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High-Level Cefixime- and Ceftriaxone-Resistant *Neisseria gonorrhoeae* in France: Novel *penA* Mosaic Allele in a Successful International Clone Causes Treatment Failure

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Recently, the first *Neisseria gonorrhoeae* strain (H041) highly resistant to the expanded-spectrum cephalosporins (ESCs) ceftriaxone and cefixime, which are the last remaining options for first-line gonorrhea treatment, was isolated in Japan. Here, we confirm and characterize a second strain (F89) with high-level cefixime and ceftriaxone resistance which was isolated in France and most likely caused a treatment failure with cefixime. F89 was examined using six species-confirmatory tests, antibiograms (33 antimicrobials), *porB* sequencing, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), multilocus sequence typing (MLST), and sequencing of known gonococcal resistance determinants (*penA*, *mtrR*, *penB*, *ponA*, and *pilQ*). F89 was assigned to MLST sequence type 1901 (ST1901) and NG-MAST ST1407, which is a successful gonococcal clone that has spread globally. F89 has high-level resistance to cefixime (MIC = 4 μg/ml) and ceftriaxone (MIC = 1 to 2 μg/ml) and resistance to most other antimicrobials examined. A novel *penA* mosaic allele (*penA-CI*), which was *penA-XXXIV* with an additional A501P alteration in penicillin-binding protein 2, was the primary determinant for high-level ESC resistance, as determined by transformation into a set of recipient strains. *N. gonorrhoeae* appears to be emerging as a superbug, and in certain circumstances and settings, gonorrhea may become untreatable. Investigations of the biological fitness and enhanced understanding and monitoring of the ESC-resistant clones and their international transmission are required. Enhanced disease control activities, antimicrobial resistance control and surveillance worldwide, and public health response plans for global (and national) perspectives are also crucial. Nevertheless, new treatment strategies and/or drugs and, ideally, a vaccine are essential to develop for efficacious gonorrhea management.

Gonorrhea is the second most prevalent bacterial sexually transmitted infection worldwide. The etiological agent of gonorrhea, *Neisseria gonorrhoeae*, has developed antimicrobial resistance (AMR) to all previous first-line drugs, e.g., penicillins, tetracycline, and fluoroquinolones (3, 18, 20, 39, 41), leaving the expanded-spectrum cephalosporins (ESCs) ceftriaxone and cefixime as the only antibiotics recommended for treatment of gonococcal infections. Unfortunately, during the past decade, susceptibility to ESCs has decreased globally (3, 5, 18, 20, 41). Treatment failures with cefixime have been verified in Japan (10, 41, 55) for many years, but recently, treatment failures have also been reported in Norway (46), Austria (45), and the United Kingdom (16). It appears that a few very successful gonococcal lineages (clones), e.g., the multilocus sequence typing (MLST) sequence type 7363 (ST7363) and ST1901, which most likely originated in Japan, are spreading globally and account for a substantial proportion of the isolates showing decreased susceptibility and resistance to ESCs and multidrug resistance in many countries (12, 15, 23, 29, 38, 42, 53; http://pubmlst.org/neisseria/; M. Ohnishi and M. Unemo, unpublished data). The recent gonorrhea treatment failures with cefixime in Norway (46), Austria (45), and the United Kingdom (16) were caused by the MLST ST1901 clone, which appears to have evolved into multiple different *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) STs. Of these, NG-MAST ST1407 is the most prevalent in Europe and has been spreading worldwide (8, 12, 15, 16, 33, 38, 42, 45, 46; http://www.ng-mast.net; Ohnishi and Unemo, unpublished). Recently, the first extensively drug-resistant (XDR) (41) gonococcal strain (H041) was isolated in Japan and characterized (27, 28). H041 has high-level resistance to both cefixime (MIC = 8 μg/ml) and ceftriaxone (the last remaining option for empirical first-line treatment; MIC = 2 to 4 μg/ml) and was most likely related to a clinical failure using ceftriaxone at 1 g intravenously. This strain was assigned to MLST ST7363 (NG-MAST ST4220). High-level resistance to all ESCs was due primarily to a novel *penA* mosaic allele (27). If these ESC-resistant strains start to spread globally, gonorrhea will become untreatable in certain circumstances and especially in some settings (27, 41). Thus, it is crucial to investigate in detail all gonococcal strains suspected to be resistant to the recommended ESCs using quality-assured methods in order to confirm resistance, predict the possibility of treatment failure and further spread of the strains, and elucidate their phenotypic and genetic characteristics, in particular, their resistance mechanisms.

The main mechanism in *N. gonorrhoeae* for decreased susceptibility and resistance to ESCs is alteration of the *penA* gene, encoding penicillin-binding protein 2 (PBP 2), which is the lethal target for these antimicrobials. Decreased susceptibility may be

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due to acquisition of a *penA* mosaic allele or alterations of A501 or possibly G545 and P551 in the transpeptidase/β-lactam binding domain of PBP 2 (1, 2, 3, 12, 15–21, 24, 27–29, 32, 33, 36–38, 41–43, 45, 46, 48, 51–54, 58). Mutations in the promoter and/or coding sequence of *mtrR* result in the overexpression of the MtrC-MtrD-MtrE efflux pump, which further decreases ESC susceptibility (1, 3, 12, 13, 19–21, 27, 35, 41, 43, 45, 46, 48, 50, 56, 58), and *porB1b* mutations that alter amino acids G101 and A102 in the PorB1b porin (the *penB* resistance determinant) result in additionally decreased ESC susceptibilities, especially for the injectable ceftriaxone (1, 3, 12, 19–21, 27, 30, 31, 35, 41, 43, 45, 46, 48, 58). Mutations in *ponA* (encoding PBP 1) and *pilQ* (encoding the pore-forming secretin PilQ protein in the type IV pili), which can be involved in high-level penicillin resistance, do not substantially enhance the MICs of ESCs in the currently circulating gonococcal strains (12, 27, 34, 54, 57, 58). At least one nontransformable resistance determinant remains unknown (12, 21, 27, 34, 43, 58).

The aims of the present study were to report the isolation, verification, and characterization of a second *N. gonorrhoeae* strain found worldwide (identified in France) with high-level cefixime and ceftriaxone resistance, which was most likely related to a treatment failure with cefixime. Thus, the present study describes the detailed phenotypic and genetic characterization of this strain, including molecular epidemiological characteristics and elucidation of the resistance mechanisms causing the high-level ESC resistance.

MATERIALS AND METHODS

Gonorrhea patient and case management. The high-level cefixime- and ceftriaxone-resistant strain 10240089 (referred to here as F89) was isolated at test of cure from a urethral specimen from a 50-year-old man who had sex with men (MSM) in June 2010 in Quimper, France. The patient had been administered cefixime at 200 mg *per os*, two doses (6 hours apart), 3 weeks earlier due to a diagnosis of culture-positive symptomatic gonorrhea (urethral discharge and dysuria); the patient repeatedly denied any sexual activity between the time of treatment and the test of cure. The patient did not have any recent travels abroad and was therefore infected in France; however, the sexual contact was not possible to trace. The patient was then successfully treated with gentamicin at 160 mg intramuscularly, single dose, which was verified by culture negativity 3 weeks later.

Species verification of high-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* strain F89. F89 was grown as previously described (49) and verified to be *N. gonorrhoeae* using six species-confirmatory tests: (i) sugar utilization, (ii) PhadeBact GC monoclonal test (Bactus AB, Solna, Sweden), (iii) PhadeBact GC monoclonal serovar test (Bactus AB, Solna, Sweden), (iv) MicroTrak *N. gonorrhoeae* culture confirmation test (Trinity Biotech, Wicklow, Ireland), (v) *porA* pseudogene PCR (14), and (vi) dual-target PCR (*porA* and *opa*) (11).

Antimicrobial susceptibility testing. MICs of ceftriaxone, cefixime, and 31 additional antimicrobials for F89 were determined using the Etest method (AB bioMérieux, Solna, Sweden) according to the manufacturer's instructions. The MIC of ceftriaxone was also determined using the agar dilution method according to the standards from the Clinical and Laboratory Standards Institute (CLSI) (9). The susceptibility to five antimicrobials (penicillin G, cefpodoxime, ceftriaxone, ciprofloxacin, and spectinomycin) was also determined using the calibrated dichotomous sensitivity (CDS) disc diffusion method (40; http://web.med.unsw.edu.au/cdstest, accessed 20 November 2011), which is used in resistance surveillance of *N. gonorrhoeae* in many countries in the World Health Organization (WHO) Western Pacific Region (WPR) (Table 1). β-Lactamase production was tested using nitrocefin discs. The 2008 WHO *N. gonorrhoeae* reference strains (48) were used as quality controls in all antimicrobial susceptibility testing.

TABLE 1 Susceptibilities of high-level cefixime- and ceftriaxone-resistant *N. gonorrhoeae* strain F89 to various antimicrobials

Class, subclass, and antimicrobial	MIC Etest[a] (S, I, or R)[b,c]	CDS (mm)[d]
β-Lactams, penicillins		
Penicillin G	1 (I)[b,c]	5 (I)
Ampicillin	0.5	ND[e]
Amdinocillin	128	ND
Oxacillin	64	ND
Piperacillin	0.125	ND
Piperacillin-tazobactam	0.016	ND
β-Lactams, monobactams, aztreonam	64	ND
β-Lactams, cephalosporins		
Cefuroxime	16 (R)[b]	ND
Cefpodoxime	16 (R)[b]	6 (R)
Ceftazidime	16 (R)[b]	ND
Cefotaxime	4 (R)[b,c]	ND
Cefixime	4 (R)[b,c]	ND
Ceftriaxone	2 (1)[a] (R)[b,c]	3 (R)
Cefepime	16 (R)[b]	ND
β-Lactams, carbapenems		
Ertapenem	0.016	ND
Meropenem	0.016	ND
Imipenem	0.5	ND
Fluoroquinolones		
Ciprofloxacin	>32 (R)[b,c]	4 (R)
Levofloxacin	8	ND
Moxifloxacin	4	ND
Macrolides		
Azithromycin	1 (R)[c]	ND
Erythromycin	2	ND
Aminoglycosides		
Gentamicin	8	ND
Kanamycin	16	ND
Tobramycin	8	ND
Aminocyclitol, spectinomycin	16 (S)[b,c]	13 (S)
Tetracyclines, tetracycline	2 (R)[c]	ND
Glycylcycline, tigecycline	0.5	ND
Folic acid antagonists, trimethoprim-sulfamethoxazole	1	ND
Rifamycins, rifampin	0.5	ND
Chloramphenicol	4	ND
Nitrofurantoin	1	ND
Fusidic acid	2	ND

[a] MICs were determined using the Etest method (AB bioMérieux, Solna, Sweden) according to the instructions from the manufacturer. Agar dilution was additionally performed for ceftriaxone (the result is in parentheses) according to the method described by the Clinical Laboratory and Standards Institute (CLSI) (9). The Etest method is capable of providing half-MIC steps; however, in accordance with the interpretation of the agar dilution method described by the CLSI (9), only whole-MIC steps are given.

[b] Where available, interpretative criteria (S, susceptible; I, intermediate susceptible; R, resistant) from the CLSI (9) were used.

[c] Interpretative criteria (susceptible, intermediate susceptible, resistant) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST; available online at www.eucast.org/Clinical breakpoints [accessed 20 November 2011]) are also reported where available.

[d] The calibrated dichotomous sensitivity (CDS) disc diffusion method (40; http://web.med.unsw.edu.au/cdstest) is used for antimicrobial resistance testing in many countries in the World Health Organization (WHO) Western Pacific Region.

[e] ND, not determined.

TABLE 2 *Neisseria gonorrhoeae* strains with different cefixime MICs and ceftriaxone MICs and containing different resistance determinants used as recipients in transformation experiments of full-length *penA* gene from F89

Strain	MLST ST[a]	NG-MAST ST[b]	MIC (μg/ml)[c]		Genotype			
			Cefixime	Ceftriaxone	*penA*[d]	*mtrR*[e]	*penB*[f]	*ponA*[g]
WHO F	New	ST3303	<0.016[e]	<0.002[e]	*penA* XV (WT)	WT[h]	WT	WT
WHO M	ST7367	ST3304	<0.016	0.016[e]	*penA* II (A345a)	A-del	Yes	L421P
WHO K	ST7363	ST1424	0.5	0.064	*penA* X (Mosaic)	A-del	Yes	L421P
35/02	ST7363	ST326	0.5	0.125	*penA* XXVIII (Mosaic)	A-del	Yes	L421P
WHO L	ST1590	ST1422	0.25	0.125	*penA* VII (A501V)	A-del	Yes	L421P

[a] MLST, multilocus sequencing typing (29).
[b] NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing (44).
[c] The Etest method reads also half-MIC steps; however, in accordance with the interpretation of the agar dilution method described by the Clinical Laboratory and Standards Institute (CLSI) (9), only whole-MIC steps are given. MICs of <0.002 μg/ml and <0.016 μg/ml were calculated as 0.001 μg/ml and 0.008 μg/ml, respectively, in the MIC ratios in Fig. 2.
[d] *penA* mosaic allele encodes a mosaic penicillin-binding protein 2 (PBP 2) that causes a decreased susceptibility to extended-spectrum cephalosporins (ESCs) (21, 27, 36, 41, 43, 58).
[e] A-del, a characteristic single-nucleotide (A) deletion in the inverted repeat of the promoter region of *mtrR* that causes overexpression of the MtrCDE efflux pump, resulting in a further decreased susceptibility to ESCs (21, 27, 41, 58).
[f] Yes, the presence of the alterations of amino acids 101 and 102 in porin PorB (*penB* determinant) that cause a decreased intake of ESCs and, accordingly, a further decreased susceptibility to ESCs (21, 27, 41, 58).
[g] The alteration of amino acid 421 in PBP 1 (encoded by *ponA*) causes decreased susceptibility to penicillins (21, 34, 58).
[h] WT, wild type.

DNA extraction. DNA was isolated in a NorDiag Bullet instrument (NorDiag ASA Company, Oslo, Norway) using a BUGS'n BEADS STI-*fast* kit (NorDiag ASA Company, Oslo, Norway) according to the manufacturer's instructions.

Genetic characterization. Molecular epidemiological characterization of F89 by means of MLST (29), *porB* gene sequencing, and NG-MAST (44) was performed as described previously. PCR amplification and sequencing of known gonococcal resistance determinants, i.e., *penA*, *mtrR*, *porB1b*, *ponA*, and *pilQ*, were done as described elsewhere (21, 48, 54).

Sequence alignments and phylogenetic analysis. Multiple-sequence alignments were performed using the Bioedit sequence alignment editor software (version 7.0.9.0). The evolutionary relationship of F89 to other *penA* mosaic strains displaying decreased ESC susceptibility and resistance (12, 27) was examined using a phylogenetic analysis of the full-length *porB* sequences with the TREECON program (version 1.3b) as previously described (44).

Transformation assays. Transformation experiments (three replicates), using purified PCR-amplified full-length *penA*, to verify that the novel *penA* mosaic allele was the causative determinant of high-level cefixime and ceftriaxone resistance in F89, were performed using one clinical strain and four of the eight 2008 WHO *N. gonorrhoeae* reference strains (48) as recipients as previously described (27, 29). The recipient strains displayed different molecular epidemiological STs, cefixime MICs, ceftriaxone MICs, and compositions of ESC resistance mechanisms, such as *penA* alleles, the *mtrR* promoter mutation, and *penB* sequences (Table 2). The full-length *penA* allele, as well as the other known gonococcal resistance determinants, i.e., the *mtrR*, *porB1b*, *ponA*, and *pilQ* genes, were sequenced in all transformants.

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the new *penA* allele of F89 (referred to as *penA-CI*; see text below) is JQ073701.

RESULTS

Phenotypic characterization of the high-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* strain F89. All conventional bacteriological tests and six species-verifying assays (see Materials and Methods) confirmed that F89 is a gonococcal strain. The serovar determination assigned F89 to serovar Bpyut.

The results of antimicrobial susceptibility testing of F89 are summarized in Table 1. Briefly, F89 displayed high-level resistance to cefixime (MIC = 4 μg/ml; actually, 3 μg/ml according to the Etest method), ceftriaxone (MIC = 1 μg/ml [agar dilution] to 2 μg/ml [Etest, 1.5 μg/ml]), and all additional ESCs tested. F89 was also resistant to most of the antimicrobials previously used for treatment of gonorrhea, including all fluoroquinolones, macrolides, tetracycline, trimethoprim-sulfamethoxazole, and chloramphenicol. Regarding other β-lactam antimicrobials, the MICs of penicillin G, ampicillin, and piperacillin, as well as the carbapenems ertapenem and meropenem, were surprisingly low (Table 1). The strain did not produce any β-lactamase. Finally, F89 was susceptible to spectinomycin, and the MICs of aminoglycosides, tigecycline, and rifampin were relatively low (as for ampicillin, piperacillin, and carbapenems, no breakpoints are available for these antimicrobials) (Table 1).

Molecular epidemiological characterization of the high-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* strain F89. Molecular epidemiological characterization of F89 assigned it to MLST ST1901 and NG-MAST ST1407. Phylogenetic analysis of full-length *porB* sequences showed that F89 is identical to previously examined *penA* mosaic ST1407 isolates (12, 27), which display decreased susceptibility and resistance to ESCs and have been cultured worldwide (data not shown).

Elucidation of the genetic resistance mechanisms causing the high-level cefixime and ceftriaxone resistance in F89. The sequencing of ESC resistance determinants showed that the strain contained a novel *penA* mosaic allele, i.e., a PBP 2 XXXIV mosaic allele (27, 33) with a single additional transversion (G → C in bp 1501 in *penA*), resulting in a A501P alteration in PBP 2 (Fig. 1), which to our knowledge has not been previously described in *N. gonorrhoeae* or in other *Neisseria* species. In Fig. 1, the *penA* alleles of H041 (27) and F89 have been designated C and CI, respectively, in accordance with the PBP 2 sequences and nomenclature proposed by Ohnishi et al. (27). Substantially higher numbers for the alleles were used to prevent confusion with the numbering of *penA* alleles reported by Allen et al. (1), some of which differ from the numbers used in the numbering system described by Ohnishi et al. (27). F89 also contained two previously verified ESC resistance determinants: (i) *mtrR* (an A deletion in the inverted repeat in the promoter, which increases expression of the MtrC-MtrD-MtrE

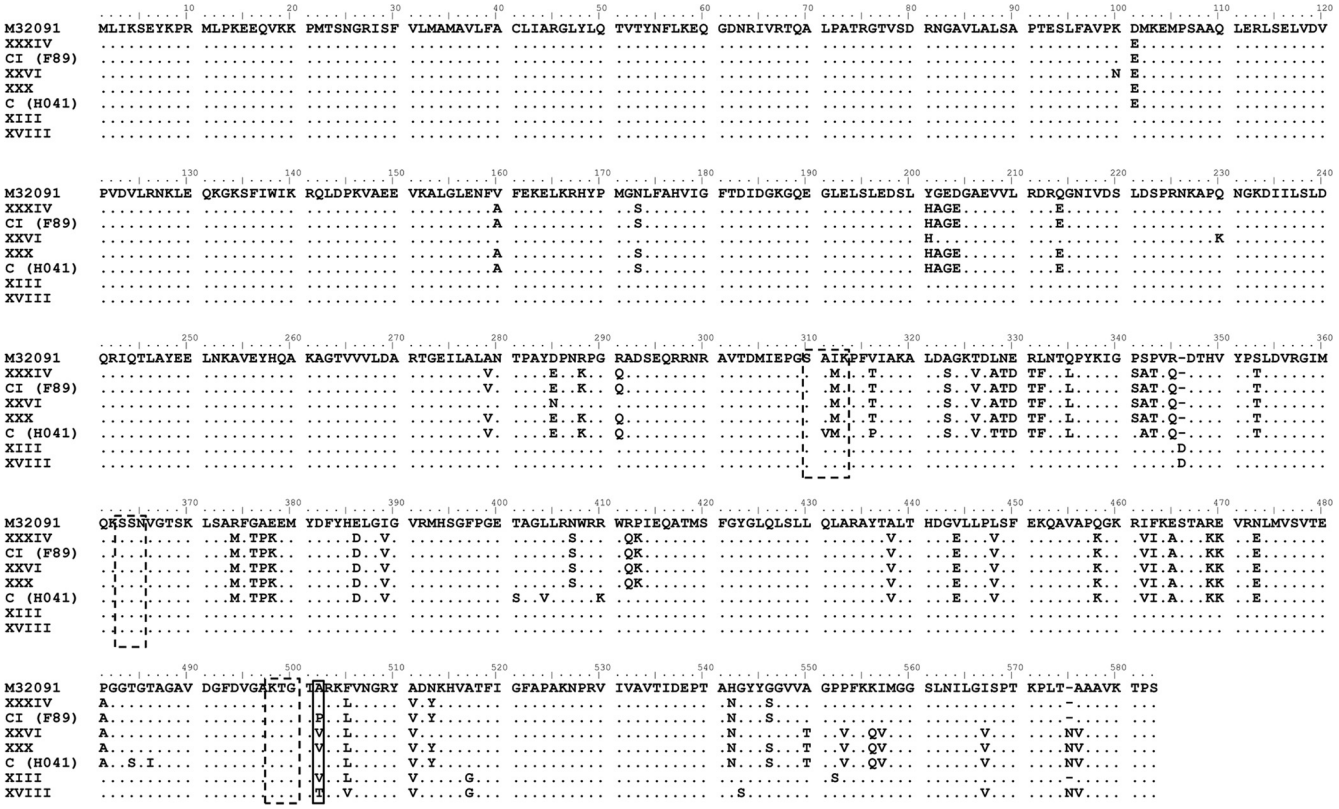

FIG 1 Alignment of PBP 2 sequences from strains of *Neisseria gonorrhoeae* with different *penA* alleles. M32091, wild-type PBP 2; XXXIV, *penA* mosaic allele previously described (27, 33); CI, *penA* mosaic allele of F89; XXVI (27, 36) and XXX (27, 51), the only previously reported true *penA* mosaic alleles containing an A501 alteration (27); C, *penA* mosaic allele of H041, which is the first high-level ceftriaxone-resistant strain found in Japan (27); and XIII and XVIII, previously described nonmosaic *penA* alleles that contain A501V (most prevalent) and A501T alterations (27, 51). Identical amino acids are designated by a dot, and alterations from the wild-type sequence are shown with a single capital letter. The three active-site motifs are marked by dashed boxes, while the location of amino acid Ala501 (position 502 in the alignment due to the D345 insertion) is marked with a solid box.

efflux pump) and (ii) *penB* (*porB1b* containing G101K and A102N alterations, which decrease diffusion of antimicrobials into the periplasm). It also contained penicillin resistance determinants such as the *ponA1* alteration (L421P), which encodes an altered form of PBP 1, and a *pilQ* VII allele (54). Accordingly, F89 was nearly identical to the previously described ST1407 strain causing treatment failures in Norway (46), with the novel *penA-CI* allele being the only new resistance determinant. Consequently, this allele was suspected to be the primary determinant responsible for the high MICs of ESCs.

To ascertain whether the increased resistance to ESCs was due to the novel *penA-CI* allele, the full-length *penA-CI* allele from F89 was amplified and used to transform four WHO reference strains and one clinical isolate, and the MICs of ceftriaxone and cefixime for the resulting sequence-verified transformants were determined. The experiments confirmed that *penA-CI* conferred high-level resistance to both cefixime and ceftriaxone (Fig. 2). Transformation of *penA-CI* into the recipient strains increased the MICs of cefixime between 8- and 500-fold (MICs = 2 to 8 μg/ml; actually, 1.5 to 6.0 μg/ml according to the Etest method) and of ceftriaxone from 31- to 500-fold (MICs = 0.5 to 4 μg/ml; actually, 0.34 to 3.3 μg/ml according to the Etest method), which were above the resistance breakpoints (>0.25 μg/ml) for both antimicrobials (9). Remarkably, transformants of WHO F, which has wild-type alleles of all known ESC and penicillin resistance determinants, displayed mean MICs of cefixime and ceftriaxone of 2 μg/ml (1.5 μg/ml, according to the Etest method) and 0.5 μg/ml (0.34 μg/ml, according to the Etest method), respectively, which represents an increase of 500-fold for the MICs of both ESCs (Fig. 2).

All transformants contained the *penA-CI* allele and had *mtrR*, *penB*, and *ponA* sequences identical to those from their respective parental strain. In all transformants except strain 35/02, the transformed *penA-CI* sequence was identical to that from F89. In strain 35/02, a few single nucleotide polymorphisms (SNPs) and one inserted codon of 3 bp in the C-terminal end of the *penA* gene (after bp 1647) differed from the *penA-CI* sequence, but most of these SNPs were synonymous and this segment of the mosaic *penA* allele most likely does not affect the MICs of cefixime or ceftriaxone. Consequently, these transformation experiments confirmed that the novel *penA-CI* mosaic allele is responsible for high-level cefixime and ceftriaxone resistance, although the MICs of the ESCs for the recipient strains vary depending on whether they contain additional resistance determinants (e.g., *mtrR* and *penB*).

DISCUSSION

This study reports the detailed phenotypic and genetic characterization and resistance mechanism of the high-level cefixime and ceftriaxone resistance in strain F89, the second *N. gonorrhoeae* strain showing high-level resistance to ESCs worldwide. F89 was

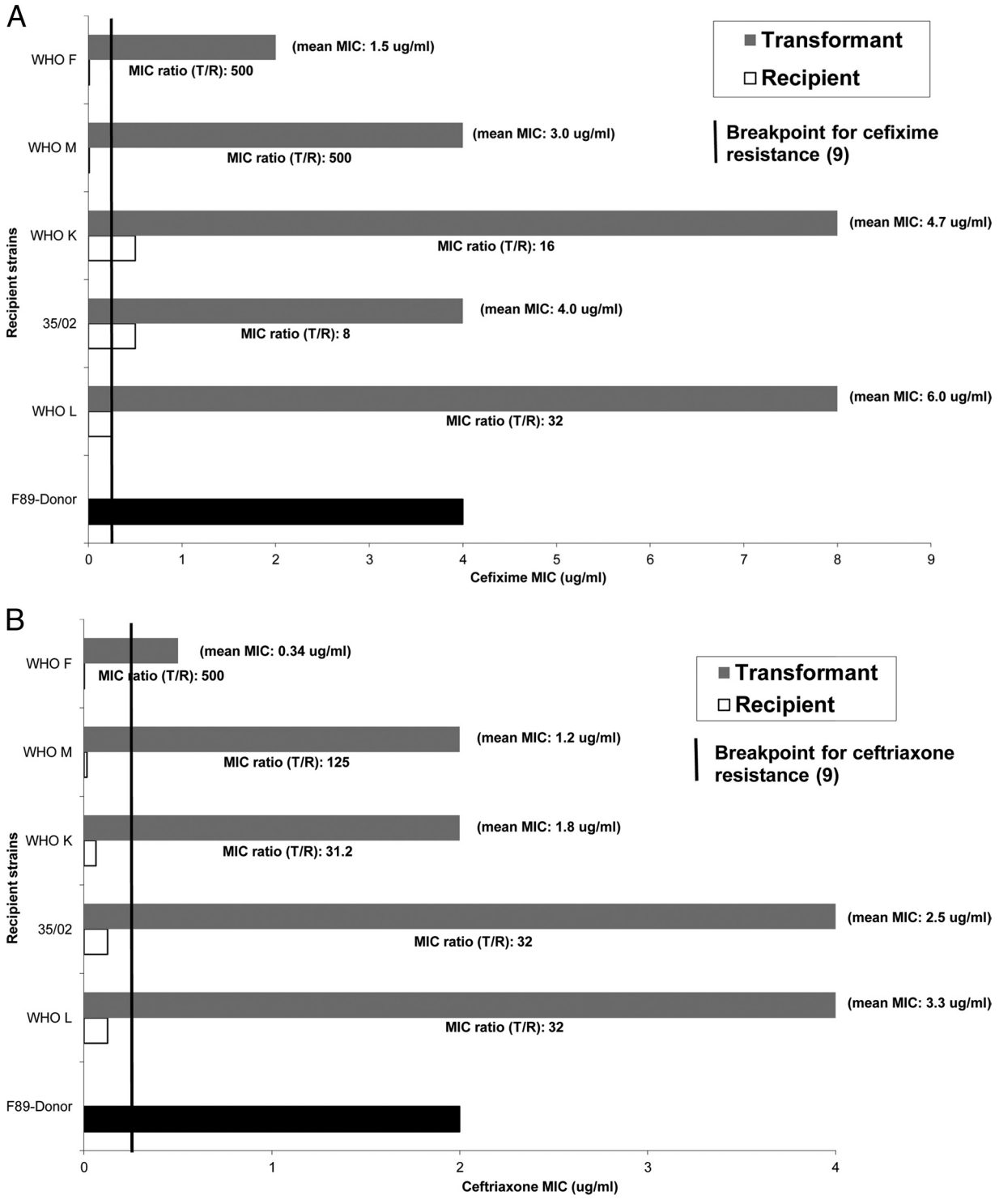

FIG 2 MICs of a set of recipient strains transformed with the full-length *penA* allele (*penA-CI*) from the high-level cefixime- and ceftriaxone-resistant *Neisseria gonorrhoeae* strain F89 (donor). The MICs of cefixime and ceftriaxone and the number and type of resistance determinants (e.g., *mtrR*, *penB*, and *ponA*) in the recipient strains varied. The MICs of cefixime (A) and ceftriaxone (B) were determined using the Etest method, and the values shown are the means of three transformation experiments (the bars give only whole-MIC steps), with the exact mean MICs in parentheses. The ratios of the MICs of the transformant to the recipient strain (T/R) are indicated.

isolated from an MSM in Quimper, France, and most likely was related to a treatment failure with cefixime. Previously, only one other strain (H041 isolated in Japan), which belongs to the internationally spreading MLST ST7363, has shown high-level resistance to cefixime and ceftriaxone (27, 28). It is of grave concern that another *N. gonorrhoeae* strain, F89, which belongs to an additional international successful clone (MLST ST1901, with ST1407 being the most prevalent NG-MAST ST in Europe), has

also developed high-level resistance to both cefixime and ceftriaxone, the last options for first-line treatment of gonorrhea. The gonococcus appears to have evolved into a superbug that may initiate a future era of untreatable gonorrhea.

Although a pretreatment isolate was not available to definitively confirm treatment failure with cefixime, it appears most likely for a number of reasons: (i) lack of sexual activity between treatment and test of cure, (ii) the very high MIC of cefixime for F89, and (iii) all pharmacokinetic and pharmacodynamic data for cefixime. According to simulations, the treatment regimen used (cefixime 200 mg *per os*, two doses, 6 h apart) results in median times of free cefixime concentration above the MIC ($fT_{>MIC}$) of 0 h for a strain, i.e., F89, with a cefixime MIC of 4 μg/ml (6). Thus, using this dosage regimen for treatment of gonorrhea caused by F89 is highly unlikely to clear the infection.

Transformation experiments with several recipient strains demonstrated that the novel *penA-CI* mosaic allele in F89 is the primary determinant for resistance to cefixime and ceftriaxone. Transfer of *penA-CI* into even a wild-type strain with no known resistance determinants resulted in MICs above the resistance breakpoints for both antibiotics; however, additional resistance determinants, especially *mtrR* and *penB*, were required to reach the same (or even higher) MICs of cefixime and ceftriaxone as the donor strain. The synergy between *penA*, *mtrR*, and *penB* has been previously reported (21, 27, 41, 58), although the synergistic effect is much more pronounced for ceftriaxone than for cefixime (58). Notably, WHO L displayed substantially higher cefixime and ceftriaxone MICs than the other recipient strains after transformation (Fig. 2). Thus, WHO L may harbor factor X, i.e., the nontransformable determinant that remains to be identified (12, 21, 27, 34, 43, 58), and it cannot be excluded that the cefixime and ceftriaxone MICs are also enhanced by the mtr_{120} mutation in WHO L (26).

The *penA-CI* sequence is nearly identical to the *penA-XXXIV* mosaic allele (27), with *penA-CI* containing only a single additional transversion (G1501C) that results in an A501P alteration in PBP 2. The *penA-XXXIV* mosaic allele was initially described in 2008 in an NG-MAST ST1407 isolate in San Francisco (33) and recently reported in Taiwan (15) and Canada (1). Whereas these strains had cefixime and ceftriaxone MICs of 0.125 to 0.25 μg/ml and 0.06 to 0.125 μg/ml, respectively (1, 15, 33), F89 had markedly higher MICs of cefixime and ceftriaxone (4 μg/ml and 1 to 2 μg/ml, respectively). Although the structure of PBP 2 with an A501P, A501V, or A501T mutation has not been reported, it has been suggested that replacement of the methyl side chain of Ala501, which is located on the β3-β4 loop very close to the KTG active-site motif of PBP 2 (Fig. 1) (43), with the more bulky side chains of valine or threonine inhibits the binding of cefixime and ceftriaxone to PBP 2 by clashing with their R1 substituents. The change of Ala501 to a proline introduces secondary structure alterations that may result in changes even more dramatic than those obtained with valine and threonine, and this is consistent with the much higher MICs conferred by the A501P mutation than the other mutations. In regard to other β-lactams, the less bulky R1 substituent of penicillin G, ampicillin, and the carbapenems may explain why they were less affected by the A501P alteration; indeed, it was shown previously that the MIC of penicillin actually decreased in strains harboring a *penA-X* A501V allele compared to the same strain with the *penA-X* allele (43). In contrast, the reason for the low MIC of piperacillin, which also contains a bulky R1 substituent, remains unknown (Table 1).

To our knowledge, the A501P alteration in PBP 2 has not been previously reported in any strain of *N. gonorrhoeae* or other *Neisseria* species. In *N. gonorrhoeae*, Ala501 alterations (A501V and A501T) have mostly been described in strains with nonmosaic *penA* alleles, e.g., XIII (A501V) and XVIII (A501T) (27, 51), having MICs of ceftriaxone of 0.06 to 0.125 μg/ml (51). In fact, only two mosaic *penA* sequences (XXVI [36] and XXX [29]), i.e., sequences containing amino acid alterations proven to decrease the susceptibility to ESCs, e.g., in A311, I312, V316, N512, and G545 (27, 36, 43), that contain an Ala501 alteration (A501V in both of these sequences) have been reported (Fig. 1) (27). The MICs of cefixime were only 0.12 to 0.25 μg/ml and 0.5 to 1.0 μg/ml for these strains, with the differences likely due to the presence or absence of other resistance determinants, e.g., *mtrR*, *penB*, and/or factor X. It has also been shown previously that transformation of FA6140, a penicillin-resistant but cephalosporin-susceptible isolate containing all known resistance determinants, including factor X, with the mosaic *penA-X* allele harboring an additional A501V alteration increases the MICs of both cefixime and ceftriaxone to levels above their resistance breakpoints (43).

Alteration of Ala501 in PBP 2 may be gonococcus specific, perhaps due to ESC selection pressure (12, 43, 53). Indeed, Takahata et al. (36) reported the finding of a spontaneous A501V mutation during transformation experiments with mosaic *penA* alleles. Moreover, isolates with nonmosaic *penA* alleles containing Ala501 mutations have been assigned to many different MLST and NG-MAST STs (12, 51, 53), which further supports the hypothesis that spontaneous Ala501 mutations have arisen in many different gonococcal clones due to ESC selective pressure. The rarity of gonococcal strains containing a *penA* mosaic allele with an Ala501 alteration may suggest that these strains have a lower biological fitness. The knowledge regarding the Ala501 alterations in mosaic as well as in nonmosaic *penA* alleles is limited, and further research is imperative. Finally, many different mosaic and nonmosaic *penA* alleles altered at Ala501 (27) have emerged and continue to emerge, and their effects on the MICs of ESCs of the corresponding gonococcal isolates can vary markedly; e.g., some mosaic and A501 altered alleles do not significantly increase the MICs of ESCs (12, 19, 41, 42, 51, 52). Therefore, it is crucial to elucidate the effects of all amino acid alterations suspected to enhance the MICs of ESCs by transformation of site-directed *penA* mutants into isogenic strain backgrounds, i.e., show causality, and not just describe statistical associations between the presence of specific amino acid alterations and elevated MICs of ESCs. It is also important to agree on the nomenclature of gonococcal *penA* alleles, e.g., as proposed by Ohnishi et al. (27), including an internationally accessible database, in order to avoid divergent numbering of identical PBP 2 alleles.

F89 was assigned to MLST ST1901 (NG-MAST ST1407), which likely originated in Japan and, along with MLST ST7363, has been successful in spreading worldwide. The treatment failures with cefixime in Norway (46), Austria (45), and, likely, the United Kingdom (16) were caused by the MLST ST1901 clone (NG-MAST ST1407), even though the United Kingdom strains belonged to NG-MAST ST3779 and ST3431. These NG-MAST STs seem to have evolved from the closely related ST1407, which all share the *tbpB110* allele and differ in only one SNP each in the *porB908* allele. Although NG-MAST ST1407 appears to be the

most prevalent NG-MAST ST of MLST ST1901 in Europe, many NG-MAST STs of the MLST clone ST1901 have been identified globally. In Japan, where the MLST ST1901 clone has been spreading for at least 10 years, many related genetic variants of NG-MAST ST1407, and possibly an additional NG-MAST clone(s) assigned to MLST ST1901, have evolved (12, 15, 16, 23, 33, 42, 45, 46; http://www.ng-mast.net; Ohnishi and Unemo, unpublished). F89 appears to represent a subclone of the MLST ST1901/NG-MAST ST1407 clone that has developed high-level resistance to cefixime and ceftriaxone. Thus, two successful *N. gonorrhoeae* clones (MLST ST7363 and MLST ST1901) are spreading worldwide and have shown their capacity to develop high-level resistance to cefixime and ceftriaxone. If strains with high-level resistance to cefixime and ceftriaxone start to spread globally, which historically has been the scenario for spread of emerged gonococcal resistance, gonorrhea might become untreatable. It is of great concern that the first two *N. gonorrhoeae* strains with high-level cefixime and ceftriaxone resistance have been identified in high-risk frequently transmitting populations, i.e., the Japanese strain H041 from a female commercial sex worker (27) and the French strain F89 from an MSM. To date, the spread of these strains has not been observed and additional treatment failures have not been identified. This fact may be due to suboptimal surveillance of gonococcal antimicrobial resistance and gonorrhea treatment failures, but it cannot be excluded that the *penA* alleles that confer high-level cefixime and ceftriaxone resistance cause a lower biological fitness that limits their further spread. Accordingly, the biological fitness of these first strains with high-level cefixime and ceftriaxone resistance is crucial to examine.

In conclusion, *N. gonorrhoeae* appears to be rapidly evolving into a superbug, in which case future gonorrhea infections may become untreatable. It is of great concern that two globally spreading MLST clones (ST7363 and ST1901) have shown an ability to develop high-level resistance to cefixime and ceftriaxone, and any future spread of these clones may need to be monitored using appropriate molecular epidemiological typing (47). For public health purposes, enhanced disease control activities, quality-assured surveillance of AMR and treatment failures worldwide, and public health action plans (global and national perspectives) are crucial. These data also highlight the urgent need for new treatment strategies, new antimicrobial agents, and, ideally, a vaccine for effective treatment of gonorrhea (4, 6, 7, 22, 25, 27, 41, 45, 46, 48).

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