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First Characterization of Heterogeneous Resistance to Imipenem in Invasive Nontypeable *Haemophilus influenzae* Isolates▽

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This study describes the first two reported invasive nontypeable *Haemophilus influenzae* (NTHI) isolates (strains 183 and 184) with heterogeneous resistance to imipenem. For both isolates, Etest showed imipenem MICs of ≥32 μg/ml. When the two strains were examined by the quantitative method of population analysis, both strain populations were heterogeneously resistant to imipenem and contained subpopulations growing in the presence of up to 32 μg of imipenem/ml at frequencies of 1.7×10^{-5} and 1.5×10^{-7}, respectively. By pulsed-field gel electrophoresis analysis, the two isolates appeared to be genetically closely related. The sequencing of the *ftsI* gene encoding penicillin-binding protein 3 (PBP 3) and comparison with the sequence of the imipenem-susceptible *H. influenzae* strain Rd identified a pattern of six amino acid substitutions shared between strains 183 and 184; an additional change was unique to strain 183. No relationship between mutations in the *dacB* gene encoding PBP 4 and imipenem resistance was found. The replacement of the *ftsI* gene in the imipenem-susceptible strain Rd (for which the MIC of imipenem is 0.38 to 1 μg/ml) with *ftsI* from strain 183 resulted in a transformant for which the MIC of imipenem ranged from 4 to 8 μg/ml as determined by Etest. The Rd/183 transformant population showed heterogeneous resistance to imipenem; it contained subpopulations growing in the presence of up to 32 μg of imipenem/ml at a frequency of 3.3×10^{-8}. The presence of additional resistance mechanisms, such as the overexpression of the AcrAB efflux pump, was investigated and does not seem to be involved. These data indicate that the heterogeneous imipenem resistance phenotype of our NTHI clone depends largely on the PBP 3 amino acid substitutions. We speculated that bacterial regulatory networks may play a role in the control of the heterogeneous expression of the resistance phenotype.

Despite the decrease in the incidence of invasive *Haemophilus influenzae* type b (Hib) disease as a result of the widespread use of Hib conjugate vaccines, *H. influenzae* remains a significant cause of invasive disease among adults, especially patients with underlying predisposing conditions (26). In adults, invasive *H. influenzae* infections, such as meningitis and septicemia, have been associated mainly with nontypeable *H. influenzae* (NTHI) (3, 5). The treatment of these life-threatening infections can be severely affected by antibiotic resistance. For years, ampicillin (alone or in combination with chloramphenicol) was regarded as the drug of choice in the treatment of invasive *H. influenzae* disease, but since the emergence of ampicillin-resistant isolates, expanded-spectrum cephalosporins have been extensively used. Carbapenems are considered a useful alternative to expanded-spectrum cephalosporins in treating invasive infections caused by ampicillin-resistant *H. influenzae* isolates (4). Until now, carbapenems have maintained a high level of activity against *H. influenzae.* Rare imipenem- or meropenem-resistant *H. influenzae* isolates have been observed in Japan, Korea, and the United States, mainly among β-lactamase-negative ampicillin-resistant isolates (1, 15, 24, 27). To our knowledge, no imipenem-resistant *H. influenzae* strains in Europe have been described. Some specific amino acid substitutions in penicillin-binding protein 3 (PBP 3), such as the N526K substitution, seem to be involved in imipenem resistance, according to the results of a site-directed mutagenesis study (21). In this study, we report the detection of two NTHI strains, isolated from patients with meningitis, that showed heterogeneous resistance to imipenem. To investigate the molecular bases of resistance, the sequences of the *ftsI* and *dacB* genes encoding PBP 3 and PBP 4, respectively, were determined. A new pattern of amino acid substitutions in PBP 3 associated with the imipenem resistance phenotype was revealed. The construction of a recombinant strain by the transformation of the imipenem-susceptible *H. influenzae* strain Rd with the *ftsI* gene coding for these PBP 3 amino acid substitutions resulted in a transformant exhibiting the heterogeneous imipenem resistance phenotype, as assessed by the quantitative method of population analysis. To investigate whether the overexpression of efflux pumps may contribute to the imipenem resistance in the NTHI isolates described herein, the effect of a broad-spectrum efflux pump inhibitor on the imipenem MIC, as well as the sequence of the *acrR* regulatory gene of the AcrAB efflux pump, was determined.

MATERIALS AND METHODS

Bacterial strains and patients. The NTHI strains 183 and 184 examined in this study were detected among 40 invasive *H. influenzae* strains collected in Italy from January 2004 to December 2006 and routinely tested for antimicrobial susceptibility. For both strains 183 and 184, the MIC of imipenem was >4 μg/ml, corresponding to the imipenem breakpoint according to the Clinical and Labo-

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ratory Standards Institute (CLSI) criteria (8). The two strains had been isolated between February and May 2005 from the cerebrospinal fluids of two patients with meningitis admitted to different hospitals in different towns in the region of Lombardy, northwestern Italy. The first patient (from whom strain 183 was obtained) was a 66-year-old man who had no demonstrable underlying conditions. The second patient (from whom strain 184 was isolated) was a 67-year-old woman with diabetes mellitus, heart disease, and chronic renal failure. Both patients received ceftriaxone for 12 to 14 days, and both fully recovered.

Four invasive NTHI imipenem-susceptible (MIC, 4 μg/ml) strains (187, 244, 248, and 251) isolated between October 2004 and July 2005 were also included in the study as controls. Bacteria were grown on chocolate agar plates supplemented with Vitox (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) at 37°C with 5% CO_2. All isolates were identified as nonencapsulated, i.e., nontypeable by PCR capsular genotyping, by following procedures reported previously (5). *H. influenzae* strain Rd (ATCC 51907) was used as the recipient strain for genetic transformation.

Antimicrobial susceptibility testing. MICs were determined by Etest (AB Biodisk, Solna, Sweden) using *Haemophilus* test medium (HTM) agar plates according to CLSI guidelines (8). *H. influenzae* ATCC 49247 and *H. influenzae* ATCC 10211 were used as quality controls. The interpretative breakpoints were based on CLSI criteria (8). For strains 183 and 184, the MICs of imipenem were also determined by the standard broth dilution method according to the CLSI procedure (8). β-lactamase activity was checked by the nitrocefin identification stick test (Oxoid Ltd., Basingstoke, Hampshire, United Kingdom).

An analysis of imipenem-resistant subpopulations of strains 183, 184, and Rd and the Rd/183 transformant was carried out by the population analysis method according to procedures described previously (28). Briefly, 10-fold serial dilutions of a starting suspension of bacteria ($\geq 10^9$ CFU/ml) were plated onto a set of HTM agar plates containing increasing concentrations of imipenem (concentration range, 0.0312 to 64 μg/ml) and onto antibiotic-free HTM agar plates. After incubation at 37°C with 5% CO_2 for 48 h, bacterial colonies were counted. Numbers of resistant bacteria were plotted against drug concentrations, providing a graphic display of the composition of each bacterial population in relation to the homogeneity or heterogeneity of the imipenem susceptibility phenotype.

PFGE. The genetic relationship between strains 183 and 184 was assessed by pulsed-field gel electrophoresis (PFGE) after the digestion of DNA with the SmaI restriction enzyme (Roche Diagnostic, Mannheim, Germany) by following procedures described previously (5).

Sequencing of the PBP genes. Alterations in PBPs of strains 183, 184, 187, 244, 248, and 251 were investigated by sequencing the *ftsI* gene encoding PBP 3 and the *dacB* gene encoding PBP 4. The *ftsI* gene was amplified by PCR using primers ftsIfrw (5′-GACGATTTGGATAACCCATA-3′; positions 1197620 to 1197639 of the *H. influenzae* strain Rd sequence [GenBank accession no. NC_000907]) and ftsIrev (5′-CTGGATAATTCTGTCTCAGA-3′; positions 1199884 to 1199865). The forward primer ftsIfrw recognized a sequence at the 3′ end of the *ftsL* gene, while primer ftsIrev annealed to a sequence at the 5′ end of the *murE* gene. To amplify the *dacB* gene, the following primer set was employed: dacBfrw (5′-TGCGACAAACAGTTCAATGAG-3′; positions 1407347 to 1407367 of the *H. influenzae* strain Rd sequence) and dacBrev (5′-TCGGGGCTTATTATTGTTCG-3′; positions 1408951 to 1408932). Both amplification reactions were performed using 1.5 U of Ex-*Taq* (Takara Bio Inc., Shiga, Japan), and PCR conditions were as follows: 30 cycles at 95°C for 1 min, 52°C for 1 min, and 72°C for 4 min, followed by a final elongation step of 72°C for 10 min. The PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Hilden, Germany). For all isolates, the sequencing of the *ftsI* gene portion encoding the transpeptidase region of PBP 3 and that of the *dacB* gene were performed with both strands by using the fluorescent dideoxy-chain terminator method with an ABI 3730 DNA sequencer (Applied Biosystems, CA). For strain 183, the sequencing of the *ftsI* gene was also carried out with the full-length gene plus upstream and downstream DNA segments.

Transformation. The *ftsI* gene amplified by PCR from NTHI strain 183 colonies grown on medium containing 32 μg of imipenem/ml was transferred into the *H. influenzae* strain Rd by electroporation as previously described (31). Considering the positions of primer set ftsIfrw/ftsIrev, the *ftsI* amplicon included the full-length *ftsI* gene plus upstream and downstream DNA segments. Briefly, 5 μl of the purified PCR product of the *ftsI* gene was added to 50 μl of *H. influenzae* Rd competent cells. Cells were then electroporated, immediately mixed with 1 ml of brain hearth infusion broth (Oxoid Ltd.) supplemented with HTM supplement (Oxoid Ltd.), and incubated at 37°C for 1.5 h. Transformants were selected on HTM agar plates containing 4 μg of imipenem/ml. To confirm *ftsI* gene transfer, PBP 3 mutations were checked by the DNA sequencing of several transformants.

TABLE 1. Antimicrobial susceptibilities of the six invasive NTHI isolates used in this study as determined by Etest

Strain	MIC (μg/ml) of:			
	Imipenem	Ampicillin	Chloramphenicol	Cefotaxime
183	≥32	0.75	0.38	0.047
184	≥32	0.5	0.5	0.125
187	1.5	0.19	0.38	0.047
244	0.5	0.25	0.38	0.016
248	0.25	0.125	0.38	0.016
251	0.5	0.125	0.25	0.016

Effect of CCCP on imipenem MICs. The effect of the broad-spectrum efflux pump inhibitor carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) on imipenem susceptibility was determined by following the procedures described previously, with minor modifications (19). CCCP (Sigma Chemical Co., St. Louis, MO) was diluted in dimethyl sulfoxide at a concentration of 100 mM and added to HTM agar to result in final concentrations of 3.12, 1.56, 0.781, and 0.390 μM CCCP. The plates were then used to perform Etest for the determination of imipenem MICs for NTHI strains 183 and 184.

Sequencing of the *acrR* gene. The *acrR* regulatory gene of the AcrAB efflux pump was amplified by using primers and PCR conditions described previously (14). The PCR product was purified and subjected to DNA sequencing.

Nucleotide sequence accession numbers. All sequences determined in this study have been submitted to the EMBL Nucleotide Sequence Database. The accession numbers are AM490810 through AM490815 for the *ftsI* gene sequences, AM490803 through AM490809 for the *dacB* gene sequences, and AM490797 through AM490802 for the *acrR* gene sequences.

RESULTS

Antimicrobial susceptibility. Table 1 shows the Etest results for the six NTHI strains evaluated in this study for susceptibility to the four classes of antimicrobial agents usually tested for isolates from cerebrospinal fluid. Both strains 183 and 184 appeared to be susceptible to ampicillin, chloramphenicol, and cefotaxime but resistant to imipenem. The imipenem MICs were ≥32 μg/ml since isolated colonies were present in the inhibition ellipses at up to 32 μg/ml. The imipenem MICs for the same isolates as determined by the standard broth dilution method were considerably lower: 2 μg/ml for strain 183 and 1 μg/ml for strain 184. All the imipenem-susceptible strains, strains 187, 244, 248, and 251, were also susceptible to ampicillin, chloramphenicol, and cefotaxime. No strain was found to produce β-lactamase.

Genetic relatedness between NTHI strains 183 and 184. By PFGE analysis, strains 183 and 184 appeared to be genetically closely related to each other, since their patterns differed by a single restriction fragment only (data not shown).

Amino acid substitutions in PBP 3. The nucleotide sequences of the *ftsI* genes of NTHI strains 183, 184, 187, 244, 248, and 251 were determined. The deduced amino acid sequences of the transpeptidase region of PBP 3 (amino acids 311 to 570) were aligned in comparison with the corresponding sequence from *H. influenzae* strain Rd. As shown in Table 2, six amino acid substitutions were shared between strains 183 and 184; an additional substitution was unique to strain 183. Among the four imipenem-susceptible isolates, only one (strain 244) showed a single amino acid substitution (L456V).

Amino acid substitutions in PBP 4. An analysis of the deduced amino acid sequences of PBP 4, encoded by the *dacB* gene, from NTHI strains 183, 184, 187, 244, 248, and 251

TABLE 2. Deduced amino acid substitutions in the transpeptidase region of PBP 3 from the six invasive NTHI isolates

Strain	MIC (μg/ml) of imipenem	PBP 3 transpeptidase region amino acid substitution[a]							
		D350N	M377I	L456V	G490E	A502V	N526K	V547I	N569S
183	≥32	*	*		*	*	*	*	*
184	≥32	*	*			*	*	*	*
187	1.5								
244	0.5			*					
248	0.25								
251	0.5								

[a] Substitutions were determined in reference to the PBP 3 sequence from strain Rd. Asterisks indicate the presence of the given substitution.

revealed considerable polymorphism in comparison with the *H. influenzae* strain Rd sequence, irrespective of the imipenem susceptibility phenotype (Table 3). Seven and four amino acid substitutions in strains 183 and 184, respectively, were found, but only two of these (D84G and E262A) were common to both strains. Moreover, the above-mentioned D84G and E262A substitutions were also found in all four imipenem-susceptible strains. A total of six substitutions in strains 244 and 251 were identified, whereas four substitutions were present in strains 187 and 248. Finally, the sequence of the control strain ATCC 10211, for which the MIC of imipenem was 1.5 μg/ml, had nine substitutions compared to the Rd sequence (Table 3).

Sequencing of the full-length *ftsI* gene from strain 183. Before the transformation of the *H. influenzae* Rd strain with the *ftsI* gene from strain 183, to verify whether the *ftsI* gene sequence of colonies of the original clinical isolate 183 grown on imipenem-free medium was identical to that of colonies of the same strain capable of growing on medium containing 32 μg of imipenem/ml, DNA segments obtained from both types of colonies were used as templates in two separate PCRs for the amplification of the *ftsI* gene. The sequencing of the resulting PCR products included the full-length *ftsI* gene plus upstream and downstream DNA. An overall identity of 100% between the two nucleotide sequences was found, irrespective of the DNA template used. Further analysis of the *ftsI* gene sequence from strain 183 in comparison with that from *H. influenzae* strain Rd showed the presence of several point mutations along the full length of the gene, even outside the transpeptidase domain region, although no mutations generating stop codons were found (data not shown). A 166-bp region upstream of the *ftsI* start codon identical to the homologous region of strain Rd (including the presence of a TAATA sequence reminiscent of the −10 consensus sequence), with the exception of one nucleotide (A was replaced with G in strain 183) immediately upstream the ATG codon, was found. No changes in the nucleotide sequence of a 100-bp region downstream of the stop codon of the *ftsI* gene were detected.

Transformation and susceptibility to imipenem. The replacement of the *ftsI* gene in *H. influenzae* Rd with the *ftsI* gene from strain 183 resulted in the selection of a transformant for which the MIC of imipenem ranged from 4 to 8 μg/ml on three different test days as determined by Etest, with isolated colonies present in the inhibition ellipses with concentrations up to these levels (Table 4). A sequence analysis of the *ftsI* gene from the Rd/183 transformant confirmed the presence of the same mutations as those in the parent strain 183.

Table 4 shows MICs of β-lactam antibiotics for the strains Rd and 183 and the Rd/183 transformant. Interestingly, the ampicillin MIC for the Rd/183 transformant increased in comparison with that for the parent strain Rd, almost reaching the ampicillin MIC for strain 183. No significant variations in the MICs of expanded-spectrum cephalosporins were observed.

Heterogeneity of the imipenem resistance phenotype. The imipenem susceptibility profiles of the NTHI strains 183 and 184, *H. influenzae* Rd, and the Rd/183 transformant were examined by the quantitative method of population analysis (Fig. 1). Both strains 183 and 184 showed heterogeneous resistance phenotypes, although strain 183 appeared to be more resistant at high concentrations of the drug. Actually, subpopulations of cells capable of growing on medium containing up to 32 μg of imipenem/ml were present at a frequency of 1.7×10^{-5} in the culture of strain 183 and 1.5×10^{-7} in the culture of strain 184. To investigate whether colonies of cells growing in the presence of high concentrations of imipenem resulted in populations with a phenotype of homogeneous or heterogeneous resistance, a colony of strain 183 was picked from HTM agar containing 16 μg of imipenem/ml and passaged in imipenem-free HTM agar. After overnight growth, this culture, which was named NTHI 183/16, was used as a starting cell suspension for further population analysis. As shown in Fig. 1, the culture of NTHI 183/16 remained heterogeneously resistant, although a small increase in the frequencies of subpopulations of cells resistant to imipenem concentrations of 16 and 32 μg/ml in comparison with the frequencies of such subpopulations of the progenitor strain 183 was observed (the frequency of strain 183/16 subpopulations resistant to 16 μg/ml was 1.3×10^{-3}, versus 7×10^{-4} for strain 183; the frequency of strain 183/16

TABLE 3. Deduced amino acid substitutions in PBP 4 from the six invasive NTHI isolates

Strain	MIC (μg/ml) of imipenem	PBP 4 amino acid substitution[a]											
		V43A	F45V	D58N	S67P	D84G	N101S	N107H	R111S	A214V	E262A	Q270K	K479N
183	>32	*	*		*	*			*		*	*	
184	>32					*	*	*			*		
187	1.5					*	*	*			*		
244	0.5	*	*	*	*	*					*		
248	0.25					*	*	*			*		
251	0.5	*	*	*	*	*					*		
ATCC 10211	1.5	*	*	*	*	*			*	*	*		*

[a] Substitutions were determined in reference to the PBP 4 sequence from strain Rd. Asterisks indicate the presence of the given substitution.

TABLE 4. Susceptibilities of strains 183 and Rd and the Rd/183 transformant to β-lactam antibiotics as determined by Etest

Strain	MIC (μg/ml) of:				Frequency of subpopulations capable of growing in the presence of the indicated imipenem concn (μg/ml)[b]		
	Imipenem[a]	Ampicillin[a]	Cefotaxime	Ceftriaxone	8	16	32
Rd	0.38–1	0.064–0.094	0.50	<0.016	$\leq 10^{-9}$	$\leq 10^{-9}$	$\leq 10^{-9}$
183	≥32	0.5–0.75	0.047	0.032	2.5×10^{-3}	7.0×10^{-4}	1.7×10^{-5}
Rd/183 transformant[c]	4–8	0.38–0.50	0.50	<0.016	2.7×10^{-4}	7.9×10^{-6}	3.3×10^{-8}

[a] MICs of imipenem and ampicillin were tested in triplicate on separate days. The values correspond to the MIC ranges.
[b] Frequencies were determined by population analysis.
[c] The source of the *ftsI* gene was strain 183.

subpopulations resistant to 32 μg/ml was 7.9×10^{-5}, versus 1.7×10^{-5} for strain 183). Notably, we checked the sequence of the full-length *ftsI* gene from the subpopulation capable of growing in the presence of 16 μg of imipenem/ml, and it was 100% identical to that previously found for both the original clinical isolate and colonies growing at 32 μg/ml.

No subpopulations of imipenem-resistant cells in the culture of strain Rd were detected since no growth in the presence of concentrations of ≥4 μg/ml was observed. The transfer of the *ftsI* gene from strain 183 to Rd conferred heterogeneous resistance to imipenem on the latter, although subpopulations of resistant cells were present at lower frequencies than those of the donor strain (Table 4).

Efflux pumps. To investigate whether efflux pumps were involved in the imipenem resistance of strains 183 and 184, the effect of the broad-spectrum efflux pump inhibitor CCCP on the imipenem MICs was tested. The highest concentration of CCCP that did not affect cell growth was 1.56 μM. Neither this nor lower concentrations (0.781 and 0.390 μM) appeared to have an influence on the imipenem MICs for both strains 183 and 184.

The nucleotide sequencing of the *acrR* genes of the AcrAB efflux pumps from strains 183, 184, 187, 244, 248, and 251 did not reveal the presence of insertions, deletions, or mutations generating frameshifts or stop codons in any isolate. Several substitutions in the deduced amino acid sequences of AcrR in

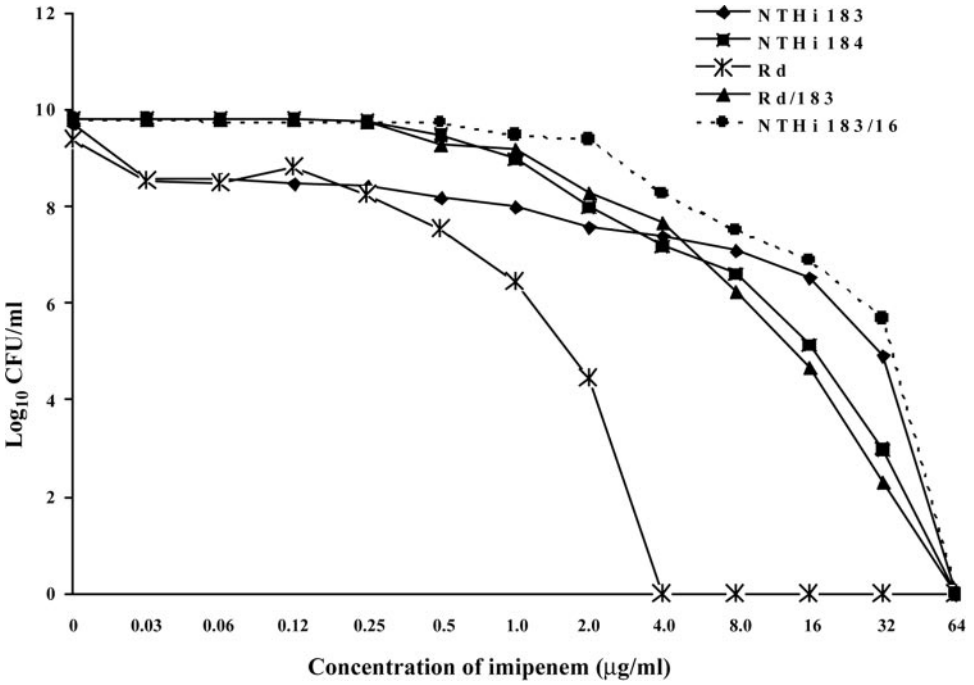

FIG. 1. Susceptibility of NTHI strains 183, 184, and Rd and the Rd/183 transformant to imipenem as demonstrated by population analysis. Tenfold serial dilutions of a starting suspension of bacteria (≥10^9 CFU/ml) were plated onto agar containing imipenem within the concentration range of 0.0312 to 64 μg/ml. After incubation at 37°C with 5% CO_2 for 48 h, bacterial colonies were counted. Strains 183 and 184 and the Rd/183 transformant demonstrated heterogeneous resistance, that is, the bacterial populations were composed of subpopulations of cells with various levels of resistance to imipenem. A colony of strain 183 capable of growing on agar containing 16 μg of imipenem/ml was picked and passaged in imipenem-free HTM agar. After overnight growth, this culture, named NTHI 183/16, was used in a starting cell suspension for population analysis, and it also showed a heterogeneous resistance phenotype. On the contrary, no subpopulations of imipenem-resistant cells capable of growing in the presence of concentrations of ≥4 μg/ml were detected among the strain Rd population. For each strain, the number of CFU per milliliter shown for each imipenem concentration is the mean of results from two experiments.

comparison with that from strain Rd were identified in all isolates, irrespective of the level of susceptibility to imipenem (data not shown). Strains 183 and 184 had nine common substitutions, but none were unique to one of the two imipenem-resistant isolates.

DISCUSSION

Heterogeneous resistance to imipenem in *H. influenzae* has not been described previously. *H. influenzae* clinical isolates with imipenem resistance have been reported very rarely, and this low frequency may be related to the heterogeneous expression of the resistance. In this study, we were able to detect two NTHI isolates showing this resistance phenotype by using Etest, probably as result of the high bacterial inoculum (3×10^8 to 4×10^8 CFU/ml) provided by this method. The presence of resistant subpopulations in the cultures of strains 183 and 184 was then confirmed by population analyses demonstrating low frequencies of bacteria able to grow in medium containing high concentrations of imipenem. Actually, when the two isolates were analyzed for susceptibility to imipenem by the standard broth dilution method based on a lower inoculum (5×10^5 CFU/ml), both appeared to be susceptible, suggesting that such resistant bacteria may not be detectable by the routine methods used in clinical microbiology laboratories.

Several previous studies have demonstrated that NTHI strains are characterized by a high degree of genetic diversity (5, 11, 18). In spite of this heterogeneity, strains 183 and 184 appeared to be genetically closely related to each other as assessed by PFGE, indicating that an NTHI clone with heterogeneous resistance to imipenem circulated and may still be circulating in different areas of northwestern Italy. Whether the strains shared the same mechanism of resistance to imipenem was investigated.

In gram-negative bacteria other than *H. influenzae*, the molecular bases of resistance to carbapenems include the plasmid- or chromosomally mediated production of new β-lactamases such as metallo-β-lactamases, alterations in outer membrane permeability due to reduced contents of specific protein channels, modifications in the contents or in the sequences of PBPs, and the increased expression of efflux pumps (12, 16, 20). Both NTHI strains described herein exhibited heterogeneous imipenem resistance phenotypes, suggesting that no enzyme-mediated mechanism was involved. As far as alterations in outer membrane permeability are concerned, *H. influenzae* has only one porin, the outer membrane protein 2 (OMP2), which produces large, highly permeable channels through which antimicrobial agents enter bacteria (2). We looked at the OMP patterns of strains 183 and 184, but no apparent changes in the intensity of the 40-kDa OMP2 protein band in comparison with those of the imipenem-susceptible strains was visible (data not shown).

Amino acid substitutions in the transpeptidase region of PBP 3, which is involved in peptidoglycan synthesis, have been correlated with non-β-lactamase-mediated resistance to most β-lactam antibiotics (7, 31). Susceptibilities to the different β-lactam antibiotics appear to be affected by single or multiple substitutions at specific positions (9, 21, 24, 29). Regarding carbapenem antibiotics, the N526K substitution seems to play a role in resistance to imipenem (21). In this study, the sequencing of the *ftsI* genes encoding PBP 3 revealed a pattern of amino acid substitutions associated with imipenem resistance. This pattern, which included the N526K substitution plus additional substitutions common to both isolates, among *H. influenzae* clinical isolates has never been previously reported. Since amino acid substitutions surrounding the binding sites of PBP 3 have previously been considered to be relevant for resistance (9), it is noteworthy that the A502V and N526K substitutions described herein surround the KTG conserved motif (at positions 512 to 514) whereas M377I is very close to the SSN motif (at positions 379 to 381). Interestingly, strain 183 was found to possess a further PBP 3 modification (the G490E substitution) near the KTG motif in addition to the pattern shared with strain 184. Considering that the culture of strain 183 contained subpopulations of imipenem-resistant cells at higher frequencies than that of strain 184, it may be supposed that the G490E substitution may contribute to the increased resistance to imipenem. Of note, despite the presence of so many substitutions in PBP 3, both isolates 183 and 184 were susceptible to ampicillin, even if the MICs were near the breakpoint.

In contrast to the results for PBP 3, the analysis of the *dacB* gene sequences encoding PBP 4 did not reveal any association between amino acid substitutions and imipenem susceptibility. The highest number of substitutions corresponded to the imipenem-susceptible strain ATCC 10211, and no substitutions were found exclusively in the two imipenem-resistant isolates. Our data confirmed the findings in previous reports showing considerable allelic variation among *dacB* genes from different *H. influenzae* isolates and indicating that there is no relationship between *dacB* mutations and β-lactam antibiotic susceptibility (29, 31).

To investigate whether mutations in the *ftsI* gene were responsible for the imipenem resistance phenotype, the PCR-amplified *ftsI* gene from strain 183 was used to transform the imipenem-susceptible Rd strain. The MIC of imipenem for the Rd/183 transformant increased 10-fold compared to that for strain Rd as determined by Etest, and the transformant showed a heterogeneous resistance phenotype as assessed by population analysis, demonstrating the involvement of PBP 3 modifications in the resistance phenotype of the parent strain 183. Notably, the MIC of ampicillin for the Rd/183 transformant also increased in comparison with that for strain Rd, almost reaching the MIC for the parent strain 183, indicating that, although the particular pattern of substitutions in PBP 3 transferred to the transformant was unable to confer resistance to ampicillin, it affected the level of susceptibility to this drug.

Since the frequency of the imipenem-resistant cells among the population of the Rd/183 transformant cells was lower than that among the population of cells of the parent strain 183, there may be additional mechanisms contributing to the resistance. In *H. influenzae*, the existence of a macrolide efflux mechanism has been recently demonstrated (22). The AcrAB pump is the primary efflux pump, and it seems to play a role in the baseline level of resistance of this organism (25, 30). It has been suggested that the AcrAB efflux pump is overexpressed as a result of mutations causing the loss of the AcrR repressor function (10). In particular, insertions in the *acrR* gene sequence generating stop codons have been indicated to contribute to the unusually high level of resistance to ampicillin observed in

some β-lactamase-negative ampicillin-resistant isolates (14). In this study, no such mutations in the *acrR* sequences of the strains 183 and 184 were identified. At the amino acid level, although many substitutions in the AcrR repressor sequences in most isolates were observed, a clear association between specific amino acid changes and imipenem susceptibility was not found. On the other hand, the imipenem MICs for the two resistant isolates did not decrease in the presence of the efflux pump inhibitor CCCP. Although the latter result suggests that pumps did not play a significant role in determining imipenem resistance in our isolates, other investigations on the possible AcrAB gene overexpression resulting from mechanisms other than the loss of the AcrR repressor function should be carried out to draw a firm conclusion about efflux pump involvement. Nevertheless, the effect of a possible efflux mechanism may be counterbalanced by the rapid influx of imipenem through the very large porin channels present in the NTHI bacterial cells.

We speculated that the genetic background of strain Rd may modulate the level of resistance to imipenem conferred by PBP 3 on the transformant. Isolates of *Streptococcus pneumoniae* carrying identical PBP sequences can show different levels of β-lactam resistance (6). Moreover, the transfer of the *pbp* genes from a clinical isolate to the laboratory strain R6 resulted in a transformant with a higher level of resistance than that of the donor strain (6). Possibly, genetic factors can either increase or decrease the level of PBP-based resistance in relation to the compatibility of the mutated PBP with other enzymes involved in peptidoglycan synthesis (6). However, *S. pneumoniae* is homogeneously resistant to β-lactam antibiotics, which means that each bacterial cell has the same degree of resistance, while the NTHI isolates described herein, including the Rd/183 transformant, were heterogeneously resistant to imipenem. What causes the heterogeneous expression of imipenem resistance in the isolates described herein remains unknown. One firm fact is that the *ftsI* gene sequences in all the analyzed colonies of strain 183, grown in medium either without imipenem or with imipenem at high concentrations, were identical, indicating that no subpopulations carrying different amino acid substitutions in PBP 3 were present within the general population of the strain. No alteration of any putative regulatory regions immediately upstream the *ftsI* gene was found, although the regulation of the genes composing the division/cell wall cluster has been found to be highly complex, with multiple promoters and multiple factors contributing to the transcriptional regulation, according to previous studies (32). Among other bacterial species, *Staphylococcus aureus* has been demonstrated to exhibit heterogeneous resistance to both methicillin (methicillin-resistant *S. aureus*) and vancomycin (heterogeneous vancomycin-intermediate *S. aureus* [hetero-VISA]) (13, 17). Of note, hetero-VISA isolates and our NTHI strains heterogeneously resistant to imipenem share some morphological and culture characteristics: cells able to grow in the presence of high concentrations of the antibiotic had lower growth rates and showed small-colony variants (data not shown). By optical microscopy, our bacterial cells appeared slightly swollen and no cell filamentation was observed, suggesting that the specific pattern of amino acid substitutions described herein did not affect septation (data not shown). In spite of a great number of studies, the mechanisms leading to the heterogeneous expression of resistance in methicillin-resistant *S. aureus* as well as in hetero-VISA have not been fully clarified. The *mecA* gene, which encodes a low-affinity PBP 2a, is the prerequisite for methicillin resistance; however, the influence of autolysins on the level of resistance as well as the possible presence of pleiotropic regulatory mechanisms that specifically control the heterogeneous resistance phenotype have been suggested previously (23). Similarly, mutations in the *ftsI* gene may be the precondition for imipenem resistance in our NTHI isolates. Whether additional levels of regulation that specifically control the heterogeneous expression of the resistance phenotype exist requires further investigations to be elucidated.

In conclusion, this is the first report of an NTHI clone with heterogeneous resistance to imipenem in a clinical setting in Italy. Our results indicate that the heterogeneous imipenem resistance phenotype of the NTHI clone depends largely on the PBP 3 amino acid substitutions, even if the mechanisms causing the heterogeneous expression of such resistance require further studies to be elucidated. Although the clinical relevance of the PBP-mediated resistance has yet to be demonstrated, we would emphasize that the frequency of *H. influenzae* isolates with heterogeneous resistance to imipenem may be underestimated, since the standard broth dilution method was unable to detect this resistance in our two strains.

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