Comparison of the Efficacies of Oral β -Lactams in Selection of *Haemophilus influenzae* Transformants with Mutated *ftsI* Genes[▽]

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Horizontal transfer of the mutated *ftsI* gene from β -lactamase-nonproducing ampicillin-resistant (BLNAR) *Haemophilus influenzae* to a susceptible strain was examined in vitro under selection with nine oral β -lactams (ampicillin, amoxicillin, cefprozil, cefuroxime, cefpodoxime, cefdinir, cefcapene, cefditoren, and tebipenem). Compared to the penicillins and the carbapenem, the cephalosporins showed a wide selection window for the genetic transfer.

β -Lactam resistance in *Haemophilus influenzae* is mediated by either the production of β -lactamase or mutations in the *ftsI* gene encoding penicillin-binding protein 3 (PBP 3) (20). The *H. influenzae* strains demonstrating ampicillin resistance due to the latter mechanism are called β -lactamase-nonproducing ampicillin-resistant (BLNAR) *H. influenzae*. The BLNAR strains remain uncommon in the United States (2, 10) and also in most European countries (1, 11). However, recent surveillance studies have revealed an exceptionally high prevalence of BLNAR strains in Japan (23% to 43%) (9, 17, 18) as well as in several other countries such as Korea (29%) and Spain (34% to 56%) (5, 11, 13). The diversity in the prevalence of the BLNAR strains is believed to be due to the different β -lactams that are prescribed for the treatment of respiratory tract infections and acute otitis media (7). In the United States and Europe, a high dose of amoxicillin-clavulanic acid is recommended as the first-choice antibiotic for the treatment of community-acquired respiratory infections. On the other hand, in Japan, the cephalosporins are often prescribed for the same treatment. The mechanisms underlying the rapid increase of the BLNAR strains in Japan and some other countries are, however, poorly understood. Recently, horizontal transfer of the *ftsI* gene was detected in *H. influenzae*, an organism that is naturally competent for transformation (19). Thus, genetic transfer of the mutated *ftsI* gene was suggested to be involved in the rapid increase of BLNAR strains. To elucidate the difference between the penicillins and cephalosporins in select-

ing BLNAR variants, we evaluated the effect of various β -lactams on the frequency of horizontal transfer of the mutated *ftsI* gene from a BLNAR strain to an antibiotic-susceptible strain.

A spontaneous rifampin-resistant *H. influenzae* Rd clone, designated Rd^{RIF} (19), and two BLNAR isolates (MSC06647 and MSC06663) were used in this study. According to the classification of the mutations in PBP 3 (21), MSC06647 and MSC06663 belong to the group II BLNAR (Asn526Lys substitution in PBP 3) and group III BLNAR (Met377Ile, Ser385Thr, Leu389Phe, and Asn526Lys substitutions in PBP 3) strains, respectively. MICs were determined by the broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (3). Susceptibilities of these strains to various antibiotics used in this study are shown in Table 1. In vitro genetic transfer experiments were performed as follows. Cells, grown on chocolate II agar (Becton Dickinson, Sparks, MD), were suspended in sBHI broth (brain heart infusion broth supplemented with 2% defibrinated, lysed horse blood and 15 μ g/ml β -NAD⁺) at a cell density of about 10⁸ CFU/ml. After preincubation at 37°C for 1 h, *H. influenzae* Rd^{RIF} and one of the BLNAR strains (MSC06647 or MSC06663) were mixed at a ratio of about 1:1. The spontaneous rifampin-resistant BLNAR mutants and β -lactam-resistant Rd^{RIF} mutants were detected by adding 50 U per ml of DNase I (Takara Bio Inc., Otsu, Japan) to the mixed culture. The cell suspensions, with or without DNase I, were incubated at 37°C for 2 h, and a portion of the culture was plated on chocolate

TABLE 1. Susceptibilities of *H. influenzae* strains to various antibiotics

Strain	MIC (μ g/ml) of drug:								
	Ampicillin	Amoxicillin	Cefprozil	Cefuroxime	Cefpodoxime	Cefdinir	Cefcapene	Cefditoren	Tebipenem
Rd ^{RIF}	0.25	0.5	2	0.5	0.03	0.25	0.008	0.008	0.06
MSC06647	2	4	4	2	1	1	0.25	0.06	1
MSC06663	1	1	4	2	8	2	2	0.5	0.25

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agar plates containing 16 µg/ml of rifampin and various concentrations of β-lactams to select transformants of the *ftsI* gene. The mechanism of the transfer of the *ftsI* gene from BLNAR strains to Rd^{RIF} was found to involve classical transformation, because the addition of DNase I totally abolished the emergence of transformants (19). The following antibiotics were purchased: rifampin, ampicillin, amoxicillin, and cefuroxime from Sigma Chemical Co. (St. Louis, MO) and cefprozil and cefdinir from Kemprotec, Ltd. (Middlesbrough, United Kingdom). Cefpodoxime, cefcapene, cefditoren, and tebipenem were synthesized at the Pharmaceutical Research Center of Meiji Seika Kaisha, Ltd. (Yokohama, Japan). Tebipenem-pivoxil (formerly L-084), a novel oral carbapenem undergoing a phase 3 clinical trial in Japan, exhibits a broad spectrum of activities against many clinically important pathogens, including *Streptococcus pneumoniae* and *H. influenzae* (8, 14). The frequency of genetic transfer of the *ftsI* gene from the BLNAR strain to a susceptible strain was determined by using the following equation: [count (CFU/ml) of rifampin- and β-lactam-resistant clones without DNase I (i.e., transformants plus spontaneous mutants) – count (CFU/ml) of rifampin- and β-lactam-resistant clones with DNase I (i.e., spontaneous mutants)]/count (CFU/ml) of Rd^{RIF}. Three independent experiments were performed, and the results are presented as mean values. Nucleotide sequencing of the *ftsI* gene for five to eight randomly selected rifampin- and β-lactam-resistant clones from each β-lactam treatment group revealed that the resistant clones possessed sequences totally or partially identical to those of the donor BLNAR strains. Most transformants (52 out of 61 transformants and 49 out of 59 transformants obtained from the experiments with MSC06647 and MSC06663, respectively) were found to possess an *ftsI* gene identical to that of the BLNAR strain. At least the mutation resulting in the Asn526Lys substitution in PBP 3 was found in every transformant.

As shown in Table 2, all β-lactams selected the transformants at some concentration after plating of the mixed culture of Rd^{RIF} and MSC06647 (group II; prevalent BLNAR type worldwide). The concentration ranges for selecting the transformants were three and two to four twofold dilutions for penicillins and cephalosporins, respectively. Among the compounds tested, cefditoren prevented the genetic transfer at the lowest concentration (0.06 µg/ml).

Table 3 summarizes the experimental results with the Rd^{RIF} and MSC06663 (group III; BLNAR type isolated only in Japan) strains. Compared with the results obtained the MSC06647 strain, the cephalosporins showed a wide selection window for the emergence of the transformants. The selection window of the transformants for the MSC06663 strain increased between 4- and 32-fold for cefuroxime, cefpodoxime, cefdinir, cefcapene, and cefditoren, while that for the penicillins remained unchanged. The transfer of the *ftsI* gene was remarkably suppressed for both ampicillin and amoxicillin at 1 µg/ml, and the degree of reduction was very similar to that found with the MSC06647 strain. Overall, cefditoren and tebipenem prevented the selection of the transformants at the lowest concentration (0.5 µg/ml).

Heterogeneity in nontypeable *H. influenzae* strains is widely recognized (4, 6). Recently, genotypically divergent BLNAR strains harboring the identical *ftsI* gene sequence were identi-

TABLE 2. Frequency of genetic transfer of the *ftsI* gene from MSC06647 (BLNAR) to Rd^{RIF}

Antibiotic	Frequency at concn (µg/ml) ^a											
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32
Ampicillin	ND	ND	ND	ND	2.8 × 10 ⁻⁶	1.7 × 10 ⁻⁷	3.7 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸		
Amoxicillin	ND	ND	ND	ND	ND	7.7 × 10 ⁻⁷	6.8 × 10 ⁻⁸	4.3 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸		
Cefprozil	ND	ND	ND	ND	ND	ND	ND	ND	ND	<2.6 × 10 ⁻⁸		
Cefuroxime	ND	ND	ND	ND	ND	ND	1.1 × 10 ⁻⁶	4.9 × 10 ⁻⁷	3.8 × 10 ⁻⁷	6.4 × 10 ⁻⁸	4.3 × 10 ⁻⁷	1.2 × 10 ⁻⁷
Cefpodoxime	ND	ND	ND	ND	ND	ND	2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸
Cefdinir	ND	ND	ND	ND	ND	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸
Cefcapene	ND	3.8 × 10 ⁻⁶	1.8 × 10 ⁻⁶	4.3 × 10 ⁻⁶	5.3 × 10 ⁻⁶	9.7 × 10 ⁻⁷	2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸
Cefditoren	2.6 × 10 ⁻⁶	1.4 × 10 ⁻⁷	<2.6 × 10 ⁻⁸	6.3 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸
Tebipenem	ND	ND	1.2 × 10 ⁻⁶	4.9 × 10 ⁻⁷	1.5 × 10 ⁻⁷	6.5 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸	<2.6 × 10 ⁻⁸			

^a The averages of three individual experiments are shown. ND, not determined due to the lack of growth-inhibitory effect on a susceptible strain.

TABLE 3. Frequency of genetic transfer of the *fisI* gene from MSC06663 (BLNAR) to Rd^{RIF}

Antibiotic	Frequency at concn (μg/ml) ^a :												
	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64
Ampicillin	ND	ND	ND	ND	2.8 × 10 ⁻⁶	3.1 × 10 ⁻⁷	2.8 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸			
Amoxicillin	ND	ND	ND	ND	ND	7.4 × 10 ⁻⁷	4.2 × 10 ⁻⁸	3.5 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸			
Cefprozil	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND			
Cefuroxime	ND	ND	ND	ND	ND	ND	3.0 × 10 ⁻⁶	1.3 × 10 ⁻⁶	1.9 × 10 ⁻⁶	1.4 × 10 ⁻⁶	8.9 × 10 ⁻⁷	4.9 × 10 ⁻⁷	1.9 × 10 ⁻⁷
Cefpodoxime	ND	ND	ND	7.0 × 10 ⁻⁶	6.2 × 10 ⁻⁶	5.3 × 10 ⁻⁶	3.5 × 10 ⁻⁶	6.3 × 10 ⁻⁷	2.2 × 10 ⁻⁷	2.7 × 10 ⁻⁸	1.1 × 10 ⁻⁶	3.3 × 10 ⁻⁷	5.1 × 10 ⁻⁸
Cefdinir	ND	ND	ND	ND	8.8 × 10 ⁻⁶	4.1 × 10 ⁻⁶	1.5 × 10 ⁻⁶	1.1 × 10 ⁻⁶	3.7 × 10 ⁻⁷	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸		
Cefcapene	ND	1.0 × 10 ⁻⁵	7.7 × 10 ⁻⁶	8.2 × 10 ⁻⁶	7.8 × 10 ⁻⁶	3.6 × 10 ⁻⁶	7.6 × 10 ⁻⁷	5.0 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸			
Cefditoren	7.1 × 10 ⁻⁶	5.7 × 10 ⁻⁶	4.0 × 10 ⁻⁶	1.6 × 10 ⁻⁶	4.1 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸			
Tebipenem	ND	ND	3.2 × 10 ⁻⁶	5.6 × 10 ⁻⁷	7.5 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸	<2.3 × 10 ⁻⁸			

^a The averages of three individual experiments are shown. ND, not determined due to the lack of growth-inhibitory effect on a susceptible strain.

fied (19). Therefore, the rapid spread of the BLNAR strains in Japan (excluding the type b strains) is not likely due to clonal dissemination, although a case of clonal spread of the BLNAR strains has been reported in the same hospital or between family members (12, 22). Previous study suggested that the horizontal transfer of the *fisI* gene in *H. influenzae* could occur in both an intraspecies and an interspecies manner (19). By using an in vitro genetic transfer method, various β -lactams were evaluated for selecting BLNAR variants derived as a result of the DNA exchange between the *H. influenzae* strains.

We found that the BLNAR variants emerged within a narrow concentration range for ampicillin, amoxicillin, and tebipenem. Moreover, ampicillin at 2 $\mu\text{g/ml}$ and amoxicillin at 4 $\mu\text{g/ml}$, concentrations that are achievable in the serum at therapeutic doses, completely inhibited the emergence of transformants from both BLNAR strains. This finding could explain, at least in part, why BLNAR strains are less prevalent in countries where amoxicillin is the first-choice antibiotic for therapeutic use. Cephalosporins, on the other hand, showed broad concentration ranges for selecting the BLNAR variants, especially when MSC06663 (BLNAR group III) was used as a donor strain. Peak serum concentrations (about 1 to 2 $\mu\text{g/ml}$) of most of the expanded-spectrum oral cephalosporins tested generally fall within or slightly above the selection window. Therefore, most cephalosporins are likely to select BLNAR strains. In fact, the BLNAR strains emerged in Japan in the 1990s closely following the introduction of the expanded-spectrum oral cephalosporins (cefepodoxime, cefdinir, cefditoren, and cefcapene), and the remarkable diffusion of BLNAR strains thenceforth observed in Japan could be related to the preferred use of oral cephalosporins for the treatment of community-acquired respiratory infections in that country. Although the horizontal transfer of the mutated *fisI* genes in clinical situations leading to the spread of the BLNAR strains is not as simple as the experimental conditions used in this study, the fact that cefditoren at the peak serum concentration or pharmacokinetic/pharmacodynamic breakpoint (16) was sufficient to prevent the emergence of transformants suggests that it might exhibit a lower propensity for the selection of BLNAR strains than other oral cephalosporins. The difference in the concentration ranges for the selection of transformants between penicillins and cephalosporins is mainly due to the reduced antibacterial activities of cephalosporins resulting from the amino acid substitutions near the SSN motif in PBP 3 (15).

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