



Carbapenem-Nonsusceptible *Haemophilus influenzae* with Penicillin-Binding Protein 3 Containing an Amino Acid Insertion

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ABSTRACT The prevalence of β -lactamase-negative ampicillin-resistant (BLNAR) *Haemophilus influenzae* has become a clinical concern. In BLNAR isolates, amino acid substitutions in penicillin-binding protein 3 (PBP3) are relevant to the β -lactam resistance. Carbapenem-nonsusceptible *H. influenzae* isolates have been rarely reported. Through antimicrobial susceptibility testing, nucleotide sequence analysis of *ftsI*, encoding PBP3, and the utilization of a collection of *H. influenzae* clinical isolates in our laboratory, we obtained a carbapenem-nonsusceptible clinical isolate (NUBL1772) that possesses 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 carbapenems. These results demonstrate that altered PBP3 containing V525_N526insM influences the reduced carbapenem susceptibility. The revertant mutant lacking the V525_N526insM exhibited lower MICs for carbapenems than NUBL1772, suggesting that this insertion affects reduced carbapenem susceptibility. The MICs of β -lactams for NUBL1772 were 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 the reduced susceptibility to carbapenems in *H. influenzae*.

KEYWORDS *Haemophilus influenzae*, carbapenems, penicillin-binding proteins

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 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 those for β -lactamase-producing ampicillin-resistant (BLPAR) and β -lactamase-negative ampicillin-resistant (BLNAR) isolates (2, 3), which can be attributed to a 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 a high frequency of 46.7% in 2012 (6).

Carbapenem-nonsusceptible *H. influenzae* isolates have been rarely reported. Al-

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

Isolate ^a	Region of source	Biotype	Patient information					MIC ($\mu\text{g/ml}$)				
			Age	Sex	Source	MLST ^b	PBP3 group ^c	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

^aAll isolates were nontypeable and β -lactamase negative.

^bMLST, multilocus sequence type.

^cPBP3 group according to amino acid mutations: II, N526K; III+, S385T, L389F, and N526K. NUBL1772 was not categorized and possessed a new insertion mutation.

though 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-nonsusceptible *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, on the basis of 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-nonsusceptible 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 of treatment for many bacterial infections (12, 13). Carbapenem-nonsusceptible *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 of $\geq 4 \mu\text{g/ml}$ or biapenem MIC of $\geq 8 \mu\text{g/ml}$). 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 patients' noses, except one isolate (from the vagina). All isolates were nontypeable and classified as biotype II (5/7) or V (2/7). All isolates were β -lactamase negative and nonsusceptible to ampicillin; five were BLNAR (ampicillin MIC, $\geq 4 \mu\text{g/ml}$). According to the Clinical and Laboratory Standards Institute (CLSI) breakpoints, only the NUBL1772 isolate showed nonsusceptibility 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 the V525_N526insM in PBP3 (9). The deduced amino acid sequence of PBP3 of NUBL1772 is aligned in Fig. 1.

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	≤0.03	0.25
Cefepime	≤0.25	2
Piperacillin-tazobactam	≤0.03	≤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

Transformation, antimicrobial susceptibility, and PBP3 competitive binding assay. Considerably elevated MICs of ampicillin and carbapenems were found for the recombinant strains harboring the entire *ftsI* gene of NUBL1772 compared to those for *H. influenzae* strain Rd (Table 3). On the basis of the competitive binding assay for the PBP3 proteins using Bocillin FL and ampicillin or carbapenems, the 50% inhibitory concentration (IC₅₀) values of ampicillin and carbapenems for NUBL1772 PBP3 were significantly higher than those for Rd PBP3, as summarized in Table 4. These findings suggest that altered PBP3 containing V525_N526insM influences the reduced carbapenem susceptibility.

The nucleotide sequence of *ftsI* and deduced amino acid sequences of PBP3 in NUBL1772 were identical to those of *H. influenzae* isolates 92 and 118 from the report by Wajima et al. (11). NUBL1772 showed antimicrobial susceptibilities that were similar

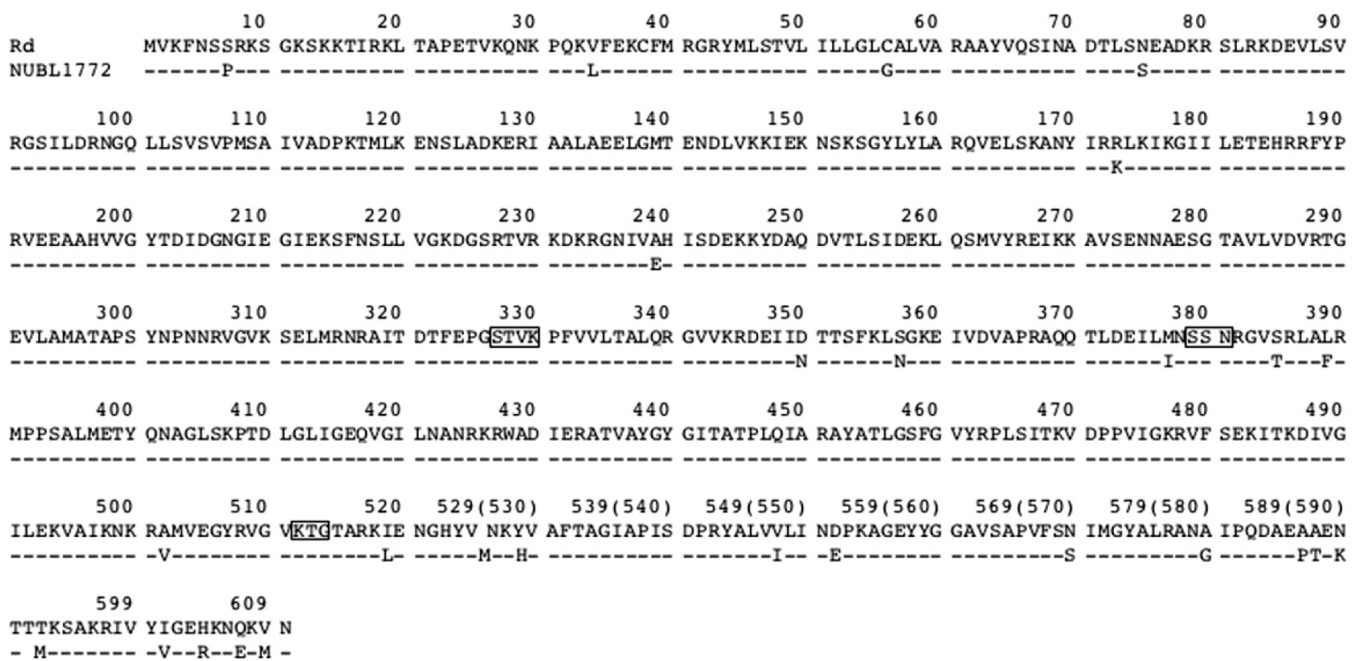


FIG 1 Deduced amino acid sequence of PBP3 from *H. influenzae* NUBL1772 compared to that of strain Rd. Dashes represent identical amino acids. Boxes highlight the three conserved motifs of the active site, specifically, Ser327-Thr-Val-Lys, Ser379-Ser-Asn, and Lys512-Thr-Gly. NUBL1772 was found to have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

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

Strain or isolate ^a	MIC ($\mu\text{g/ml}$)				
	Ampicillin	Meropenem	Doripenem	Imipenem	Biapenem
Rd	0.25	0.03	0.06	0.5	0.5
NUBL1772	16	1	4	16	16
Rd (NUBL1772 <i>ftsI</i>)	8	0.5	2	4	8
Rd (Rd <i>ftsI</i> V525_N526insM)	0.25	0.03	0.06	0.5	0.5
Rd (NUBL1772 <i>ftsI</i> M526del)	2	0.12	0.5	1	2

^aRd recombinants contained the corresponding *ftsI* genes.

to those of *H. influenzae* isolates 92 and 118 reported by Wajima et al. (11) within their description.

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

In terms of computer modeling, the effect of the altered PBP3 containing V525_N526insM on reduced susceptibility to carbapenems was investigated (see Fig. S1 in the supplemental material). The PBP3 protein of *Escherichia coli* (PDB accession 4BJP) was used as a template. N526 was located on the loop between $\beta 3$ and $\beta 4$. 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 $\beta 3$ and $\beta 4$, and this resulted in increased flexibility of the loop, which was found to be important for the generation of high-level β -lactam resistance (16–18). Similarly, the insertion in NUBL1772 might impart flexibility to the loop between $\beta 3$ and $\beta 4$, 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 the 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* has 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 ($\beta 2b$ – $\beta 2c$ in *E. coli*, $\beta 2a$ – $\beta 2d$ in *N. gonorrhoeae*, and $\beta 3$ – $\beta 4$ 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 $\beta 3$ and $\beta 4$, that the loop was slightly expanded and shifted toward the active

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

Antimicrobial	IC ₅₀ ($\mu\text{g/ml}$) \pm SD		P value
	Rd	NUBL1772	
Ampicillin	0.14 \pm 0.03	5.13 \pm 1.48	0.028
Meropenem	0.10 \pm 0.02	0.15 \pm 0.03	0.049
Doripenem	0.12 \pm 0.05	0.73 \pm 0.33	0.035
Imipenem	0.20 \pm 0.15	3.22 \pm 0.59	0.001
Biapenem	0.25 \pm 0.08	5.86 \pm 2.58	0.020

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

Strain or isolate	Relative mRNA level of <i>acrB</i>	<i>P</i> value	Initial velocity (fluorescence intensity/min) of ethidium bromide accumulation	<i>P</i> value
Rd	1.11 ± 0.65	0.038	1.63 ± 0.32	0.026
NUBL1772	4.80 ± 1.98		0.89 ± 0.18	

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 coexists 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 strain Rd. NUBL1772 had nucleotide deletions at position 442 to 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 fact that 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* in NUBL1772 was significantly elevated compared to that in strain 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 strain Rd, as shown in Table 5. These results suggest that the disruption of AcrR affects the reduced carbapenem susceptibility of NUBL1772.

Kaczmarek et al. (21) and Seyama et al. (22) reported some amino acid sequences that predict the early termination of AcrR in *H. influenzae*. 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 -lactams 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 strain Rd (Table 2) indicates that the resistance might be mediated by the disruption of AcrR. One limitation is that small molecules such as β -lactams appear to abolish the effect of any efflux system on the basis of the rapid influx through porin channels (24). Many studies concerning *acrR* sequence analysis in BLNAR isolates 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 rates and maximum cell densities of NUBL1772 and the strain Rd recombinant carrying the entire *ftsI* gene of NUBL1772 were lower than those of strain Rd (see 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 resistance in BLNAR isolates is dependent on clonal dissemination or horizontal gene transfer (25–31). When *H.*

influenzae isolates possessing the same *ftsI* genes 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. (11) 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 carbapenems can be considered the last-line treatment upon clinical failure (34, 35). NUBL1772 showed nonsusceptibility 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 the susceptibility to piperacillin-tazobactam was not well investigated, to the best of our knowledge, no piperacillin-tazobactam-resistant *H. influenzae* has been reported to date (6). The prevalence of fluoroquinolone-resistant *H. influenzae* remains 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 carbapenems are considered last-resort drugs, 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 the reduced susceptibility to carbapenems in *H. influenzae*, on the basis of 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 carbapenems 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 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 the bacteriological testing of them. According to the results of antimicrobial susceptibility testing, we selected seven isolates with reduced susceptibility to carbapenems (MIC of imipenem of ≥ 4 $\mu\text{g/ml}$ or MIC of biapenem of ≥ 8 $\mu\text{g/ml}$) for further investigation. The 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). The production of β -lactamase was tested using Cefinase disks (Becton Dickinson and Company, Franklin Lakes, NJ), and the 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 broth (Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 10 $\mu\text{g/ml}$ β -NAD (Nacalai Tesque, Kyoto, Japan) and 10 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$ β -NAD, 15 $\mu\text{g/ml}$ hemin, and 5 g/liter Bacto yeast extract (Becton, Dickinson and Company, Franklin Lakes, NJ) was typically used. Chocolate II agar (Becton, Dickinson and Company, Franklin Lakes, NJ) or

HTM agar was used as the agar for bacterial growth. Cultures were incubated at 37°C with 5% CO₂ for 20 to 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 *ftsI*_{frw} and *ftsI*_{rev} 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 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, the grouping proposed by Skaare et al. was determined to be more relevant to reduced carbapenem susceptibility (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, and N526K), group III-like (S385T, R517H), and group III-like+ (S385T, L389F, and 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 in the supplemental material. 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 *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), pBAD18-Cm containing Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526del was constructed. Colonies of recombinants containing Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526del 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 Sall sites. These PCR products of *KanR2* and *murE* were inserted sequentially into the KpnI-Sall sites of pBAD18-Cm containing Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526del.

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 the recombinant construction or *E. coli* Rosetta 2 (DE3) pLysS (Merck, Darmstadt, Germany) for the 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 *ftsI*_{frw} and *ftsI*_{rev}, as described by Cerquetti et al. (7), from NUBL1772 chromosomal DNA. Rd *ftsI* with V525_N526insM or NUBL1772 *ftsI* M526del was amplified using *ftsI*-*KanR2*-*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* M526del. 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 strain 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 liter of LB medium containing 100 μg/ml kanamycin and incubated at 37°C. When the optical density at 600 nm (OD₆₀₀) reached 0.5, the production of PBP3 was induced by adding 1.0 mM isopropyl β-D-1-thiogalactopyranoside, and the 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 sonicating. The cell lysate was centrifuged at 30,000 × 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 bicinchoninic acid 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. The final concentrations of ampicillin and biapenem were 0.06 to 2 μM; other carbapenems were used at 0.03 to 1 μM, and Bocillin FL was used at 5 μM. These reaction mixtures were boiled in SDS sample buffer to stop the reactions. Fluorescent-labeled PBP3 was quantified using an LAS 4010 (GE Healthcare Japan, Tokyo, Japan). Using Kaleida graph version 4.5 (Synergy Software, Reading, PA), the IC₅₀s of β-lactams for PBP3 were determined.

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

Reverse transcription-quantitative PCR. *H. influenzae* Rd or NUBL1772 was grown to the logarithmic growth phase (OD₆₀₀ of 0.3). RNA was extracted by using a 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 that of the housekeeping gene, *gyrA*, using the 2^{-ΔΔCT} method (41). The *acrB* genes were amplified by PCR with a sense primer (5'-AGTGCCTAGTAGTTCGAC-3') and a reverse primer (5'-GCTCCACCTGAAGAAGAGG-3'). The *gyrA* genes were amplified by PCR using the primers *gyrA*fw and *gyrA*rev described by Giufre et al. (42).

Ethidium bromide efflux assays. Ethidium bromide (EB) efflux assays were performed according to a 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 a 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 seven housekeeping genes, specifically, *adk*, *atpG*, *frdB*, *fuck*, *mdh*, *pgi*, and *recA*, as previously described (44). The ST was determined on the basis of the *H. influenzae* MLST website (<https://pubmlst.org/hinfluenzae/>) at the University of Oxford (45). For cases in which an 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 to 34.9 s.

Growth kinetics. The growth kinetics of *H. influenzae* NUBL1772 and the 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 1,000-fold in 5 ml HTM broth and measured every 1 h for 24 h at an OD₆₀₀ using an OD-Monitor C&T (Taitec, Koshigaya, Japan). Incubation was performed at 37°C at ambient atmosphere with shaking at 180 rpm.

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 number(s). The GenBank accession numbers for nucleotide sequences from NUBL1772 are as follows: *ftsI*, [LC279277](https://pubmlst.org/hinfluenzae/); *acrR*, [LC279278](https://pubmlst.org/hinfluenzae/).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00671-18>.

SUPPLEMENTARY FILE 1, PDF file, 7.1 MB.

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