Horizontal Gene Transfer of *ftsI*, Encoding Penicillin-Binding Protein 3, in *Haemophilus influenzae*[▽]

Sho Takahata,* Takashi Ida, Nami Senju, Yumiko Sanbongi, Aiko Miyata, Kazunori Maebashi, and Shigeru Hoshiko

Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222-8567, Japan

Received 12 December 2006/Returned for modification 20 January 2007/Accepted 15 February 2007

Horizontal gene transfer has been identified in only a small number of genes in *Haemophilus influenzae*, an organism which is naturally competent for transformation. This report provides evidence for the genetic transfer of the *ftsI* gene, which encodes penicillin-binding protein 3, in *H. influenzae*. Mosaic structures of the *ftsI* gene were found in several clinical isolates of *H. influenzae*. To identify the origin of the mosaic sequence, complete sequences of the corresponding gene from seven type strains of *Haemophilus* species were determined. Comparison of these sequences with mosaic regions identified a homologous recombination of the *ftsI* gene between *H. influenzae* and *Haemophilus haemolyticus*. Subsequently, ampicillin-resistant *H. influenzae* strains harboring identical *ftsI* sequences were genotyped by pulsed-field gel electrophoresis (PFGE). Divergent PFGE patterns among β -lactamase-nonproducing ampicillin-resistant (BLNAR) strains from different hospitals indicated the potential for the genetic transfer of the mutated *ftsI* gene between these isolates. Moreover, transfer of the *ftsI* gene from BLNAR strains to β -lactamase-nonproducing ampicillin-susceptible (BLNAS) *H. influenzae* strains was evaluated in vitro. Coincubation of a BLNAS strain (a rifampin-resistant mutant of strain Rd) and BLNAR strains resulted in the emergence of rifampin- and cefdinir-resistant clones at frequencies of 5.1×10^{-7} to 1.5×10^{-6} . Characterization of these doubly resistant mutants by DNA sequencing of the *ftsI* gene, susceptibility testing, and genotyping by PFGE revealed that the *ftsI* genes of BLNAR strains had transferred to BLNAS strains during coincubation. In conclusion, horizontal transfer of the *ftsI* gene in *H. influenzae* can occur in an intraspecies and an interspecies manner.

Haemophilus influenzae is naturally competent for DNA uptake from the surrounding environment. Recognition and efficient uptake of species-specific DNA molecules is mediated by the presence of uptake signal sequences (USSs) in donor DNA. The consensus sequence of the USS in *H. influenzae* was identified as 5'-AAGTGC GGT-3' (6). A recent study has revealed that USSs exist in the genomes of other species of the family Pasteurellaceae, suggesting that this element is utilized for the uptake and exchange of DNA between *H. influenzae* and related species (2). A total of 1,471 copies of the USS were found in the *H. influenzae* Rd strain by searching its complete genome (15). However, horizontal gene transfer in *H. influenzae* has been reported for a small number of genes. For instance, the genetic transfer of *ompP2*, a gene encoding major outer membrane protein P2, was reported among clinical isolates of *H. influenzae* (7, 16). Intergeneric lateral transfer of the tryptophanase gene cluster between *Escherichia coli* and *H. influenzae* was also identified (11).

Penicillin-binding protein 3 (PBP 3), which is encoded by the *ftsI* gene, is a transpeptidase needed for cross-linking of the septal cell wall peptidoglycan during cell division. The essentiality of this enzyme in *H. influenzae* was demonstrated by targeted gene disruption (18). A remarkable increase of β -lactamase-nonproducing ampicillin-resistant (BLNAR) *H. influenzae* strains has been reported in Japan (14). These resistant

strains have been shown to possess various mutations in the *ftsI* gene, leading to amino acid substitutions responsible for β -lactam resistance, such as substitutions at positions 526 (Asn→Lys), 517 (Arg→His), 377 (Met→Ile), 385 (Ser→Thr), and 389 (Leu→Phe) in the PBP 3 protein (12, 19). Due to their rapid increase and nationwide spread, it is difficult to consider that BLNAR strains emerged independently by accumulating multiple mutations in the *ftsI* gene. Moreover, totally identical *ftsI* sequences from different BLNAR isolates have frequently been observed (14). These observations drove us to hypothesize that the genetic transfer of the *ftsI* gene occurs in *H. influenzae* strains. In this study, evidence for the genetic transfer of the *ftsI* gene in *H. influenzae* was obtained by carefully analyzing the nucleotide sequence of the *ftsI* genes from various clinical isolates and also by in vitro gene transfer experiments.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are listed in Table 1. *H. influenzae* Rd (ATCC 51907) and type strains of *Haemophilus* species (*H. haemolyticus* ATCC 33390, *H. parainfluenzae* ATCC 33392, *H. aphrophilus* ATCC 33389, *H. paraphrophilus* ATCC 29241, *H. segnis* ATCC 33393, *H. parahaemolyticus* ATCC 10014, and *H. paraphrohaemolyticus* ATCC 29237) were obtained from the American Type Culture Collection. A spontaneous rifampin-resistant derivative of *H. influenzae* Rd, designated *H. influenzae* Rd^{RIF}, was obtained by selecting a clone grown on chocolate agar containing 25 μ g/ml of rifampin. Complete sequencing of the *rpoB* gene in this mutant revealed a point mutation, G436T, that resulted in the replacement of Val-146 to Phe-146 in the RpoB protein. Other *H. influenzae* and *H. haemolyticus* strains were clinical isolates collected in Japan between 1997 and 2003 (12, 14). The isolates were identified through their requirements for β -NAD⁺ (V factor) and hemin (X factor) for growth, by the nucleotide sequence of the 16S rRNA gene (4), and also by hemolytic activity assessment.

* Corresponding author. Mailing address: Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd., 760 Morooka-cho, Kohoku-ku, Yokohama 222-8567, Japan. Phone: 81-45-545-3139. Fax: 81-45-541-1768. E-mail: sho_takahata@meiji.co.jp.

[▽] Published ahead of print on 26 February 2007.

TABLE 1. Strains used in this study

Strain no.	Strain	Relevant properties	MIC (μ g/ml)			Reference or source
			Ampicillin	Amoxicillin-clavulanic acid	Cefdinir	
1	<i>H. influenzae</i> MSC02070	BLNAR (<i>ftsI</i> sequence 1); hospital A	8	16	8	
2	<i>H. influenzae</i> MSC01869	BLPACR (<i>ftsI</i> sequence 1); hospital B	>64	16	8	
3	<i>H. influenzae</i> MSC01400	BLNAR (<i>ftsI</i> sequence 1); hospital C	8	16	8	
4	<i>H. influenzae</i> MSC01855	BLNAR (<i>ftsI</i> sequence 1); hospital D	4	8	8	
5	<i>H. influenzae</i> MSC07237	BLNAR (<i>ftsI</i> sequence 2); hospital E	4	16	16	14
6	<i>H. influenzae</i> MSC02104	BLPACR (<i>ftsI</i> sequence 2); hospital F	>64	32	16	14
7	<i>H. influenzae</i> MSC02149	BLNAR (<i>ftsI</i> sequence 2); hospital G	8	32	16	14
8	<i>H. influenzae</i> MSC02022	BLNAR (<i>ftsI</i> sequence 3); hospital H	1	2	2	
9	<i>H. influenzae</i> MSC01363	BLNAR (<i>ftsI</i> sequence 3); hospital H	2	4	1	
10	<i>H. influenzae</i> MSC01432	BLNAR (<i>ftsI</i> sequence 3); hospital H	1	4	2	
11	<i>H. influenzae</i> MSC06647 (HI00128)	BLNAR (PBP 3, ³⁷⁷ M ³⁸⁵ S ³⁸⁹ L ⁵⁰² T ⁵¹⁷ R ⁵²⁶ K)	2	4	1	12
12	<i>H. influenzae</i> MSC06651 (HI00132)	BLNAR (PBP 3, ³⁷⁷ T ³⁸⁵ T ³⁸⁹ F ⁵⁰² A ⁵¹⁷ H ⁵²⁶ N)	2	8	8	12
13	<i>H. influenzae</i> MSC06663 (HI00144)	BLNAR (PBP 3, ³⁷⁷ T ³⁸⁵ T ³⁸⁹ F ⁵⁰² A ⁵¹⁷ R ⁵²⁶ K)	1	8	2	12
14	<i>H. influenzae</i> Rd ^{RIF}	Rifampin resistant; RpoB(Val146Phe)	0.25	0.25	0.25	This study
15	<i>H. influenzae</i> MSC07169	Clinical isolate (source, rhinopharynx)	1	1	1	
16	<i>H. influenzae</i> MSC07771	Clinical isolate (source, sputum)	2	4	1	
17	<i>H. haemolyticus</i> MSC07286	Clinical isolate (source, rhinopharynx)	2	2	2	
18	<i>H. haemolyticus</i> ATCC 33390	Type strain of <i>H. haemolyticus</i>	0.5	1	0.12	
19	<i>H. parainfluenzae</i> ATCC 33392	Type strain of <i>H. parainfluenzae</i>	0.5	0.5	0.12	
20	<i>H. aphrophilus</i> ATCC 33389	Type strain of <i>H. aphrophilus</i>	0.5	0.5	0.12	
21	<i>H. paraphrophilus</i> ATCC 29241	Type strain of <i>H. paraphrophilus</i>	0.25	0.25	0.06	
22	<i>H. segnis</i> ATCC 33393	Type strain of <i>H. segnis</i>	0.5	0.5	0.06	
23	<i>H. parahaemolyticus</i> ATCC 10014	Type strain of <i>H. parahaemolyticus</i>	0.25	0.5	0.016	
24	<i>H. paraphrohaemolyticus</i> ATCC 29237	Type strain of <i>H. paraphrohaemolyticus</i>	0.25	0.5	0.016	

Media, antimicrobial susceptibility testing, and antibacterial agents. Chocolate II agar plates (Becton Dickinson, Sparks, MD) and brain heart infusion broth supplemented with 2% defibrinated, lysed horse blood plus 15 μ g/ml β -NAD⁺ (sBHI broth) were routinely used for bacterial growth. MICs were determined by the broth dilution method according to the guidelines of the Clinical and Laboratory Standards Institute (3). The following antibiotics were used in this study: rifampin (Sigma Chemical Co., St. Louis, MO), cefdinir (Kemprotec, Ltd., Middlesbrough, United Kingdom), and fosfomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan).

Determining the sequence of the *ftsI* genes from type strains of *Haemophilus* species. Reported nucleotide sequences of the *ftsI* genes from species of the family *Pasteurellaceae* (*H. influenzae* Rd, *H. ducreyi* 35000HP, *H. somnus* 2336, *Mannheimia succiniciproducens* MBEL55E, *Pasteurella multocida* Pm70, and *Actinobacillus pleuropneumoniae* 4074 [GenBank accession nos. NC 00097, NC 002940, NZ AACJ01000008, NC 006300, NC 002663, and NZ AACK01000005, respectively]) were aligned in order to search for conserved regions. Degenerate oligonucleotide primers specific for the conserved regions upstream and downstream of the coding region of the *ftsI* gene were constructed. One of two upstream forward primers, primer PH1 (5'-TTGGTAAAGCNATTMWGCC-3') and primer PH2 (5'-GWAGTGCVRTATTACG-3'), was used for PCR amplification, along with a downstream reverse primer, primer PH3 (5'-TGATAA TCNARATGATCRGC-3'). Primers PH2 and PH3 were used to amplify the full-length *ftsI* genes of *H. haemolyticus*, *H. parainfluenzae*, *H. aphrophilus*, and *H. paraphrophilus*. Primers PH1 and PH3 were used for *H. segnis*. The following two primers were constructed for specific amplification of the *ftsI* genes from *H. parahaemolyticus* and *H. paraphrohaemolyticus*: 5'-GTGAGGCAGAAATAGAGG-3' and 5'-AAAGGTTCTGCCATCCAC-3'. PCR amplifications were performed as follows: 30 s of denaturation at 98°C and 30 cycles of denaturation at 95°C for 30 s, annealing at 45 to 55°C for 30 s, and extension at 72°C for 3 min. The cycling reaction was performed with a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). Sequencing was carried out with a BigDye Terminator (version 1.1) cycle sequencing kit and a 3730 DNA analyzer (Applied Biosystems). The sequences were confirmed by sequencing at least two independent PCR products for each strain.

Nucleotide sequencing of the *ftsI* genes from clinical isolates. The full-length *ftsI* genes were amplified by PCR with the following primers: 5'-CTCGTTATC CGTTACAGCAG-3' and 5'-GCCAACC GTGTGATGAAAC-3'. PCR amplifications were performed as follows: 2 min of denaturation at 94°C and 30 cycles of denaturation at 94°C for 30 s, annealing at 53°C for 30 s, and extension at 72°C

for 3 min. The cycling reaction and DNA sequencing were performed as described above.

PFGE. The preparation of the plugs containing bacteria and pulsed-field gel electrophoresis (PFGE) were performed as described previously (13), with slight modifications. Briefly, cells grown in sBHI broth were collected and suspended in 10 mM EDTA buffer (pH 8.0). The suspension was mixed with an equivalent volume of 2% low-melting-point agarose to produce agarose plugs. After solidification, the plugs were incubated in 100 mM EDTA buffer containing 50 μ g lysozyme per ml at 37°C for 1 h. Then the plugs were treated with 250 mM EDTA buffer containing 10 mg proteinase K per ml and 1% *N*-lauroylsarcosine overnight at 50°C. After the plugs were washed with extensive volumes of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA buffer, the DNA was digested with SmaI. The resulting DNA fragments were subjected to PFGE in 0.5 $\times$ TBE (0.045 M Tris-borate, 1 mM EDTA) buffer with a CHEF Mapper electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The DNA fragments were visualized with ethidium bromide.

In vitro genetic transfer. Cells grown on chocolate II agar were suspended in sBHI broth at a density of about 10⁸ CFU/ml. After preincubation at 37°C for 1 h, *H. influenzae* Rd^{RIF} and one of the BLNAR strains (strain MSC06647, MSC06651, or MSC06663) were mixed at a ratio of 1:1. The effect of DNase I on transfer was assessed by adding 100 U of the enzyme (Takara Bio Inc., Otsu, Japan). The cell suspensions were incubated at 37°C for 2 h and plated on chocolate agar containing 16 μ g/ml of rifampin and 0.5 μ g/ml of cefdinir. Nucleotide sequencing of the *ftsI* gene from doubly resistant mutants was performed as indicated above.

Nucleotide sequence accession numbers. The complete DNA sequences of the *ftsI* genes from type strains of *Haemophilus* species and from clinical isolates of *H. influenzae* and *H. haemolyticus*, as determined in the present study, appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession nos. AB267855 to AB267861 (for the type strains) and AB267863 to AB267867 (for the clinical isolates).

RESULTS

Determination of *ftsI* gene sequences from *Haemophilus* species. Analysis of the nucleotide sequences of the *ftsI* genes from 621 *H. influenzae* isolates revealed that at least 10 strains

<i>H. influenzae</i>	1	MVKFNSSRSGKSKK--TIRKLAPETVQKNPKQVFEKCFMRGYMLSTVILLGLCALVARAAYVQSINADTLSNEADKRSRLRKDEVLSVRGSIL	95
<i>H. haemolyticus</i>	1	A.....A.H...KI.....V..WV.AFF..A.....VDSS...G.....	95
<i>H. parainfluenzae</i>	1	..R...K.P.P.--AQ.AS..P...KM.....L...LIA.SF.F..G.....V.SE...G.....D.....	95
<i>H. aphrophilus</i>	1	K.V.PI.P.-T.KSLSHSGSA..P...KI.Y..S.LKA..ISALS..FM..I...TQ.....IV...S.ID.....Q.IQF.....	96
<i>H. paraphrophilus</i>	1	K.V.PI.P.-T.KSLSHSGSA..P...KI.Y..S.LKA..ISALS..FM..I...TQ.....IV...S.ID.....Q.IQF.....	96
<i>H. segnis</i>	1	K.I.PIQA..NNPKPSPQNTSS..P...KM.Y.RS.LK...LWALTSVF..M...Q...M.I...S.MG.....TQ.IQF.....	97
<i>H. parahaemolyticus</i>	1	MARG..QKDKK.PFFDFFKRKQE.NSD-----ENQEEKASYLPK.FCVIWW.VGSAVF...LQ.VV..V.HSPR.IK..NN...TI.LPFT....	92
<i>H. paraphrohaemolyticus</i>	1	MARAI.PKNNKNPRF.LFKRKKH.TENNA.LAE.ENE.AV.PSY.PR.FIMLW.VVIIIVS...YKVVAI.V.ESPR.IS..NN...TI.LPFK....	100
<i>H. influenzae</i>	96	DRNGQLLSVSVFMSAIVADPKTMLKENSIA-----	125
<i>H. haemolyticus</i>	96	N.V.....A.....	125
<i>H. parainfluenzae</i>	96	E.RL...A.D-----	125
<i>H. aphrophilus</i>	97	YSV...FIFD...K-----	126
<i>H. paraphrophilus</i>	97	YSV...FIFD...K-----	126
<i>H. segnis</i>	98	YSV...FIFD...K-----	127
<i>H. parahaemolyticus</i>	93	...KF...A.YS.TI..REYFDSKIKRSEETLIALAETGSSSKMVKSIQQFLDKKNLSAEKVDIDPRAINTQAPKYWTLLSESTGISPEKLVEV	192
<i>H. paraphrohaemolyticus</i>	101	...KF...A.YS.TI..REYFDSKLSRSPDTWKALAIETGSSRSKIISVSRFLDKKNNSSEKVEFDPRSILNTQDPKYWTLLSESTEVSAEQLVV	200
<i>H. influenzae</i>	126	-----DKERIAALAEELGMTENDLVKKIEKNSKGYLYLARQVELSKANYIRRLKIKIGILETEHRRFYPRVBEAAHVGYTIDIG-	206
<i>H. haemolyticus</i>	126	-----T...Q...S.....SN.D...K.....N.....	206
<i>H. parainfluenzae</i>	126	-----K...N.N.PAE.....A..F.....A.....D.....S...P.....QLI.F.N...	206
<i>H. aphrophilus</i>	127	-----WQK...A..ISYGN...RV..DP..RFV..S..ISPTL.Q.VKD...T..V.KS.A.....T..LI..N...	207
<i>H. paraphrophilus</i>	127	-----WQK...A..ISYGN...RV..DP..RFV..S..ISPTL.Q.VKD...T..V.KS.A.....T..LI..N...	207
<i>H. segnis</i>	128	-----WQK...A..ISYGN...RV..DP..RFV..S..ISPTL.Q.VKE...T...KM.A.....T..LI..N...	208
<i>H. parahaemolyticus</i>	193	RQNPNYSYFVQLENAAEKLERDKWQ.F.KSVKVPYSEMMDRLY.AHRQRFT...HETE.I.R.AQE..LNA.V.RE.S...L...SQLI.F...DK	292
<i>H. paraphrohaemolyticus</i>	201	RQNPNYSYFVQLENAAEKLERDKWQ.F.KSVKVPY..EMMDRLY.AHRQRFT..V.HETE.I.R.AQE..LDA.V.RE.S...L...SQL.F...DK	300
<i>H. influenzae</i>	207	NGIEGIEKSFNSLLVGKDGSRTRVKDKRGNIVAHISDEKKYDAQDVTLSIDEKLQSMVYREIKKAVSENNAESGTAVLVDVVRTGEVLAMATAPSYNPNR	306
<i>H. haemolyticus</i>	207	M.....D.....E.A.....	306
<i>H. parainfluenzae</i>	207	K...V.A...M.....A.V.....V.E.A.....T.....	306
<i>H. aphrophilus</i>	208	S.....D.M...S.L.Y...F...EN...V.....M.....A..K.....N.....	307
<i>H. paraphrophilus</i>	208	S.....D.M...S.L.Y...F...EN...V.....M.....A..K.....N.....	307
<i>H. segnis</i>	209	A.....D.M...S.M.Y...RF...EN...V.....M.....A..K.....N.....	308
<i>H. parahaemolyticus</i>	293	H.S.L.R..DA..I..S.KQVI...A...IEN.R...E.NP...V...QD...A...N.K...I.....Q...I..N...F....	392
<i>H. paraphrohaemolyticus</i>	301	H.S.L.R..DA..I..S.KQVI...A...IEN.R...E.NP...V...QD...N...K...I.....Q...I..N...F....	400
<i>H. influenzae</i>	307	VGKSELMRNRAITDTFEPG*STVKKFFVVLTAQRGVVKRDEIDTTSFKLSGKEIVDVAPRAQQTLDIELMSSNMRGVSRLALRMPSPALMETYQNAGLS	406
<i>H. haemolyticus</i>	307	R.....N.G.TV.....I.....	406
<i>H. parainfluenzae</i>	307	AN..D.K.....A.R...V.N.G.TV.....P.....I.....	406
<i>H. aphrophilus</i>	308	T...T.....E.A.R...N.GPLI.N.H.VK...DRLS..G..E.....D...S...G	407
<i>H. paraphrophilus</i>	308	T...T.....E.A.R...N.GPLI.N.H.VK...DRLS..G..E.....D...S...G	407
<i>H. segnis</i>	309	A.R...N.GPLI.N.H.VK...D.LS..G..E.....	408
<i>H. parahaemolyticus</i>	393	SEF.P.....T.....N..TY...V.N.HP.VVN.HT.R...DSLITG..QK...I.....ST..VD..SKV.FG	492
<i>H. paraphrohaemolyticus</i>	401	SEF.P.....T.....N..TY...V.N.HP.VVN.HT.R...DSLITG..QK...I.....ST..VD..SKV.FG	500
<i>H. influenzae</i>	407	KPTDLGLIGEQVGILNANRKRWDIERATVAYGYGITATPLQIARAYATLGSFGVYRPLSITKVDPPVIGKRVFSEKITKDIVGILEKVAIKNK---RAM	503
<i>H. haemolyticus</i>	407	R.....S.....I.....Q.....M.....	503
<i>H. parainfluenzae</i>	407	.D.E...R.V.....S.....I.....Q.....M.....	503
<i>H. aphrophilus</i>	408	S.L...H.A.S...N...LN...V...I.....N...M.REV.NMM...G---L	504
<i>H. paraphrophilus</i>	408	S.L...H.A.S...N...LN...V...I.....N...M.REV.NMM...G---L	504
<i>H. segnis</i>	409	S.L...A.S...N...LN...V...I.....N...M.REV.NMM...G---L	505
<i>H. parahaemolyticus</i>	493	.D.G...GEQRG--T.GD...S...L...LNV...L...I.....E..LP...R.V.HMM.S..Q.GEGGA...	590
<i>H. paraphrohaemolyticus</i>	501	.D.G...GEQRG--T.GD...S...L...LNV...L...I.....E..LP...R.V.HMM.S..Q.GEGGA...	598
<i>H. influenzae</i>	504	VEGYRVGVKTG*ARKIENGHYVNKYVAFTAGIAPISDPYALVVLINDPKAGEYYGAVSAPVFSNIMGYALRANAIPQDAEAENTTTKSAKRIVYIGE	603
<i>H. haemolyticus</i>	504	I.....S.....I.....Q.....S...T...G.AP...PT.K.ARRTVRLSDQKF...	603
<i>H. parainfluenzae</i>	504	I.....S.....I.....Q.....S...T...G.AP...PT.K.ARRTVRLSDQKF...	603
<i>H. aphrophilus</i>	505	I.....K.L...R.D...Y...V...Q.....I.....S...T...NVAP.GVEQDK.ARRTVRLSDKKPS	604
<i>H. paraphrophilus</i>	505	I.....K.L...R.D...Y...V...Q.....I.....S...T...NVAP.GVEQDK.ARRTVRLSDKKPS	604
<i>H. segnis</i>	506	I.....K.L...R.D...Y...T...Q.....I.....S...S...NV.P.GVETDK..KRTVRLSSKKSS	605
<i>H. parahaemolyticus</i>	591	.D...A.....L.K.Q..E.L.Y...L..A.N..F...V.E.SKDK.....Q...T.K.YN.KP.NLTE	674
<i>H. paraphrohaemolyticus</i>	599	.D...A.....L.K.Q..E.L.Y...L..A.N..F...V.E.SKDK.....Q...T.K.YN.KP.NLTE	682
<i>H. influenzae</i>	604	HKNQKVN	610
<i>H. haemolyticus</i>	604		610
<i>H. parainfluenzae</i>	604	KM.	606
<i>H. aphrophilus</i>	605	GV.	607
<i>H. paraphrophilus</i>	605	GV.	607
<i>H. segnis</i>	606	EV.	608

FIG. 1. Deduced amino acid sequences of PBP 3 from seven *Haemophilus* species. The complete PBP 3 sequences for *H. haemolyticus* ATCC 33390, *H. parainfluenzae* ATCC 33392, *H. aphrophilus* ATCC 33389, *H. paraphrophilus* ATCC 29241, *H. segnis* ATCC 33393, *H. parahaemolyticus* ATCC 10014, and *H. paraphrohaemolyticus* ATCC 29237 were aligned with that of *H. influenzae* Rd. Three conserved motifs in the active site are boxed, and an asterisk shows the position of the catalytic serine residue. Dots, identical amino acids.

FIG. 2. Schematic representation of the *ftsI* genes of *H. influenzae* and *H. haemolyticus* strains. The nucleotide sequence divergences between regions of the *ftsI* genes compared with the sequences of the corresponding regions in the *ftsI* gene of *H. influenzae* Rd are shown as percentages. Solid bars, nucleotides that differ from the corresponding nucleotides in *H. influenzae* strains. The sites and orientations of the USSs are shown as arrows under the *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 sequences.

harbored mosaic structures of the *ftsI* gene which contained a sequence divergent from that of the Rd strain. The nucleotide sequences of these regions showed no similarity with those of the reported *ftsI* genes. To identify the origin of the mosaic sequence, the complete sequence of the *ftsI* genes of *Haemophilus* species, whose natural host is only humans, was determined. Figure 1 shows the deduced amino acid sequences of PBP 3 for type strains of seven *Haemophilus* species. The PBP 3 sequences of *H. haemolyticus* and *H. parainfluenzae* showed high degrees of homology with that of *H. influenzae*. The identities of the amino acid sequences were 92.5% and 83.1%, respectively. The *ftsI* genes of *H. parahaemolyticus* and *H. paraphrohaemolyticus* were quite different from that of *H. influenzae* and much closer to those of *H. ducreyi* and *Actinobacillus pleuropneumoniae*.

Recombination of the *ftsI* gene between *H. influenzae* and *H. haemolyticus*. Comparison of the mosaic *ftsI* genes with the corresponding genes of *Haemophilus* species (*H. parainfluenzae*, *H. haemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, *H. parahaemolyticus*, and *H. paraphrohaemolyticus*) indicated that homologous recombination between *H. haemolyticus* and *H. influenzae* had occurred in some isolates (Fig. 2). While the overall divergence of the nucleotide sequences between *H. haemolyticus* ATCC 33390 and *H. influenzae* Rd was 12.7% (233 bp of 1,833 bp), both genes possessed two USSs at the same position. One of the *H. influenzae* isolates (isolate MSC07169) possessed a sequence at the 3'-terminal region (positions 1647 to 1833) almost identical to that of *H. haemolyticus* ATCC 33390. Another *H. influenzae* isolate (isolate MSC07771) was also found to have an *H. haemolyticus*-derived sequence at positions 402 to 831. Furthermore, an *H. haemolyticus* isolate (isolate MSC07286) harboring a partial *ftsI* gene of *H. influenzae* at the 3'-terminal region (positions 897 to 1833) was also identified among the culture collection. These

data provide direct evidence for the homologous recombination of the *ftsI* gene between *H. influenzae* and *H. haemolyticus*.

Evidence for intraspecies genetic transfer of the *ftsI* gene. To investigate the genetic transfer of the *ftsI* gene between *H. influenzae* strains, particularly among ampicillin-resistant variants, BLNAR and β -lactamase-positive ampicillin-clavulanic acid-resistant (BLPACR) strains possessing *ftsI* genes with identical nucleotide sequences were selected for genotyping by PFGE (Table 1, strains 1 to 10). Four strains isolated from different hospitals (strains MSC02070, MSC01869, MSC01400, and MSC01855) possessed identical nucleotide sequences for *ftsI* (sequence 1). Another *ftsI* sequence (sequence 2) was found in three strains isolated from three separate hospitals (strains MSC07237, MSC02104, and MSC02149). Strains MSC02022, MSC01363, and MSC01432 were isolated at the same hospital and harbored *ftsI* genes with identical sequences (sequence 3). As shown in Fig. 3A, strains isolated from different hospitals harboring identical *ftsI* genes exhibited distinctive PFGE patterns (lanes 1 to 4 and lanes 5 to 7), indicating that these isolates are phylogenetically independent, despite the identity of their *ftsI* genes. On the other hand, three BLNAR strains harboring identical *ftsI* genes from the same hospital showed the same PFGE pattern (lanes 8 to 10), suggesting their clonal spread.

In vitro transfer of *ftsI* gene from BLNAR to BLNAS. A spontaneous rifampin-resistant mutant of *H. influenzae* Rd, designated Rd^{RIF} (a β -lactamase-nonproducing ampicillin-susceptible [BLNAS] strain), and three BLNAR isolates (isolates MSC06647, MSC06651, and MSC06663) were used to evaluate the genetic transfer of the *ftsI* gene in vitro. The spontaneous rates of rifampin resistance in the BLNAR strains were 4.3×10^{-9} to 8.7×10^{-9} . After the cultures of Rd^{RIF} and one of the BLNAR strains were mixed, mutants resistant to both rifampin and cefdinir were selected. Doubly resistant mutants were ob-

FIG. 3. PFGE patterns of BLNAR and BLPACR isolates, and strains used for in vitro gene transfer. (A) Lane numbers correspond to the strain numbers in Table 1. Identical *ftsI* genes were identified among strains 1 to 4 (*ftsI* sequence 1), 5 to 7 (*ftsI* sequence 2), and 8 to 10 (*ftsI* sequence 3). (B) Lane numbers 11 to 14 correspond to the strain numbers in Table 1. Lanes 15, 16, and 17, PFGE patterns of rifampin- and cefdinir-resistant mutants from MSC06647 (Rd^{RIF}/MSC06647), MSC06651 (Rd^{RIF}/MSC06651), and MSC06663 (Rd^{RIF}/MSC06663), respectively. Lanes M, molecular size marker (bacteriophage lambda ladder DNA).

tained after the coinoculation of Rd^{RIF} with MSC06647, MSC06651, and MSC06663 at frequencies of 5.1×10^{-7} , 1.2×10^{-6} , and 1.5×10^{-6} , respectively (Table 2). Treatment with DNase I at 100 U totally abolished the emergence of doubly resistant mutants (below the lower limit of detection). Nucleotide sequencing of the *ftsI* genes from doubly resistant mutants revealed that the mutants possessed sequences identical to that of each parent BLNAR strain (data not shown). As shown in Table 3, the susceptibilities of the mutant clones (clones Rd^{RIF}/MSC06647, Rd^{RIF}/MSC06651, and Rd^{RIF}/MSC06663) to rifampin and fosfomycin were equivalent to those of Rd^{RIF}. Moreover, as predicted from the *ftsI* sequence, the susceptibility of these mutants to cefdinir was reduced to a level similar to that of each of the parent BLNAR strains, suggesting that doubly resistant mutants were derivatives of the Rd^{RIF} strain that acquired the *ftsI* gene from each BLNAR strain. The genotypes of the doubly resistant mutants were confirmed by PFGE (Fig. 3B). Doubly resistant mutants (lanes 15 to 17) had the same pattern with Rd^{RIF} (lane 14) and had patterns that were divergent from those of the BLNAR strains (lanes 11 to 13).

TABLE 2. Transfer of *ftsI* genes from BLNAR to Rd^{RIF} (BLNAS)

Strains (recipient × donor)	Frequency of rifampin- and cefdinir-resistant cells ^a	
	Without DNase I	With DNase I
Rd ^{RIF} × MSC06647	5.1×10^{-7}	$<2.6 \times 10^{-8}$
Rd ^{RIF} × MSC06651	1.2×10^{-6}	$<2.6 \times 10^{-8}$
Rd ^{RIF} × MSC06663	1.5×10^{-6}	$<2.5 \times 10^{-8}$

^a The averages of three individual experiments are shown.

TABLE 3. Susceptibilities of parent strains and doubly resistant derivatives

Strains	MIC (μg/ml)		
	Rifampin	Cefdinir	Fosfomycin
Rd	0.5	0.25	2
Rd ^{RIF}	>128	0.25	2
MSC06647	0.25	1	0.12
MSC06651	0.5	8	256
MSC06663	0.5	2	0.06
Rd ^{RIF} /MSC06647	>128	2	2
Rd ^{RIF} /MSC06651	>128	8	1
Rd ^{RIF} /MSC06663	>128	8	2

DISCUSSION

The nucleotide sequences of the *ftsI* gene of BLNAR strains are quite different from that of the *H. influenzae* Rd strain. For instance, the sequences of *ftsI* from strains MSC02070 (sequence 1; GenBank accession no. AB267863), MSC07237 (sequence 2; GenBank accession no. AB257096), and MSC02022 (sequence 3; GenBank accession no. AB267864), the strains used in PFGE analysis, differed from that of Rd by 2.7% (49 bp of 1,833 bp), 3.0% (55 bp of 1,833 bp), and 5.6% (102 bp of 1,833 bp), respectively. The existence of genotypically divergent strains harboring identical *ftsI* genes (Fig. 3A) strongly suggests the occurrence of genetic transfer of the *ftsI* gene in *H. influenzae*. The existence of USSs in the middle of and just downstream of the *ftsI* gene supports the efficient uptake and recombination of this gene (Fig. 2), resulting in horizontal gene transfer among species.

Extensive sequence analysis of the *ftsI* genes from numerous clinical isolates of *H. influenzae* identified a mosaic structure of the *ftsI* gene in some strains. Complete sequences of the *ftsI* genes from *Haemophilus* species other than *H. influenzae*, such as *H. parainfluenzae*, *H. haemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, and *H. segnis*, were determined and compared with that of the mosaic *ftsI* gene. Homologous recombination of the *ftsI* gene derived from *H. haemolyticus* was identified in some isolates (Fig. 2). Moreover, an *H. haemolyticus* strain harboring a partial *ftsI* gene from *H. influenzae* was also found. This is the first report describing the homologous recombination of the gene encoding a PBP in *H. influenzae*. It has been reported that other naturally competent gram-negative bacteria, *Neisseria* species (*Neisseria gonorrhoeae* and *N. meningitidis*), develop resistance to β-lactams by acquiring a mosaic-like structure of PBP 2, encoded by the *penA* gene. PBP 2 of *Neisseria* species is a homologue of PBP 3 in *H. influenzae* (36% amino acid sequence identity). It has been suggested that the horizontal genetic exchange of *penA* genes between commensal *Neisseria* species, such as *N. cinerea*, *N. perflava*, and *N. flavescens*, resulted in the mosaic structure of PBP 2 in *N. gonorrhoeae* and *N. meningitidis* (17). As *H. haemolyticus* is often recovered from the oropharyngeal area (10), it is possible that genetic exchange between colocalized *H. influenzae* and *H. haemolyticus* isolates or other *Haemolyticus* species takes place.

The molecular evolution of BLNAR strains has been considered to occur through the acquisition of point mutations in the *ftsI* gene by antibiotic pressure. However, our results raise the possibility of the development of resistance through ho-

FIG. 4. Partial alignment of *ftsI* genes of *H. influenzae* Rd, four BLNAR strains (strains MSC06651, MSC06663, MSC02070, and MSC07237), and *H. haemolyticus* ATCC 33390. The nucleotide sequences from positions 1001 to 1200 and positions 1501 to 1600 are shown. Sequences coding for the conserved motifs, SSN and KTG, are boxed. Amino acid sequences associated with β -lactam resistance are shown above the corresponding nucleotide sequences. Dots, identical nucleotides.

mologous recombination from related species, which is a well-recognized mechanism known in *N. gonorrhoeae* (17) and *Streptococcus pneumoniae* (5, 9). As shown in Fig. 4, the nucleotide sequences of BLNAR strains are highly similar to that of *H. haemolyticus* around the region encoding the conserved SSN motif. Indeed, PBP 3 of *H. haemolyticus*, as well as those of BLNAR strains, possesses 377-Ile, which is identified as the amino acid residue associated with β -lactam resistance. Therefore, the *ftsI* gene of *H. haemolyticus* and/or related species might be the source of BLNAR. On the other hand, no sequence homology was observed in the region downstream of the KTG motif between BLNAR strains and *H. haemolyticus* or other *Haemophilus* species. These observations suggest that primary mutations of BLNAR, i.e., Asn526Lys and Arg517His, have emerged from the point mutation during antibiotic pressure.

A recent study of the bacterial flora in patients with acute upper respiratory tract infections and healthy subjects revealed that *H. influenzae* strains are frequently detected in the nasopharynxes from both groups aged 6 years or younger (8). Therefore, the colocalization of or coinfection with multiple *H. influenzae* strains could happen in some children, giving the opportunity for *H. influenzae* strains to exchange DNA. In Japan, oral cephalosporins are extensively used for the treatment of upper respiratory tract infections in children. BLNAR strains harboring mutations in PBP 3 are resistant to most of the oral cepheims but are not resistant to cefditoren. The use of those cepheims with poor activities against BLNAR strains not only results in the failure of the treatment (i.e., the failure to eliminate BLNAR strains) but also leads to the selection of BLNAR strains mediated by genetic transfer of the mutated *ftsI* gene to BLNAS (i.e., the failure to prevent the emergence of BLNAR strains).

Direct evidence for horizontal gene transfer of the *ftsI* gene

between *H. influenzae* strains was obtained from in vitro gene transfer experiments. Coincubation of BLNAS and BLNAR strains resulted in the transfer of the *ftsI* gene from BLNAR to a BLNAS strain and recombination (Table 2). Addition of DNA fragments of the *ftsI* gene derived from BLNAR strains by PCR amplification, at final concentrations of 1 to 10 ng/ml, to the culture of BLNAS (strain Rd^{RIF}) had equivalent effects on the emergence of rifampin- and cefdinir-resistant mutants as coincubation of BLNAR and BLNAS strains (data not shown). Treatment with 100 U of DNase I abolished the emergence of resistant mutants not only after the transformation of DNA fragments to Rd^{RIF} strain but also after the coincubation of BLNAS and BLNAR strains. Although cell-to-cell contact has previously been implicated in chromosomal gene transfer (1), the mechanism of in vitro gene transfer of the *ftsI* gene was found to involve classical transformation.

In conclusion, horizontal transfer of the *ftsI* gene in *H. influenzae* was identified for the first time. The transfer of mutated *ftsI* genes might be associated with the rapid increase in BLNAR strains in Japan. Additionally, in vitro gene transfer experiments will be useful in evaluating the evolution of BLNAR strains.

ACKNOWLEDGMENT

We thank Kimiko Ubukata (Kitasato University) for kindly providing clinical isolates of *H. influenzae*.

REFERENCES

- Albritton, W. L., J. K. Setlow, and L. Slaney. 1982. Transfer of *Haemophilus influenzae* chromosomal genes by cell-to-cell contact. *J. Bacteriol.* **152**:1066-1070.
- Bakkali, M., T.-Y. Chen, H. C. Lee, and R. J. Redfield. 2004. Evolutionary stability of DNA uptake signal sequences in the Pasteurellaceae. *Proc. Natl. Acad. Sci. USA* **101**:4513-4518.
- Clinical and Laboratory Standards Institute. 2006. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.

4. Dewhirst, F. E., B. J. Paster, I. Olsen, and G. J. Fraser. 1992. Phylogeny of 54 representative strains of species in the family *Pasteurellaceae* as determined by comparison of 16S rRNA sequences. *J. Bacteriol.* **174**:2002–2013.
5. Dowson, C. G., A. Hutchison, J. A. Brannigan, R. C. George, D. Hansman, J. Linares, A. Tomasz, J. M. Smith, and B. G. Spratt. 1989. Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. USA* **86**:8842–8846.
6. Goodgal, S. H., and M. A. Mitchell. 1990. Sequence and uptake specificity of cloned sonicated fragments of *Haemophilus influenzae* DNA. *J. Bacteriol.* **172**:5924–5928.
7. Hiltke, T. J., A. T. Schiffmacher, A. J. Dagonese, S. Sethi, and T. F. Murphy. 2003. Horizontal transfer of the gene encoding outer membrane protein P2 of nontypeable *Haemophilus influenzae*, in a patient with chronic obstructive pulmonary disease. *J. Infect. Dis.* **188**:114–117.
8. Konno, M., S. Baba, H. Mikawa, K. Hara, F. Matsumoto, K. Kaga, T. Nishimura, T. Kobayashi, N. Furuya, H. Moriyama, Y. Okamoto, M. Furukawa, N. Yamanaka, T. Matsushima, Y. Yoshizawa, S. Kohno, K. Kobayashi, A. Morikawa, S. Koizumi, K. Sunakawa, M. Inoue, and K. Ubukata. 2006. Study of upper respiratory tract bacterial flora: first report. Variations in upper respiratory tract bacterial flora in patients with acute upper respiratory tract infection and healthy subjects and variations by subject age. *J. Infect. Chemother.* **12**:83–96.
9. Laible, G., B. G. Spratt, and R. Hakenbeck. 1991. Interspecies recombinational events during the evolution of altered PBP 2X genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Mol. Microbiol.* **5**:1993–2002.
10. Martel, A. Y., G. St-Laurent, L. A. Dansereau, and M. G. Bergeron. 1989. Isolation and biochemical characterization of *Haemophilus* species isolated simultaneously from the oropharyngeal and anogenital areas. *J. Clin. Microbiol.* **27**:1486–1489.
11. Martin, K., G. Morlin, A. Smith, A. Nordyke, A. Eisenstark, and M. Golomb. 1998. The tryptophanase gene cluster of *Haemophilus influenzae* type b: evidence for horizontal gene transfer. *J. Bacteriol.* **180**:107–118.
12. Osaki, Y., Y. Sanbongi, M. Ishikawa, H. Kataoka, T. Suzuki, K. Maeda, and T. Ida. 2005. Genetic approach to study the relationship between penicillin-binding protein 3 mutations and *Haemophilus influenzae* β -lactam resistance by using site-directed mutagenesis and gene recombinants. *Antimicrob. Agents Chemother.* **49**:2834–2839.
13. Saito, M., A. Umeda, and S. Yoshida. 1999. Subtyping of *Haemophilus influenzae* strains by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* **37**:2142–2147.
14. Sanbongi, Y., T. Suzuki, Y. Osaki, N. Senju, T. Ida, and K. Ubukata. 2006. Molecular evolution of β -lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. *Antimicrob. Agents Chemother.* **50**:2487–2492.
15. Smith, H. O., M. L. Gwinn, and S. L. Salzberg. 1999. DNA uptake signal sequences in naturally transformable bacteria. *Res. Microbiol.* **150**:603–616.
16. Smith-Vaughan, H. C., K. S. Sriprakash, J. D. Mathews, and D. J. Kemp. 1997. Nonencapsulated *Haemophilus influenzae* in aboriginal infants with otitis media: prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer. *Infect. Immun.* **65**:1468–1474.
17. Spratt, B. G., L. D. Bowler, Q. Y. Zhang, J. Zhou, and J. M. Smith. 1992. Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. *J. Mol. Evol.* **34**:115–125.
18. Trepod, C. M., and J. E. Mott. 2005. Elucidation of essential and nonessential genes in the *Haemophilus influenzae* Rd cell wall biosynthetic pathway by targeted gene disruption. *Antimicrob. Agents Chemother.* **49**:824–826.
19. Ubukata, K., Y. Shibasaki, K. Yamamoto, N. Chiba, K. Hasegawa, Y. Takeuchi, K. Sunakawa, M. Inoue, and M. Konno. 2001. Association of amino acid substitutions in penicillin-binding protein 3 with β -lactam resistance in β -lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Antimicrob. Agents Chemother.* **45**:1693–1699.