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MECHANISMS OF RESISTANCE

Neisseria cinerea with High Ceftriaxone MIC Is a Source of Ceftriaxone and Cefixime Resistance-Mediating *penA* Sequences in *Neisseria gonorrhoeae*

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ABSTRACT Mosaic *penA* alleles have caused most of the cephalosporin resistance in *Neisseria gonorrhoeae*, but their evolution is mostly unknown. The *penA* gene from *Neisseria cinerea* strain AM1601 (ceftriaxone MIC, 1.0 μg/ml) caused ceftriaxone resistance (MIC, 1 μg/ml) in a ceftriaxone-susceptible gonococcal strain. The 3′-terminal half of AM1601 *penA* was almost identical to that of the ceftriaxone-resistant gonococcal GU140106 and FC428 strains. *N. cinerea* can serve as a reservoir of ceftriaxone resistance-mediating *penA* sequences that can be transferred to gonococci.

KEYWORDS *Neisseria cinerea*, *Neisseria gonorrhoeae*, antimicrobial resistance, cefixime, ceftriaxone, *penA*, penicillin-binding protein 2

Resistance to the extended-spectrum cephalosporin (ESC) ceftriaxone in *Neisseria gonorrhoeae* has sporadically emerged worldwide. The main ESC resistance determinant is mosaic *penA* alleles, encoding penicillin-binding protein 2 (PBP2) (1–10). These gonococcal mosaic *penA* alleles are proposed to have evolved through transformation of partial *penA* sequences from commensal *Neisseria* species (1, 2, 8, 11, 12); however, detailed knowledge is lacking. The ceftriaxone-resistant gonococcal strains H041 (4), A8806 (6), GU140106 (7), and FC428 (8) possessed different mosaic *penA* alleles. However, A8806 (6), GU140106 (7), and FC428 (8) possessed identical or almost identical 3′-terminal halves of *penA*, although the central region of *penA* showed substantially less nucleotide sequence similarity (8). The conserved 3′-terminal part of $penA_{FC428}$ was very different from the FA1090 wild-type *penA* (8), illustrating that the origin of gonococcal ceftriaxone resistance exists in other species.

Here, we investigated *Neisseria cinerea* AM1601, which was isolated in 2016 from a patient with bacteremia in Aichi, Japan (13), and has high MICs for ceftriaxone (1.0 μg/ml) and cefixime (2.0 μg/ml), as determined by an agar dilution method according to CLSI guidelines (14). To verify the species of *N. cinerea* AM1601, ribosomal multilocus sequence typing was performed. Briefly, the single-nucleotide polymorphisms (SNPs) on 53 *rps* alleles were extracted from the AM1601 genome sequence generated by a MiSeq sequencer (Illumina, San Diego, CA, USA) and were compared with those on 53 *rps* alleles from various *Neisseria* species (15). The results indicated that *N. cinerea* AM1601 clustered with six *N. cinerea* reference strains (see Fig. S1 in the supplemental material) (15, 16).

To show that *penA* of *N. cinerea* AM1601 is responsible for ceftriaxone resistance, a full-length AM1601 *penA* gene was PCR amplified using PrimeStar HS Premix (TaKaRa Bio, Shiga, Japan), genomic DNA, and the primers 5′-ATGTTGATTAAGAGCGAATAT

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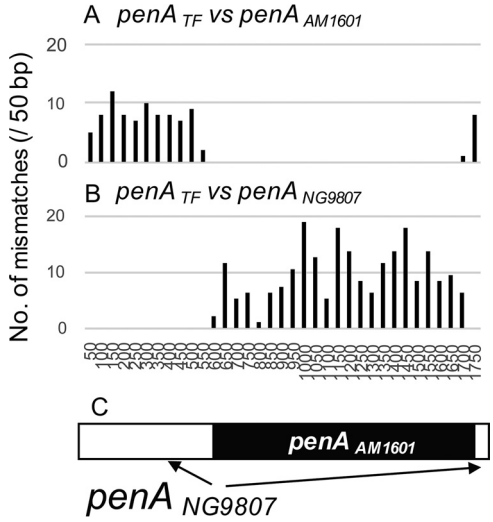

FIG 1 Mosaic *penA* allele generated by transformation of *Neisseria cinerea* $penA_{AM1601}$ into *Neisseria gonorrhoeae* NG9807 (with wild-type *penA*). The *penA* allele of the NG9807 transformant ($penA_{TF}$) was compared with the donor allele of *N. cinerea* AM1601 ($penA_{AM1601}$) and the recipient wild-type allele of *N. gonorrhoeae* NG9807 ($penA_{NG9807}$). (A) Number of mismatches in each 50 bp between alleles $penA_{TF}$ and $penA_{AM1601}$. (B) Number of mismatches in each 50 bp between alleles $penA_{TF}$ and $penA_{NG9807}$. (C) Schematic representation of the mosaic $penA_{TF}$ by a white rectangle (remaining from $penA_{NG9807}$) and a black rectangle (derived from $penA_{AM1601}$).

AAG-3′ and 5′-TTAAGACGGTGTTTTGACGG-3′. The primers were designed on the basis of the *N. cinerea* AM1601 *penA* sequence determined by whole-genome sequencing and confirmed by conventional Sanger sequencing, as described previously (8). After purification with the High Pure PCR product purification kit (Roche Diagnostics GmbH, Mannheim, Germany), the PCR product was transformed into NG9807, as described previously (17). The ceftriaxone-susceptible gonococcal strain NG9807 (MIC of 0.016 μg/ml, with the wild-type *penA*) was used as a recipient for transformation (17). NG9807 has a single-nucleotide (A) deletion in the inverted repeat of the *mtrR* promoter, a PBP1 L421P alteration, and *penB* alterations at PorB1b G120 and A121 (4). The transformation frequency was estimated as 1 in 10^7 recipient cells. The full-length *penA* gene in the NG9807 transformant was sequenced using conventional Sanger sequencing.

Transformation of $penA_{AM1601}$ into the ceftriaxone-susceptible gonococcal strain NG9807 caused resistance to ceftriaxone (64-fold MIC increase, from 0.016 to 1 μg/ml) and cefixime (128-fold MIC increase, from 0.016 to 2 μg/ml) (14, 18). Only ~62% of the donor $penA_{AM1601}$ was incorporated into the recipient $penA_{NG9807}$ (Fig. 1), resulting in a mosaic *penA* allele. To characterize the *penA* sequence of *N. cinerea* AM1601, we compared the *penA* gene of AM1601 with the *penA* genes of ceftriaxone-resistant *N. gonorrhoeae* strains using ClustalW. The levels of similarity between *N. cinerea* $penA_{AM1601}$ and ceftriaxone-resistant gonococcal $penA_{H041}$ (4), $penA_{GU140106}$ (7), and $penA_{FC428}$ (8) were 92.7%, 90.3%, and 92.9%, respectively. The mismatches between $penA_{AM1601}$ and $penA_{GU140106}$ accumulated in the 5′-terminal half, whereas there were no SNPs in the 3′-terminal half, including the PBP2 β-lactam-active motifs (Fig. 2A). Similarly, the $penA_{AM1601}$ 3′-terminal half was highly homologous to the corresponding $penA_{FC428}$ sequence (only one synonymous SNP) (Fig. 2B). Accordingly, the AM1601 PBP2 shared a trait with those of the ceftriaxone-resistant gonococcal strains GU140106 (7) and FC428 (8). Importantly, the PBP2 forms of all of these strains possessed V311 and S483, which are two of the three mutated amino acids causing high-level ceftriaxone resistance in *N. gonorrhoeae* H041 (19). However, the *penA* 3′-terminal region that was conserved between $penA_{AM1601}$ and $penA_{H041}$ was smaller (Fig. 2). To further characterize $penA_{AM1601}$, it was compared with another *penA* (GenBank accession number AB904039), from a *Neisseria* strain that has high ESC MICs (ceftriaxone MIC, 2 μg/ml; cefixime MIC, 4 μg/ml). The strain was verified as *N. cinerea*

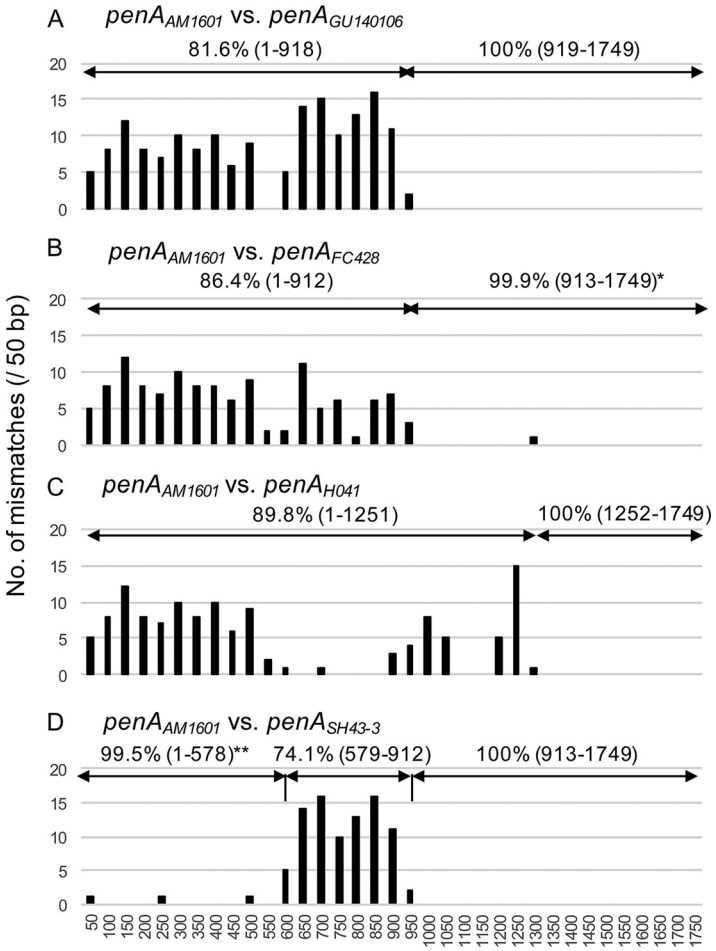

FIG 2 Sequence comparison of *penA* genes from ceftriaxone-resistant *Neisseria cinerea* and *Neisseria gonorrhoeae* strains isolated in Japan. Pairwise comparisons of *penA* nucleotide sequences for *penA* genes from *N. cinerea* AM1601 and *N. gonorrhoeae* GU140106 (7) (A), *N. gonorrhoeae* FC428 (8) (B), *N. gonorrhoeae* H041 (4) (C), and *N. cinerea* SH43-3 (D) are shown. The mismatched bases in each 50 bp of the *penA* genes were counted. The similarity of each region is indicated, with the nucleotide positions evaluated in parentheses. *, one synonymous mismatch, at nucleotide position 1296; **, three mismatches, at nucleotide positions 48, 219, and 489.

by *rps* gene comparison in this study (Fig. S1). *N. cinerea* SH43-3 was isolated in 2013 from an asymptomatic female sex worker, in Kyoto, Japan, during a routine examination for sexually transmitted infections (gonococcus-negative pharyngeal specimen). The 5′-terminal and 3′-terminal parts were very similar (99.5%) and identical (100%), respectively. Nearly all of the mismatches accumulated in the central part of *penA*, indicating recombination event(s) (Fig. 2D).

Commensal *Neisseria* species, including *N. cinerea*, are members of the human oropharyngeal microflora (20–23). These *Neisseria* species might be genetic reservoirs of resistance determinants for β-lactam antimicrobials (including ESCs) that can be transferred to the pathogenic species *Neisseria meningitidis* and gonococci (1, 11, 12, 23–26). We demonstrate that *N. cinerea* strains with high ceftriaxone MICs (1 to 2 μg/ml) possess ceftriaxone resistance-mediating *penA* sequences that can be transferred to gonococci by transformation and result in ceftriaxone and cefixime resistance. The 3′-terminal half of mosaic *penA* in the transformant, which was transferred from *N. cinerea*, has also been described in ceftriaxone-resistant clinical gonococcal strains, i.e., A8806 (6), GU140106 (7), and FC428 (8), isolated in 2013 to 2015 in Australia and Japan. Accordingly, the 3′-terminal half of *N. cinerea penA* has caused ceftriaxone resistance in genetically different gonococcal strains in different countries. This indicates that *N.*

cinerea strains represent an origin of the ceftriaxone resistance-mediating *penA* sequences in the gonococcal strains A8806 (6), GU140106 (7), and FC428 (8). However, an unknown origin of ceftriaxone resistance-mediating *penA* sequences might also exist, from which genetic material has been transferred to both ceftriaxone-resistant gonococcal strains and ceftriaxone-resistant *N. cinerea* strains, which might be supported by the mismatches in the central region of *penA* in AM1601 versus SH43-3. Further investigations of commensal *Neisseria* species are imperative.

In conclusion, *N. cinerea* can serve as a reservoir of ceftriaxone resistance-mediating *penA* sequences that are transferred to and cause ceftriaxone and cefixime resistance in clinical gonococcal strains. Examinations of commensal *Neisseria* species are crucial to understand, and ideally to mitigate, the emergence and evolution of resistance to ESCs in gonococci. This will provide new insights regarding interspecies sharing and reservoirs of resistance determinants for other antimicrobials in commensal bacteria.

Accession number(s). The *N. cinerea* AM1601 *penA* sequence was deposited in the DDBJ (accession number LC316656).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02069-17.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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