Statistical significance was indicated by a P-value of <0.05. All statistical analyses were performed using IBM SPSS version 24 statistical software (IBM corp., Armonk, NY).

Accession numbers

Gen Bank/ENA/DDBJ accession numbers of nucleotide sequences are as follows: *ftsI* of NUBL1772, LC279277; *acrR* of NUBL1772, LC279278.

Acknowledgments

We thank all members of Professor Arakawa's laboratory for technical advice and discussion. We thank Meiji Seika Pharma Co, Ltd. for providing biapenem. This work was supported by a grant (H24-Shinkou-Ippan-010) from the Ministry of Health, Labour and Welfare of Japan, in part by a Grant-in-Aid for Scientific Research (C) from the Japan Society for the Promotion of Science and in part by the Research Program on Emerging/Re-emerging Infectious Disease Project of Japan of the Agency for Medical Research and Development, AMED. The funders had no role in this study design, data collection and interpretation, or the decision to submit the work for publication. The manuscript was edited by Editage, an English language editing company.

Reference

1. Hasegawa K, Kobayashi R, Takada E, Ono A, Chiba N, Morozumi M, Iwata S, Sunakawa

K, Ubukata K, Nationwide Surveillance for Bacterial M. 2006. High prevalence of type b beta-lactamase-non-producing ampicillin-resistant Haemophilus influenzae in meningitis: the situation in Japan where Hib vaccine has not been introduced. J Antimicrob Chemother 57:1077-82.

2. Jorgensen JH. 1992. Update on mechanisms and prevalence of antimicrobial resistance in Haemophilus influenzae. Clin Infect Dis 14:1119-23.

3. Sykes RB, Matthew M, O'Callaghan CH. 1975. R-factor mediated beta-lactamase production by Haemophilus influenzae. J Med Microbiol 8:437-41.

4. Mendelman PM, Chaffin DO, Stull TL, Rubens CE, Mack KD, Smith AL. 1984. Characterization of non-beta-lactamase-mediated ampicillin resistance in Haemophilus influenzae. Antimicrob Agents Chemother 26:235-44.

5. 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 45:1693-9.

6. Shiro H, Sato Y, Toyonaga Y, Hanaki H, Sunakawa K. 2015. Nationwide survey of the development of drug resistance in the pediatric field in 2000-2001, 2004, 2007, 2010, and 2012: evaluation of the changes in drug sensitivity of Haemophilus influenzae and patients' background factors. J Infect Chemother 21:247-56.

7. Cerquetti M, Giufre M, Cardines R, Mastrantonio P. 2007. First characterization of heterogeneous resistance to imipenem in invasive nontypeable Haemophilus influenzae isolates. Antimicrob Agents Chemother 51:3155-61.

8. 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 49:2834-9.

9. Skaare D, Anthonisen IL, Kahlmeter G, Matuschek E, Natas OB, Steinbakk M, Sundsfjord A, Kristiansen BE. 2014. Emergence of clonally related multidrug resistant Haemophilus influenzae with penicillin-binding protein 3-mediated resistance to extended-spectrum cephalosporins, Norway, 2006 to 2013. Euro Surveill 19.

10. Cherkaoui A, Diene SM, Renzoni A, Emonet S, Renzi G, Francois P, Schrenzel J. 2017. Imipenem heteroresistance in nontypeable Haemophilus influenzae is linked to a combination of altered PBP3, slow drug influx and direct efflux regulation. Clin Microbiol Infect 23:118 e9-118 e19.

11. Wajima T, Seyama S, Nakamura Y, Kashima C, Nakaminami H, Ushio M, Fujii T, Noguchi N. 2016. Prevalence of macrolide-non-susceptible isolates among beta-lactamase-negative ampicillin-resistant Haemophilus influenzae in a tertiary care hospital in Japan. J Glob Antimicrob Resist 6:22-26.

12. El-Gamal MI, Oh CH. 2010. Current status of carbapenem antibiotics. Curr Top Med Chem 10:1882-97.

13. Patel G, Bonomo RA. 2013. "Stormy waters ahead": global emergence of carbapenemases. Front Microbiol 4:48.

14. CLSI. 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard: ninth edition, M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.

15. Sanbongi Y, Suzuki T, Osaki Y, Senju N, Ida T, Ubukata K. 2006. Molecular evolution of beta-lactam-resistant Haemophilus influenzae: 9-year surveillance of penicillin-binding

protein 3 mutations in isolates from Japan. Antimicrob Agents Chemother 50:2487-92.
16. Contreras-Martel C, Dahout-Gonzalez C, Martins Ados S, Kotnik M, Dessen A. 2009. PBP active site flexibility as the key mechanism for beta-lactam resistance in pneumococci. J Mol Biol 387:899-909.
17. Contreras-Martel C, Job V, Di Guilmi AM, Vernet T, Dideberg O, Dessen A. 2006. Crystal structure of penicillin-binding protein 1a (PBP1a) reveals a mutational hotspot implicated in beta-lactam resistance in Streptococcus pneumoniae. J Mol Biol 355:684-96.
18. Job V, Carapito R, Vernet T, Dessen A, Zapun A. 2008. Common alterations in PBP1a from resistant Streptococcus pneumoniae decrease its reactivity toward beta-lactams: structural insights. J Biol Chem 283:4886-94.
19. Alm RA, Johnstone MR, Lahiri SD. 2015. Characterization of Escherichia coli NDM isolates with decreased susceptibility to aztreonam/avibactam: role of a novel insertion in PBP3. J Antimicrob Chemother 70:1420-8.
20. Powell AJ, Tomberg J, Deacon AM, Nicholas RA, Davies C. 2009. Crystal structures of penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of Neisseria gonorrhoeae reveal an unexpectedly subtle mechanism for antibiotic resistance. J Biol Chem 284:1202-12.
21. 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 48:1630-9.
22. Seyama S, Wajima T, Nakaminami H, Noguchi N. 2016. Clarithromycin Resistance Mechanisms of Epidemic beta-Lactamase-Nonproducing Ampicillin-Resistant Haemophilus influenzae Strains in Japan. Antimicrob Agents Chemother 60:3207-10.
23. Seyama S, Wajima T, Nakaminami H, Noguchi N. 2017. Amino Acid Substitution in the Major Multidrug Efflux Transporter Protein AcrB Contributes to Low Susceptibility to Azithromycin in Haemophilus influenzae. Antimicrob Agents Chemother 61.
24. Sanchez L, Pan W, Vinas M, Nikaido H. 1997. The acrAB homolog of Haemophilus influenzae codes for a functional multidrug efflux pump. J Bacteriol 179:6855-7.
25. 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 66:788-96.
26. 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.
27. 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 19:224-30.
28. Skaare D, Allum AG, Anthonisen IL, Jenkins A, Lia A, Strand L, Tveten Y, Kristiansen BE. 2010. Mutant ftsI genes in the emergence of penicillin-binding protein-mediated beta-lactam resistance in Haemophilus influenzae in Norway. Clin Microbiol Infect 16:1117-24.
29. 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.
30. Sondergaard A, Lund M, Norskov-Lauritsen N. 2015. TEM-1-encoding small plasmids impose dissimilar fitness costs on Haemophilus influenzae and Haemophilus parainfluenzae. Microbiology 161:2310-5.
31. 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 51:1589-95.
32. Lieberthal AS, Carroll AE, Chonmaitree T, Ganiats TG, Hoberman A, Jackson MA, Joffe MD, Miller DT, Rosenfeld RM, Sevilla XD, Schwartz RH, Thomas PA, Tunkel DE. 2013. The diagnosis and management of acute otitis media. Pediatrics 131:e964-99.
33. Wald ER, Applegate KE, Bordley C, Darrow DH, Glode MP, Marcy SM, Nelson CE, Rosenfeld RM, Shaikh N, Smith MJ, Williams PV, Weinberg ST, American Academy of P. 2013. Clinical practice guideline for the diagnosis and management of acute bacterial sinusitis in children aged 1 to 18 years. Pediatrics 132:e262-80.
34. Kitamura K, Iino Y, Kamide Y, Kudo F, Nakayama T, Suzuki K, Taiji H, Takahashi H, Yamanaka N, Uno Y. 2015. Clinical practice guidelines for the diagnosis and management of acute otitis media (AOM) in children in Japan - 2013 update. Auris Nasus Larynx 42:99-106.
35. Yamanaka N, Iino Y, Uno Y, Kudo F, Kurono Y, Suzaki H, Haruna S, Hotomi M, Horiguchi S, Mashima Y, Matsubara S, Nakayama T, Hirakawa K, Okamoto Y, Drafting Committee for Acute Rhinosinusitis Management Guideline JRS. 2015. Practical guideline for management of acute rhinosinusitis in Japan. Auris Nasus Larynx 42:1-7.
36. Seyama S, Wajima T, Yanagisawa Y, Nakaminami H, Ushio M, Fujii T, Noguchi N. 2017. Rise in Haemophilus influenzae With Reduced Quinolone Susceptibility and Development of a Simple Screening Method. Pediatr Infect Dis J 36:263-266.
37. Zhang Y, Zhang F, Wang H, Zhao C, Wang Z, Cao B, Du Y, Feng X, Hu Y, Hu B, Ji P, Liu Z, Liu Y, Liao W, Lu J, Sun H, Wang Z, Xu X, Xu X, Yang Q, Yu Y, Zhang R, Zhuo C. 2016. Antimicrobial susceptibility of Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis isolated from community-acquired respiratory tract infections in China: Results from the CARTIPS Antimicrobial Surveillance Program. J Glob Antimicrob Resist 5:36-41.
38. Fukasawa C, Hoshino T, Kutsuna S, Sawada K, Sato H, Ishiwada N. 2013. [Concentration of tazobactam/piperacillin in the cerebrospinal fluid of patients with Haemophilus influenzae type B meningitis]. Kansenshogaku Zasshi 87:590-5.
39. Falla TJ, Crook DW, Brophy LN, Maskell D, Kroll JS, Moxon ER. 1994. PCR for capsular typing of Haemophilus influenzae. J Clin Microbiol 32:2382-6.
40. Mitchell MA, Skowronek K, Kauc L, Goodgal SH. 1991. Electroporation of Haemophilus influenzae is effective for transformation of plasmid but not chromosomal DNA. Nucleic Acids Res 19:3625-8.
41. Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-8.
42. Giufre M, Carattoli A, Cardines R, Mastrantonio P, Cerquetti M. 2008. Variation in expression of HMW1 and HMW2 adhesins in invasive nontypeable Haemophilus influenzae isolates. BMC Microbiol 8:83.
43. Kamicker BJ, Sweeney MT, Kaczmarek F, Dib-Hajj F, Shang W, Crimin K, Duignan J, Gootz TD. 2008. Bacterial efflux pump inhibitors. Methods Mol Med 142:187-204.

44. Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, Popovic T, Spratt BG. 2003. Characterization of encapsulated and noncapsulated Haemophilus influenzae and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol 41:1623-36.
45. Jolley KA, Maiden MC. 2010. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics 11:595.
46. Curran R, Hardie KR, Towner KJ. 1994. Analysis by pulsed-field gel electrophoresis of insertion mutations in the transferrin-binding system of Haemophilus influenzae type b. J Med Microbiol 41:120-6.
47. Kakuta R, Yano H, Hidaka H, Kanamori H, Endo S, Ichimura S, Ogawa M, Shimojima M, Ozawa D, Inomata S, Tanouchi A, Kaku M, Katori Y. 2016. Molecular Epidemiology of Ampicillin-resistant Haemophilus influenzae Causing Acute Otitis Media in Japanese Infants and Young Children. Pediatr Infect Dis J 35:501-6.
48. Saito M, Umeda A, Yoshida S. 1999. Subtyping of Haemophilus influenzae strains by pulsed-field gel electrophoresis. J Clin Microbiol 37:2142-7.
49. San Millan A, Garcia-Cobos S, Escudero JA, Hidalgo L, Gutierrez B, Carrilero L, Campos J, Gonzalez-Zorn B. 2010. Haemophilus influenzae clinical isolates with plasmid pB1000 bearing blaROB-1: fitness cost and interspecies dissemination. Antimicrob Agents Chemother 54:1506-11.

Figure Legends

FIG 1 Deduced amino acid sequence of PBP3 from *H. influenzae* NUBL1772 compared to that of Rd. A dash symbol represents identical amino acids. Boxes highlight the three conserved motifs of the active site, specifically, Ser327-Thr-Val-Lys, Ser379-Ser-Asn, and Lys512-Thr-Gly. NUBL1772 was found to have S385T and L389F alterations. The number of amino acids in PBP3 from NUBL1772, having insertion V525_N526insM, was altered and is described in parentheses. The number of amino acids in PBP3 from NUBL1772, having deletion T591del, was restored.

Tables

TABLE 1 Characterization of seven *H. influenzae* isolates with reduced carbapenem susceptibility

Isolates[a]	Region	Biotype	Age	Sex	Source	MLST	PBP3 Group[b]	MIC (μg/ml)				
								Ampicillin	Meropenem	Doripenem	Imipenem	Biapenem
NUBL970	Kanagawa	II	5	M	Nose	1763	II	2	0.06	0.25	4	4
NUBL1767	Ibaragi	II	0	F	Nose	57	III+	8	0.12	4	4	8
NUBL1771	Kanagawa	V	1	M	Nose	1764	III+	8	1	4	4	8
NUBL1772	Kanagawa	II	1	M	Nose	57		16	1	4	16	16
NUBL1775	Saitama	II	7	F	Vagina	1765	III+	2	1	4	4	8
NUBL1777	Nagano	V	7	M	Nose	1766	III+	16	0.12	0.4	2	16
NUBL2814	Chiba	II	5	M	Nose	1683	II	2	0.25	1	4	8

[a] All isolates were nontypeable and β-lactamase negative.

[b] PBP3 group according to amino acid mutations, as follows: II, N526K; III+, S385T+ L389F+ N526K. NUBL1772 was not categorized and possessed a new insertion mutation.

MLST, multilocus sequence type.

564

TABLE 2 MICs for *H. influenzae* NUBL1772

Antimicrobial	MIC (μg/ml)	
	Rd	NUBL1772
Ampicillin	0.25	16
Ampicillin-sulbactam	0.25	16
Amoxicillin	0.5	32
Amoxicillin-clavulanic acid	0.5	32
Cefotaxime	0.06	1
Ceftriaxone	$\leq$ 0.03	0.25
Cefepime	$\leq$ 0.25	2
Piperacillin-tazobactam	$\leq$ 0.03	$\leq$ 0.03
Aztreonam	0.06	4
Meropenem	0.03	1
Doripenem	0.06	4
Imipenem	0.5	16
Biapenem	0.5	16
Clarithromycin	8	16
Ciprofloxacin	0.015	0.03
Levofloxacin	0.015	0.03

565

566

TABLE 3 MICs for Rd recombinants carrying *ftsI* genes related to NUBL1772

Strain/Isolate[a]	MIC (μg/ml)				
	Ampicillin	Meropenem	Doripenem	Imipenem	Biapenem
Rd	0.25	0.03	0.06	0.5	0.5
NUBL1772	16	1	4	16	16
Rd (NUBL1772 *ftsI*)	8	0.5	2	4	8
Rd (Rd *ftsI* V525_N526insM)	0.25	0.03	0.06	0.5	0.5
Rd (NUBL1772 *ftsI* M526 del)	2	0.12	0.5	1	2

[a] Rd recombinant contained the corresponding *ftsI* gene.

TABLE 4 Binding of Bocillin FL and β-lactams to recombinant PBP3 proteins

Antimicrobial	IC_{50} (μg/ml) ± SD		P-value
	Rd	NUBL1772	
Ampicillin	0.14 ± 0.03	5.13 ± 1.48	0.028
Meropenem	0.10 ± 0.02	0.15 ± 0.03	0.049
Doripenem	0.12 ± 0.05	0.73 ± 0.33	0.035
Imipenem	0.20 ± 0.15	3.22 ± 0.59	0.001
Biapenem	0.25 ± 0.08	5.86 ± 2.58	0.020

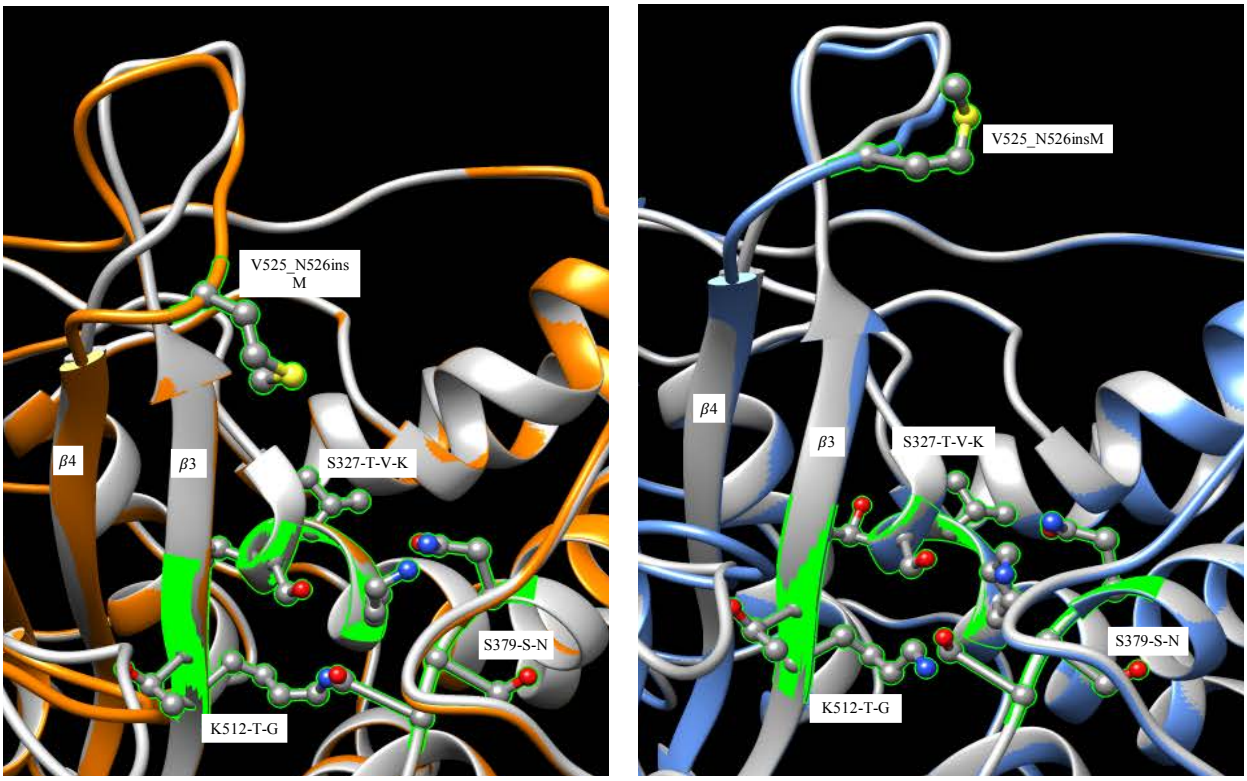
569

TABLE 5 Transcription of *acrB* and efflux pump activity in *H. influenzae* NUBL1772 compared to that in Rd

Strain/Isolate	Relative mRNA levels of *acrB*	P-value	Initial velocity of ethidum bromide accumulation	P-value
Rd	1.11 ± 0.65	0.038	1.63 ± 0.32	0.026
NUBL1772	4.80 ± 1.98		0.89 ± 0.18	

570

```
                10         20         30         40         50         60         70         80         90
Rd         MVKFNSSRKS GKSKKTIRKL TAPETVKQNK PQKVFEKCFM RGRYMLSTVL ILLGLCALVA RAAYVQSINA DTLSNEADKR SLRKDEVLSV
NUBL1772   ------P--- ---------- ---------- ---L------ ---------- -----G---- ---------- ----S----- ----------

       100        110        120        130        140        150        160        170        180        190
RGSILDRNGQ LLSVSVPMSA IVADPKTMLK ENSLADKERI AALAEELGMT ENDLVKKIEK NSKSGYLYLA RQVELSKANY IRRLKIKGII LETEHRRFYP
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- --K------- ----------

       200        210        220        230        240        250        260        270        280        290
RVEEAAHVVG YTDIDGNGIE GIEKSFNSLL VGKDGSRTVR KDKRGNIVAH ISDEKKYDAQ DVTLSIDEKL QSMVYREIKK AVSENNAESG TAVLVDVRTG
---------- ---------- ---------- ---------- --------E- ---------- ---------- ---------- ---------- ----------

       300        310        320        330        340        350        360        370        380        390
EVLAMATAPS YNPNNRVGVK SELMRNRAIT DTFEPGSTVK PFVVLTALQR GVVKRDEIID TTSFKLSGKE IVDVAPRAQQ TLDEILMNSS NRGVSRLALR
---------- ---------- ---------- ---------- ---------- ---------N ------N--- ---------- ------I--- ----T---F-

       400        410        420        430        440        450        460        470        480        490
MPPSALMETY QNAGLSKPTD LGLIGEQVGI LNANRKRWAD IERATVAYGY GITATPLQIA RAYATLGSFG VYRPLSITKV DPPVIGKRVF SEKITKDIVG
---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------

       500        510        520   529(530)   539(540)   549(550)   559(560)   569(570)   579(580)   589(590)
ILEKVAIKNK RAMVEGYRVG VKTGTARKIE NGHYV NKYV AFTAGIAPIS DPRYALVVLI NDPKAGEYYG GAVSAPVFSN IMGYALRANA IPQDAEAAEN
---------- -V-------- --------L- -----M--H- ---------- -------I-- -E-------- ---------S ---------G ------PT-K

       599        609
TTTKSAKRIV YIGEHKNQKV N
- M------- -V--R--E-M -
```



A B

FIG S1 Modeling of penicillin-binding protein (PBP) 3 structure. Modelling of the structure of PBP3 Rd and NUBL1772 was performed with the UCSF Chimera package including MODELLER (1, 2). Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311). The optimal homologous template was searched by HOMCOS (3) and the PBP3 protein from *Escherichia coli* (PDB: 4BJP) was used as a template. (A) PBP 3 of NUBL1772 (orange) superposed onto that of Rd (gray). The structure of the active site (S327-T-V-K, S379-SN and K512-T-G) was found to be unchanged. V525_N526insM N526 was found to be located on the loop between β3 and β4. The loop was lengthened and shifted toward the active site. (B) PBP3 of Rd *ftsI* with V525_N526insM (cornflower blue) superposed onto that of Rd (gray). The structure of the active site was found to be unchanged. V525_N526insM N526 was located on the loop between β3 and β4. The loop was slightly lengthened and shifted toward

the active site.

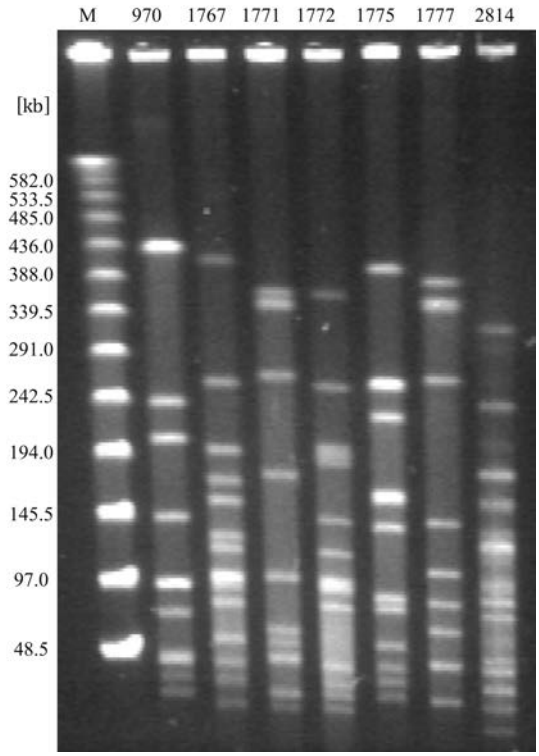


FIG S2 Pulsed-field gel electrophoresis (PFGE) band patterns of seven *H. influenzae* isolates with reduced carbapenem susceptibility. Numbers above the gel image show NUBL numbers of each isolate. M, CHEF DNA size standard Lambda Ladder.

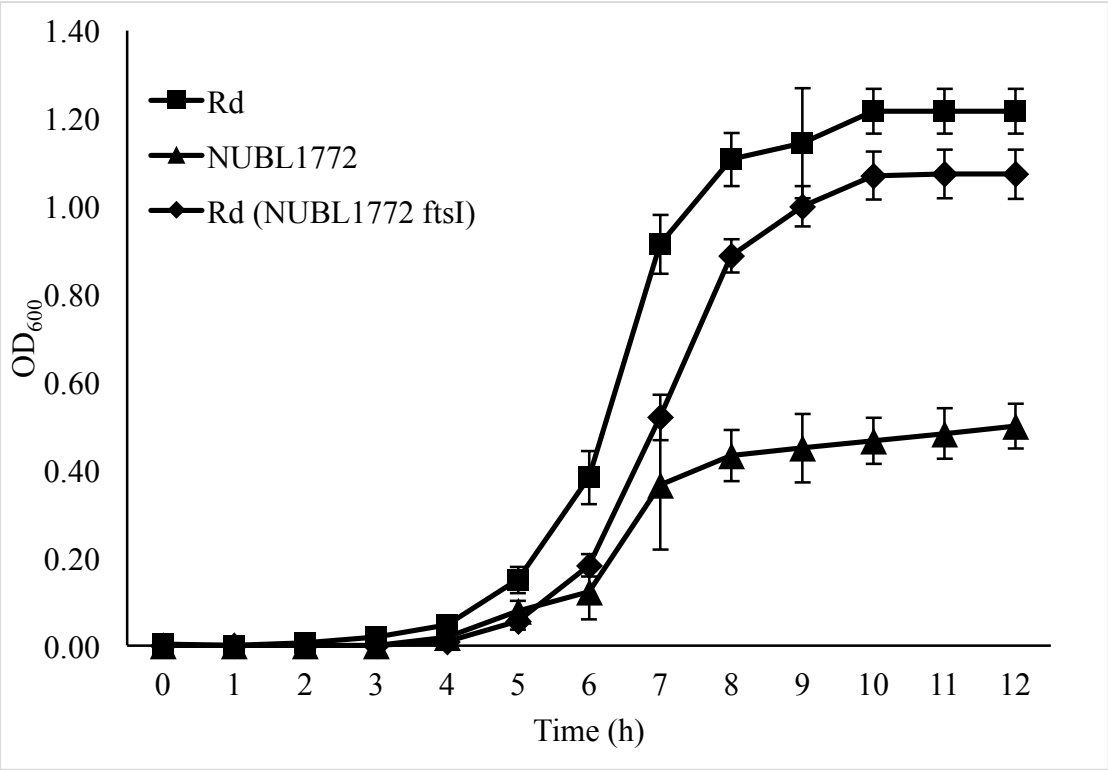


FIG S3 Growth curve comparison of *H. influenzae* Rd, NUBL1772 and Rd recombinant carrying the whole *ftsI* gene of NUBL1772. Error bars indicate the standard deviations of the means. The growth curves were determined by three independent experiments. OD_{600}, optical density at absorbance of 600 nm. Rd (NUBL1772 ftsI), Rd recombinant carrying the whole *ftsI* gene of NUBL1772.

TABLE S1 DNA primers used for cloning and mutagenesis with *ftsI*

Designation	Forward primer	Reverse primer
Cloning of Rd *ftsI* for recombination	CTAGCTAGCGACGATTTGGATAACCCATA	CGGGGTACCTTAATTCACTTTTTGATTCTTG
Cloning of NUBL1772 *ftsI* for recombination	CTAGCTAGCGACGATTTGGATAACCCATA	CGGGGTACCTTAATTCATTTTTTCATTCTTA
Introduction of V525_N526insM for Rd *ftsI*	ATGTAATGAATAAGTATGTGGCATTTACT	ACTTATTCATTACATAATGTCCATTTTCA
Introduction of M526 del for NUBL1772 *ftsI*	ATTATGTGAATAAACACGTTGCATTT	GTTTATTCACATAATGACCATTCTC
Cloning of KanR2 gene in pBAD18-Kan	CGGGGTACCAGCCACGTTGTGTCTCAAA	CGCGGATCCTTAGAAAAACTCATCGAGCATC
Cloning of *murE*	CGCGGATCCTATGGTGGTGCGGTTTCTG	CGCGTCGACTTATTTTAAGAAATCAAGTGC
Cloning of Rd *ftsI* for expression	TCCCCCGGGTCTATTAATGCCGATACG	CGCGGATCCTTAATTCACTTTTTGATTCTTG
Cloning of NUBL1772 *ftsI* for expression	TCCCCCGGGTCTATTAATGCGGATACG	CGCGGATCCTTAATTCATTTTTTCATTCTTACGC

Reference

1. **Webb B, Sali A.** 2014. Comparative Protein Structure Modeling Using MODELLER. Curr Protoc Bioinformatics **47:**5 6 1-32.
2. **Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE.** 2004. UCSF Chimera--a visualization system for exploratory research and analysis. J Comput Chem **25:**1605-1612.
3. **Kawabata T.** 2016. HOMCOS: an updated server to search and model complex 3D structures. J Struct Funct Genomics **17:**83-99.