

Mosaic-Like Structure of Penicillin-Binding Protein 2 Gene (*penA*) in Clinical Isolates of *Neisseria gonorrhoeae* with Reduced Susceptibility to Cefixime

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Neisseria gonorrhoeae strains with reduced susceptibility to cefixime (MICs, 0.25 to 0.5 µg/ml) were isolated from male urethritis patients in Tokyo, Japan, in 2000 and 2001. The resistance to cepheims including cefixime and penicillin was transferred to a susceptible recipient, *N. gonorrhoeae* ATCC 19424, by transformation of the penicillin-binding protein 2 gene (*penA*) that had been amplified by PCR from a strain with reduced susceptibility to cefixime (MIC, 0.5 µg/ml). The sequences of *penA* in the strains with reduced susceptibilities to cefixime were different from those of other susceptible isolates and did not correspond to the reported *N. gonorrhoeae penA* gene sequences. Some regions in the transpeptidase-encoding domain in this *penA* gene were similar to those in the *penA* genes of *Neisseria perflava* (*N. sicca*), *Neisseria cinerea*, *Neisseria flavescens*, and *Neisseria meningitidis*. These results showed that a mosaic-like structure in the *penA* gene conferred reductions in the levels of susceptibility of *N. gonorrhoeae* to cepheims and penicillin in a manner similar to that found for *N. meningitidis* and *Streptococcus pneumoniae*.

Gonococcal infections have existed as sexually transmitted diseases since early times and have never been regarded as intractable diseases. In Japan, the numbers of gonococcal infections, including those resistant to antimicrobial therapy, have gradually increased since the mid-1990s (11).

Penicillins and tetracyclines are used for the treatment of gonococcal urethritis worldwide. After the emergence and worldwide spread of penicillin- and tetracycline-resistant *Neisseria gonorrhoeae* strains, fluoroquinolones were recommended as the primary therapy for uncomplicated gonorrhea in many countries (24). Fluoroquinolones have been used extensively for the treatment of gonococcal urethritis due to their high degrees of efficacy against the disease. Intense selective pressure resulting from the continual exposure of *N. gonorrhoeae* to fluoroquinolones resulted in the emergence of resistant strains with altered GyrA and ParC proteins (3, 6, 21, 22, 23). In recent years, expanded-spectrum oral cepheims have been widely used instead of fluoroquinolones for the treatment of gonorrhea in Japan. However, the emergence and spread of gonococci resistant to oral cepheims have been reported (1, 13).

N. gonorrhoeae has three penicillin-binding proteins (PBPs), denoted PBPs 1, 2, and 3. PBPs 1 and 2 of *N. gonorrhoeae* are the major targets of β-lactam antibiotics. PBP 2, encoded by the *penA* gene, has an approximately 10-fold higher affinity for penicillin than PBP 1 (7). In previous reports, insertion of the Asp-345A codon into the *penA* gene has been proved to make

a major contribution to the reduction of the affinity of gonococcal PBP 2 to penicillin (5). Other reports showed that C-terminal amino acid residues of the *penA* transpeptidase domain were also altered in penicillin-resistant *N. gonorrhoeae* (8, 18, 19). Enhancement of the efflux pump by mutations in the *mrR* and *penB* loci was reported to be due to β-lactam resistance (9, 10).

In 2000 we isolated gonococcal strains with reduced susceptibilities to penicillin and cepheims including cefixime, which is recommended as therapy for gonococcal urethritis, during an investigation into the cause of clinical failure in patients with gonococcal urethritis treated with oral cepheims. This study was conducted to investigate the susceptibilities to various antimicrobials of clinical isolates of *N. gonorrhoeae* recently isolated in Japan and to clarify the mechanism of reduced susceptibility to cefixime in *N. gonorrhoeae*.

MATERIALS AND METHODS

Bacteria and media. The *N. gonorrhoeae* strains used in this study were clinical strains isolated from male urethritis patients at the School of Medicine, Jikei University, and related hospitals in 2000 (February to July) and 2001 (February to March). The specimens were directly streaked onto Thayer-Martin selective agar (Becton Dickinson, Cockeysville, Md.) in the hospitals. The plates were placed in a Bio-Bag environmental chamber (type C; Becton Dickinson) and immediately transported to the laboratory, where they were incubated at 35°C for 48 h in a 5% CO₂ atmosphere. The organisms were identified by Gram staining and by oxidase and catalase tests. The identities of isolates cultured on Chocolate II agar (Becton Dickinson) were further confirmed with a Gonochek-II kit (EY Laboratories, San Mateo, Calif.). *N. gonorrhoeae* isolates were maintained at –80°C in modified skim milk (15) until antimicrobial susceptibility testing. The isolates were tested for β-lactamase production by a nitrocefin method. In the antibiotic susceptibility test, 53 and 24 strains isolated in 2000 and 2001, respectively, were used. The *penA* sequences of six of the

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TABLE 1. Oligonucleotides used in this study

Oligo-nucleotide	Sequence
NGPA-F	5'-TCGGGCAATACCTTTATGGTGGAAACAT-3'
NGPA-R	5'-ACAACGGCGGGGATATAACT-3'
Fs1	5'-CAAAGATAGAAGCAGCCTGTGT-3'
Fs2	5'-GATATTGACGGCAAAGGTCAGGAAGGT-3'
Fs3	5'-CTTTGGATGTGCGGGCATTATGCA-3'
Rs1	5'-GCCGTCGGTATATTCGCCCTGA-3'
Rs2	5'-CAGCCAAAGGGGTTAACTTGCTGAAC-3'
Rs3	5'-TTCTCAACAAACCTGCAGTTTC-3'
Rs4	5'-CTTTGCCGTTTTCGGGGGCTTTATTGC-3'
Aat	5'-CATCCAAAGAGGGGTAAACATGGGTGACG-3'
r1	5'-GCGCAACCGTGCCGTAACCGATATGATCG-3'

clinical gonococcal isolates described above (strains NG-3, NG-12, NG-25, NG-46, NG-48, and NG-83) were used. The bacteria were grown at 37°C under a 5% CO₂ atmosphere on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) including 5% sheep defibrinated blood (Nippon Bio-Test Laboratories Inc., Tokyo, Japan) for 48 h.

Susceptibility testing and antimicrobials. The MICs were determined by an agar dilution method according to the approved guidelines of the National Committee for Clinical Laboratory Standards (14). The following reference antimicrobials were used: penicillin G (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan); piperacillin, tazobactam-piperacillin, and cefteteram (Toyama Chemical Co., Ltd., Tokyo, Japan); ceftriaxone (Nippon Roche Co., Ltd., Tokyo, Japan); flomoxef (Shionogi Pharmaceutical Co., Ltd., Osaka, Japan); aztreonam (Eizai Co., Ltd., Tokyo, Japan); spectinomycin and minocycline (Sigma Chemical Co., St. Louis, Mo.); cefixime and cefdinir (Fujisawa Co., Ltd., Osaka, Japan); cefpodoxime (GlaxoSmithKline Japan, Tokyo, Japan); cefodizime (Kyorin Pharmaceutical Co., Ltd., Tokyo, Japan); and levofloxacin (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). Cefixime, cefdinir, cefpodoxime, and levofloxacin were extracted from commercially available capsules or tablets. The purities of these four agents were above 99.8%, as measured by high-performance liquid chromatography (HPLC).

Genetic transformation. Genomic DNA was prepared from an *N. gonorrhoeae* strain with reduced susceptibility to cefixime (strain NG-3). The *penA* amplicon used for transformation was amplified by PCR as follows. Bacteria were suspended in 50 µl of distilled water, subjected to one freeze-thaw cycle, heated at 100°C for 3 min, and then centrifuged at 10,000 × g for 5 min. The full-length gene was amplified by PCR from the supernatant with oligonucleotides NGPA-F and NGPA-R (Table 1) and Ex *Taq* polymerase (Takara Shuzo, Kyoto, Japan). PCR was performed as follows: 5 min of denaturation at 94°C and 35 cycles of

TABLE 3. MICs of various antibiotics for *N. gonorrhoeae* ATCC 19424, transformant S1-05, and NG-3

Antibiotic	MIC (µg/ml)			MIC ratio ^a
	ATCC 19424	Transformant (S1-05)	NG-3	
Penicillin G	0.004	0.25	2	64
Piperacillin	0.0005	0.001	0.0625	2
Tazobactam-piperacillin	0.0005	0.002	0.125	4
Cefixime	0.001	0.0625	0.5	64
Cefteteram	0.004	0.125	0.5	32
Cefdinir	0.001	0.125	1	128
Cefpodoxime	0.001	0.125	2	128
Ceftriaxone	0.00025	0.002	0.0625	8
Cefodizime	0.00025	0.004	0.125	16
Aztreonam	0.008	1	8	128
Flomoxef	0.0313	0.5	2	16
Levofloxacin	<0.004	<0.004	8	NC ^b
Spectinomycin	2	1	4	0.5

^a MIC for transformant/MIC for ATCC 19424.

^b NC, not calculated.

denaturation at 94°C for 1 min, annealing at 60°C for 0.5 min, and extension at 72°C for 2 min, concluding with a final extension at 72°C for 5 min. Transformation for homologous recombination of the *penA* gene was done with the PCR amplicon and by incubation under static conditions. Transformants were selected on plates containing cefixime at a concentration of 0.0313 µg/ml.

Nucleotide sequence of *N. gonorrhoeae penA* gene. The full-length *penA* gene was amplified by PCR with oligonucleotides NGPA-F and NGPA-R (Table 1). The amplicons were purified with a PCR product presequencing kit (Amersham Pharmacia Biotech, Tokyo, Japan). The cycling reaction was performed with Thermo Sequenase DNA polymerase (Amersham Pharmacia Biotech) and oligonucleotides Fs1, Fs2, Fs3, Rs1, Rs2, Rs3, and Rs4 (Table 1). Sequencing was carried out with a DSO-1000 sequencer (Shimadzu, Kyoto, Japan). Primer Fs1-3 was used for sequencing of the forward sequence, and primer Rs1-4 was used for sequencing of the reverse sequence (Table 1).

Restriction fragment length polymorphism analysis of *penA* gene. The amplicon obtained by PCR with primers Aat and r1 (Table 1) was digested with the restriction endonuclease *AatII* (New England Biolabs, Inc., Beverly, Mass.). Restriction digests were analyzed by electrophoresis on 4% agarose gels (Agarose X; Nippon Gene, Toyama, Japan). Primer Aat makes a site that is digested with *AatII* if the GAC codon Asp-345A is inserted in the *penA* gene.

TABLE 2. Susceptibilities of clinical isolates of *N. gonorrhoeae* from male urethritis patients in 2000 and 2001

Antibiotic	MIC (µg/ml)				MIC ratio ^a	
	2000 (n = 53)		2001 (n = 24)		50%	90%
	50%	90%	50%	90%		
Penicillin G	0.5	2	1	2	2	1
Piperacillin	0.0313	0.0625	0.0625	0.125	2	2
Tazobactam-piperacillin	0.0625	0.125	0.0625	0.125	1	1
Cefixime	0.008	0.25	0.0313	0.25	4	1
Cefteteram	0.0156	0.5	0.0625	0.25	4	0.5
Cefdinir	0.0156	1	0.0313	1	2	1
Cefpodoxime	0.0156	1	0.125	1	8	1
Ceftriaxone	0.004	0.0625	0.0156	0.0313	4	0.5
Cefodizime	0.0156	0.0625	0.0313	0.0625	2	1
Aztreonam	0.125	4	0.5	4	4	1
Flomoxef	0.5	2	2	4	4	2
Levofloxacin	0.25	4	4	>4	16	NC ^b
Spectinomycin	4	4	ND ^c	ND		
Minocycline	0.25	0.5	ND	ND		

^a MIC for 2001/MIC for 2000.

^b NC, not calculated.

^c ND, not determined.

1

MetLeuIleLysSerGluTyrLysProArgMetLeuProLysGluGluGln

A CAAAGATAGAAAGCAGCCTGTGTGCCGAATCGGATTCCTGCATCAGGATAATAATAACGAGAAGTAAAAATGTTGATTAAAGCGAATATAAGCCCGGATGCTGCCCAAGAGAGCAG

B -----

C -----

18

ValLysLysProMetThrSerAsnGlyArgIleSerPheValLeuMetAlaMetAlaValLeuPheAlaCysLeuIleAlaArgGlyLeuTyrLeuGlnThrValThrTyrAsnPheLeu

A GTCAAAAACCGGATGACCACTAACGGACGGATTAGCTTCCTGCTGATGGCAATGGCGGCTCTTGTTCGCTGTCTGATTGCCCGGGGCTGTACTCGACAGCGTAACTATAACTTTTGG

B -----

C -----

58

LysGluGlnGlyAspAsnArgIleValArgThrGlnAlaLeuPheAlaThrArgGlyThrValSerAspArgAsnGlyAlaValLeuAlaLeuSerAlaProThrGluSerLeuPheAla

A AAAGAACAGGGCGCAACCGGATTGTGCGGACTCAAGCATTGCGGGTACACGCGTACGGTTTCGGACCGGAACGGTGGCGTTTGGCGTTGAGCGCGCCGACGGAGTCCCTGTTGGC

B -----

C -----

98

ValProLysAspMetLysGluMetProSerAlaAlaGlnLeuGluArgLeuSerGluLeuValAspValProValAspValLeuArgAsnLysLeuGluGlnLysGlyLysSerPheIle

A GTGCCAAAGATATGAAGGAAATGCCGCTCGCCGCCAATTGGAACGCTGTCCAGCTTGTGATGTCGCGGTCGATGTTTGGAGGACAACTCGACAGAGAGGCAAGCTGTTTATT

B --A-----G-----T-----T-----A-----C

C -----

138

TrpIleLysArgGlnLeuAspProLysValAlaGluGluValLysAlaLeuGlyLeuGluAsnPheValPheGluLysGluLeuLysArgHisTyrProMetGlyAsnLeuPheAlaHis

A TGGATCAAGCGAGCTCGATCCCAAGTGTCCGAGAGGTCAAGCCTTGGGTTTGGAAACTTTGTATTGAAAAGAAATTAACACGCCATTCACCGATGGCAACCTGTTTGCACAC

B -----

C -----

178

ValIleGlyPheThrAspIleAspGlyLysGlyGlnGluGlyLeuGluLeuSerLeuGluAspSerLeuTyrGlyGluAspGlyAlaGluValValLeuArgAspArgGlnGlyAsnIle

A GTCATCGATTTACCGATATTGACGGCAAGGTTCAGGAGGTTTGGAACTTTCGCTGAAAGACAGCCTGTATGGCGAAGACGGCGCGGAAGTGTGTTTFCGGGACCGCGAGGCAATATT

B -----

C -----

218

ValAspSerLeuAspSerProArgAsnLysAlaProGlnAsnGlyLysAspIleIleLeuSerLeuAspGlnArgIleGlnThrLeuAlaTyrGluGluLeuAsnLysAlaValGluTyr

A GTGGACAGCTTGGACTCCCGCGCAATAAAGCACCGCAACCGCAAGACATCATCTTCCCTCGATCAGAGGATTCAGACCTTGGCCTATGAAGAGTTGAACAGCGGTCGAATAC

B -----

C -----

258

HisGlnAlaLysAlaGlyThrValValLeuAspAlaArgThrGlyGluIleLeuAlaLeuAlaAsnThrProAlaTyrAspProAsnArgProGlyArgAlaAspSerGluGlnArg

A CATCAGGCAAAAGCGGAAACGGTGGTGGTTTGGATGCCCGACGGGGGAAATCTCGCCTTGGCCAAACGCGCCCTACGATCCCAACAGACCCCGCGGCGAGCAGCGAAGCAGCGG

B -----

C -----

298

ArgAsnArgAlaValThrAspMetIleGluProGlySerAlaIleLysProPheValIleAlaLysAlaLeuAspAlaGlyLysThrAspLeuAsnGluArgLeuAsnThrGlnProTyr

A CGCAACCGTCCGCTAACCGATATGATCGAACCTGGTTCGGCAATCAAACCGTTCGATGTCGGAAGCATTGGATCGGGCAAAACCGATTGGAACGACGGCTGAAATACCGACCTTAT

B -----

C -----

338

LysIleGlyProSerProValArg... AspThrHisValTyrProSerLeuAspValArgGlyIleMetGlnLysSerSerAsnValGlyThrSerLysLeuSerAlaArgPheGlyAla

A AAAATCGGACCGTCCCGTCCGCGCC... GATACCCATGTTACCCCTCTTGGATGTCCCGGCATTTATGAGAAATCTCCAACGTCGGCACAACAACAGTCTGCGCTGTTCGGCGCC

B -----

C -----

377

GluGluMetTyrAspPheTyrHisGluLeuGlyIleGlyValArgMetHisSerGlyPheProGlyGluThrAlaGlyLeuLeuArgAsnTrpArgArgTrpArgProIleGluGlnAla

A GAAGAAATGTATGACTTCTATCATGAATFGGSCATCGGTGTGCGTATGCACTCGGGCTTCCCGGGGAACTGCAGGTTTGTGAGAAATGGCGCAGTGGCGGCCCATCGAACAGGG

B -----

C -----

417

ThrMetSerPheGlyTyrGlyLeuGlnSerLeuLeuAlaAlaGlnAlaThrAlaLeuThrHisAspGlyValLeuLeuProLeuSerPheGluLysGlnAlaValAlaPro

A ACCGATGCTTTCGGTTACGGTCTGCAATTGAGCCTGCTGCAATTGGCGCGCGCTATACCGCACTGACGCACGACGGCCTTGTGCTGCGCTCAGCTTTGAGAAGCAGGGCGGTTGCCGCG

B -----

C -----

457

GlnGlyLysArgIlePheLysGluSerThrAlaArgGluValArgAsnLeuMetValSerValThrGluProGlyGlyThrGlyThrAlaGlyAlaValAspGlyPheAspValGlyAla

A CAAGGCAAAACGATATTCAGAAATCGACCGCGCGGAGGTACGCAATCTGATGGTTCCGTAACCGAGCCGGCGGACCGGTACGGCGGGTGGGTTGACCGGTTTCGATGTCCGGCT

B -----

C -----

497

LysThrGlyThrAlaArgLysPheValAsnGlyArgTyrAlaAspAsnLysHisValAlaThrPheIleGlyPheAlaProAlaLysAsnProArgValIleValAlaValThrIleAsp

A AAAACCGGACCGGGCGCAAGTTCGTAACCGGGCTTATCCCGACACAAACACGTCGCTACCTTTATCGGTTTTCGCCCCCAAAAACCCCGCTGTGATTTGGCGGTAACCATCGAC

B -----

C -----

537

GluProThrAlaHisGlyTyrGlyValValAlaGlyProProPheLysLysIleMetGlyGlySerLeuAsnIleLeuGlyIleSerProThrLysProLeuThr... AlaAla

A GAACCGACTGCCACGGGTATTACGGCGCGGTAGTGGCAGGCGCGCCTTCAAAAAAATTTGGCGCGCAGCCTGAACATCTTGGCATTTCCCGGACCAAGCAGCTGACC...GCGCA

B -----

C -----

576

AlaValLysThrProSer

A GCGGTCAAAACACCGCTTAATCCGAGTATCAACGAGATGTTTATGTTTCAGCAAGTTAAGCCCTTGGCTGAAACCGGCATCCCGACCTGTCTGTGCAAAACCGGGCAGGGCGTTTG

B -----

C -----

A TTGCATTGACAGCGGTCAAATCAA

B -----

C -----

Nucleotide sequence accession number. The *penA* sequence of *N. gonorrhoeae* NG-3 has been deposited in the DDBJ data library under accession number AB071984.

RESULTS

Antimicrobial susceptibility and β -lactamase production.

The MICs of various antimicrobials and β -lactamase production were determined for 53 and 24 clinical isolates recovered in 2000 and 2001, respectively. The MICs at which 50% of isolates are inhibited (MIC₅₀s) and the MIC₉₀s of various antimicrobials for the clinical isolates are shown in Table 2. Nine of 53 strains (17.0%) isolated in 2000 and 4 of 24 strains (16.7%) isolated in 2001 showed reduced susceptibilities to cefixime (MICs, 0.25 and 0.5 μ g/ml, respectively). These strains also exhibited reduced susceptibilities to penicillin and other β -lactams, and some of them were cross-resistant to fluoroquinolones, spectinomycin, and minocycline. There were no apparent differences in the MIC₉₀s of any antimicrobials for the strains isolated in 2000 and 2001. However, the MIC₅₀s of some β -lactams for the isolates recovered in 2001 were four- to eightfold higher than those for the isolates recovered in 2000. β -Lactamase production was not detected in any of the clinical isolates tested.

Antimicrobial susceptibility of the transformant with the *penA* gene derived from an *N. gonorrhoeae* strain with reduced susceptibility to cefixime. To investigate whether a reason for the reduced susceptibility to cefixime was alteration of PBP 2, the *penA* gene derived from strain NG-3, which had reduced susceptibility to cefixime, was transformed into *N. gonorrhoeae* ATCC 19424 (cefixime MIC, 0.001 μ g/ml). After transformation of the *penA* gene, many transformants were obtained on plates containing 0.0313 μ g of cefixime per ml. These transformants had similar susceptibility profiles. Table 3 shows the susceptibilities of the recipient (ATCC 19424) and one of the transformants (S1-05). The MICs of cefixime and ceftriaxone for the transformant were 0.0625 and 0.002 μ g/ml, respectively. The susceptibilities of the transformant to penicillin G, cefixime, cefdinir, cefpodoxime, and aztreonam were reduced 64- to 128-fold, and those to piperacillin and ceftriaxone were reduced 2- to 8-fold. There were some discrepancies in antimicrobial susceptibilities between the transformant and a clinical isolate, NG-3, the donor of the resistance gene.

Sequences of *penA* genes in strains with reduced susceptibilities to cefixime. The full-length *penA* sequences were determined by using five strains (strains NG-3, NG-25, NG-46, and NG-48, isolated in 2000, and strain NG-83, isolated in 2001) with reduced susceptibilities to cefixime (MICs, 0.5 and 0.25 μ g/ml for the strains isolated in 2000 and 2001, respectively) and one cefixime-susceptible strain (strain NG-12, isolated in 2000; cefixime MIC, 0.008 μ g/ml). Figure 1 shows the full-length sequences of the *penA* genes of NG-3 (Fig. 1B) and NG-12 (Fig. 1C). In cefixime-susceptible strain NG-12, the

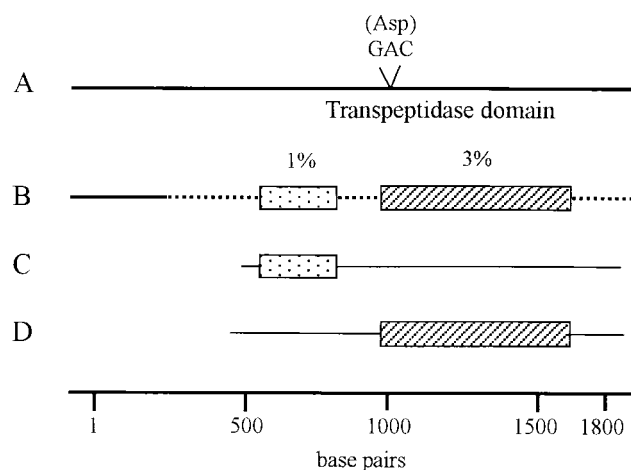


FIG. 2. Schematic representation of mosaic-like *penA* genes of *Neisseria* strains. The *penA* gene of *N. gonorrhoeae* and the coding region for PBP 2 are represented in the diagram. The *penA* genes of cefixime-susceptible *N. gonorrhoeae* strain NG-12 (A), an *N. gonorrhoeae* strain with reduced susceptibility to cefixime (strain NG-3) (B), *N. cinerea* strain LPN3173 (C), and *N. perflava* (*N. sicca*) strain 1654/1659 (D) are shown. The nucleotide sequence divergences (in percent) between regions of the *N. gonorrhoeae* NG-3 *penA* genes and the corresponding regions in the *penA* genes of *N. cinerea* LPN3173 (▨) and *N. perflava* (*N. sicca*) 1654/1659 (▩) are shown.

penA gene sequence corresponded to that of penicillin-susceptible *N. gonorrhoeae* LM306 (GenBank accession no. M320921; Fig. 1A) except for an extra aspartate codon and an extra 2 bp. The *penA* gene of strain NG-3, which had reduced susceptibility to cefixime, did not have the extra codon (Fig. 1B), and the sequence was not consistent with the sequence reported in the database. Of 581 amino acids in the PBP 2 sequence of NG-3, 59 (10.2%) amino acids were different from the sequence of NG-12, in addition to 1 amino acid insertion and a defect.

As a result of restriction fragment length polymorphism analysis, it was found that the *penA* genes of all strains for which cefixime MICs were below 0.125 μ g/ml had an extra aspartate (GCA) codon (data not shown). The sequences of the *penA* genes of strains NG-25, NG-46, NG-48, and NG-83 (strains with reduced susceptibilities to cefixime) were the same as that of NG-3 except for a few mutations (data not shown). These *penA* genes had a mosaic-like structure that included regions that were quite similar to each region of the *penA* genes of *Neisseria perflava* (*Neisseria sicca*) and *Neisseria cinerea* (Fig. 2) as well as to those of *Neisseria flavescens* and *Neisseria meningitidis* (data not shown). This mosaic-like structure was mainly observed in the region of the transpeptidase-encoding domain of *penA*. The active-site serine residue (Ser-X-X-Lys) as well as the Ser-X-Asn and the Lys-Thr-Gly motifs were conserved in the *penA* sequence.

FIG. 1. Nucleotide sequence of *penA* gene of *N. gonorrhoeae*. The sequences of the *penA* genes of penicillin-susceptible strain LM306 (GenBank accession no. M32091) (A), the strain with reduced susceptibility to cefixime (strain NG-3) (B), and cefixime-susceptible strain NG-12 (C) are shown. The insertion of an extra aspartate (Asp-345A) is shown in cefixime-susceptible strain NG-12 but is not shown in the strain with reduced susceptibility to cefixime, NG-3. Asp*, Asp-345A. The Ser-X-X-Lys, Ser-X-Asn, and Lys-Thr-Gly conserved motifs are indicated by underlining.

DISCUSSION

In Japan, the emergence of resistance to cepheims in *N. gonorrhoeae* is a serious concern. A more serious problem, however, is that these isolates are already resistant to non- β -lactam antimicrobials (1, 13). *N. gonorrhoeae* strains with reduced susceptibilities to cefixime from male urethritis patients at hospitals in Tokyo were also resistant to non- β -lactam antimicrobials, including fluoroquinolones. From the results of susceptibility testing with the strains isolated in 2000 and 2001, it was revealed that the numbers of strains with reduced susceptibilities to β -lactams, such as cefixime, ceftazidime, cefdinir, cefpodoxime, and aztreonam, had increased. Similar results were obtained with ceftazidime-resistant *N. gonorrhoeae* strains isolated in Kitakyushu, Japan, for which the cefixime MICs were 0.125 to 0.5 $\mu\text{g/ml}$ (13).

It has been reported that *N. gonorrhoeae* strains with reduced susceptibilities to cepheims evolved by the acquisition of β -lactamases, target modification (alteration of PBPs), alteration of outer membrane transport, or enhancement of MtrCDE efflux pumps (10). β -Lactamase production did not contribute to the resistance in the strains tested in this study because β -lactamase activity was not detected in any of the strains. Transformation of the *penA* gene from a strain with reduced susceptibility to cefixime showed that the reduction in susceptibility to β -lactams was caused by PBP alterations. However, the reasons for the differences in the ratios of the MICs for the transformants to the MICs for the recipients between some β -lactams and the differences in susceptibilities between transformants and clinical isolates have not been identified. The latter reasons for these differences were considered enhancement of efflux pumps, alteration of outer membrane transport, and other PBP mutations.

In previous reports, insertion of the Asp-345A codon into the *penA* gene has proved to make a major contribution to the reduction of the affinity of gonococcal PBP 2 to penicillin (5). In this study, all strains for which cefixime MICs were below 0.125 $\mu\text{g/ml}$ had an extra aspartate codon (Asp-345A) and showed reduced susceptibilities to penicillin, as reported previously (5). On the other hand, this extra codon was not detected in the strains for which cefixime MICs were 0.25 and 0.5 $\mu\text{g/ml}$.

The sequence of the *penA* gene of one strain, NG-3, with reduced susceptibility to cefixime (MIC, 0.5 $\mu\text{g/ml}$) was not completely consistent with the sequence reported in the database and had a mosaic-like structure that included a region whose sequence was quite similar to the sequences of the *penA* genes of *N. perflava* (*N. sicca*) and *N. cinerea* (Fig. 2) as well as those of *N. flavescens* and *N. meningitidis* (data not shown). Similar results have been reported from studies of the sequences of the *penA* genes of penicillin-resistant strains of *N. meningitidis* and *Neisseria* spp. (2, 4, 12, 16, 18, 20). One of the donors conferring the *penA* penicillin resistance gene to *N. meningitidis* has been identified as the naturally penicillin-resistant species *N. flavescens* (20). An *N. gonorrhoeae penA* gene with a mosaic-like structure that confers reduced susceptibility to cefixime might have been constructed by a medley of partial *penA* genes from *N. perflava* (*N. sicca*), *N. cinerea*, *N. flavescens*, and *N. meningitidis*. The reduction of susceptibility to cepheims, including cefixime, in this study might have evolved

by genetic exchange between commensal resistant *Neisseria* spp. and the original susceptible gonococci.

N. gonorrhoeae is one of the bacteria isolated from patients with sexually transmitted diseases. It has recently been reported that, in Japan, *N. gonorrhoeae* has been isolated from areas unrelated to the urethra, such as the pharynx (17). In the present study it was clear that the source of infection was oral sex for two of four patients from whom *N. gonorrhoeae* strains for which the cefixime MIC was 0.5 $\mu\text{g/ml}$ were isolated. We speculate that a *penA* gene with a novel type of mosaic-like structure might have emerged by the transduction of regions from the *penA* genes of *Neisseria* spp. Due to the diversity of commercial sex, *N. gonorrhoeae* can inhabit the pharynx, and gene transformation between *N. gonorrhoeae* and other *Neisseria* spp. might proceed.

Our preliminary study with *penA* genes from isolates with reduced susceptibilities to cefixime (cefixime MICs, 0.0625 to 0.125 $\mu\text{g/ml}$) recovered in 2001 showed that the *penA* genes of these strains also had a mosaic-like structure and did not have the Asp-345A codon insert. This *penA* gene was different from that found in strain NG-3 in the present study (data not shown). The preliminary information presented above and the results obtained in this study suggest that the complicated process concerning the evolution of resistance in *N. gonorrhoeae* might be developing, and more attention should be paid to the emergence of resistance in *Neisseria* spp., including *N. gonorrhoeae*.

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