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Citation	Johnson, S. R., Y. Grad, S. R. Ganakammal, M. Burroughs, M. Frace, M. Lipsitch, R. Weil, and D. Trees. 2014. "In Vitro Selection of Neisseria Gonorrhoeae Mutants with Elevated MIC Values and Increased Resistance to Cephalosporins." Antimicrobial Agents and Chemotherapy 58 (11) (September 8): 6986–6989. doi:10.1128/aac.03082-14. http://dx.doi.org/10.1128/AAC.03082-14.
Published Version	doi:10.1128/AAC.03082-14
Citable link	http://nrs.harvard.edu/urn-3:HUL.InstRepos:26883711
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In Vitro Selection of *Neisseria gonorrhoeae* Mutants with Elevated MIC Values and Increased Resistance to Cephalosporins

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Strains of *Neisseria gonorrhoeae* with mosaic *penA* genes bearing novel point mutations in *penA* have been isolated from ceftriaxone treatment failures. Such isolates exhibit significantly higher MIC values to third-generation cephalosporins. Here we report the *in vitro* isolation of two mutants with elevated MICs to cephalosporins. The first possesses a point mutation in the transpeptidase region of the mosaic *penA* gene, and the second contains an insertion mutation in *pilQ*.

Increases in MICs and antibiotic resistance have been observed among clinical isolates of *Neisseria gonorrhoeae* and have resulted in a greater incidence of treatment failure (1–6). β-Lactam resistance results collectively from mutations in four genes—*penA* (7, 8), *ponA* (9, 10), *mtrR* (11–13), and the *porBIb* allele of the porin gene (14, 15)—and increased cephalosporin resistance requires the presence of a mosaic *penA* gene (16–18), a variant presumably resulting from gene transfer and recombination (19). Mutations within the mosaic *penA* gene can result in still greater resistance to cephalosporins (5, 6).

The *penA* and *ponA* genes of 11 gonococcal isolates with MICs to penicillin and ceftriaxone ranging from 4.0 to 8.0 μg/ml and 0.008 to 0.060 μg/ml, respectively, were sequenced using GenomeLab DTCS-Quickstart kits (Beckman Coulter, Brea, CA), with product analysis performed on a CEQ3000 instrument, and the data were analyzed using DNAStar software. Isolates with MICs to ceftriaxone ranging from 0.008 to 0.015 μg/ml demonstrated a single additional codon that coded for the insertion of an aspartic acid residue, while all four of the isolates with MICs ranging from 0.03 to 0.06 μg/ml possessed mosaic *penA* genes. In all of the isolates sequenced, the *ponA* gene contained a single point mutation (Table 1) as described elsewhere (10). The MtrR phenotype of all of the isolates was established by determining their resistance to 1.2 μg/ml of erythromycin and 600 μg/ml of Triton X-100 (12). Two isolates with nonmosaic *penA* genes and two with mosaic *penA* genes (Table 1) were chosen for further study as to their potential to mutate to greater resistance to cephalosporins. Gradient plates of GC base agar with Isovitalex (Becton, Dickinson and Company, Sparks, MD) and either 4.0 or 6.0 μg/ml of cefpodoxime in the agar substratum were seeded with 5×10^{10} CFU of each of the isolates to determine the range of concentrations over which mutants could be selected. Mutants were obtained for both of the mosaic *penA* strains SPL4 and SPN284 on both concentrations of cefpodoxime. No mutants were obtained for the two nonmosaic *penA* strains SPN275 and SPN280. Mutants derived from SPL4 and SPN284 were tested for MICs by the agar dilution method (20). The growth patterns observed on the gradient plates and the MICs obtained for the mutants (Table 2) suggested that 3.0 to 3.5 μg/ml of cefpodoxime was the concentration likely to be optimal for the selection of mutants. The actual frequencies of mutation to elevated resistance to cefpodoxime (Cpd^r) were determined by seeding GC base agar containing 3.0 μg/ml of cefpodoxime with approximately 2.0×10^9 CFU as determined by viable count on plain GC base agar and were found to be 2.1×10^{-9} Cpd^r/CFU for SPL4 and 1.9×10^{-9} Cpd^r/CFU for SPN284. By comparison, the frequency of mutation to resistance to rifampin (30 μg/ml) was similarly determined to be 3.0×10^{-9} Rif^r/CFU for both SPN284 and SPL4. The MIC values to ceftriaxone for 10 mutants derived from SPL4 were found to be 0.25 to 0.5 μg/ml. Similar results were obtained for most of the mutants derived from SPN284 (Table 2), but a single mutant was found to have an MIC to ceftriaxone of 1.0 μg/ml. Because of the unusually high MIC to ceftriaxone, this mutant, designated SPN284 3-1, and a mutant derived from SPL4 with an MIC to ceftriaxone of 0.5 μg/ml and designated SPL4 3-4 were retained for further characterization. DNA isolated from SPN284 3-1 and from SPL4 3-4 was used to transform the mosaic *penA* strain 3502 to resistance to 4.5 μg/ml of cefpodoxime as described elsewhere (20, 21). Resistant transformants appeared at a frequency of 2×10^{-3} transformants/donor CFU. Three transformants obtained with SPN284 3-1 DNA and three obtained with SPL4 3-4 DNA exhibited MICs identical to those of the donor strains (Table 1). No detectible level of resistance could be transformed into 28Bl, which lacks the genetic background of 3502 (Table 1). To determine the mutation responsible for increased resistance in SPN284 3-1, DNA from the three transformants was purified and subjected to genomic sequencing at the Broad Institute from modified Illumina (Illumina, Inc., San Diego, CA) libraries (22) using either an Illumina HiSeq 2000 or Gallx instrument. Sequence alignments and analysis were carried out with BWA version 0.5.9-r16 9 (23), SAMtools (24), Galaxy (25–27), and SeaView version 4.2.6 (28, 29). In each trans-

Received 16 April 2014 Returned for modification 25 May 2014
Accepted 12 August 2014
Published ahead of print 8 September 2014
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doi:10.1128/AAC.03082-14

TABLE 1 Properties of the strains used in this study

Strain (references)	Source	MIC (μg/ml)[a]					Genotype	
		Pen	Cro	Cfx	Cpd	Ery/Tx[b]	*penA*	*ponA*[c]
SPN275	GISP[d] isolate	8.0	0.008	0.015	0.06	R	Insertion	Leu-Pro
SPN280	GISP isolate	8.0	0.015	0.03	0.03	R	Insertion	Leu-Pro
SPN284	GISP isolate	4.0	0.06	0.25	2.0	R	Mosaic	Leu-Pro
SPL4	GISP isolate	8.0	0.03	0.25	2.0	R	Mosaic	Leu-Pro
3502	Reference isolate	4.0	0.03	0.25	1.0	R	Mosaic	Leu-Pro
28Bl (20, 21)	DGI isolate (CDC, 1974)	0.015	0.008	0.015	0.06	S	WT[e]	WT
SPN284 3-1	Spontaneous mutant of SPN284	16.0	1.0	>1.0	>8.0	R	Mosaic	Leu-Pro
SPL4 3-4	Spontaneous mutant of SPL4	32.0	0.5	1.0	>8.0	R	Mosaic	Leu-Pro
3502T1	Transformant SPN284 3-1 × 3502	32.0	1.0	>1.0	>8.0	R	Mosaic	Leu-Pro
3502T2	Transformant SPN284 3-1 × 3502	32.0	1.0	>1.0	>8.0	R	Mosaic	Leu-Pro
3502T4	Transformant SPN284 3-1 × 3502	32.0	1.0	>1.0	>8.0	R	Mosaic	Leu-Pro
3502L3-1	Transformant SPL4 3-4 × 3502	32.0	0.5	1.0	>8.0	R	Mosaic	Leu-Pro
3502L3-2	Transformant SPL4 3-4 × 3502	32.0	0.5	1.0	>8.0	R	Mosaic	Leu-Pro
3502L3-3	Transformant SPL4 3-4 × 3502	32.0	0.5	1.0	>8.0	R	Mosaic	Leu-Pro
3502L3-4	Transformant SPL4 3-4 × 3502	32.0	0.5	1.0	>8.0	R	Mosaic	Leu-Pro

[a] Pen, penicillin; Cro, ceftriaxone; Cfx, cefixime; Cpd, cefpodoxime.
[b] Resistance to 600 μg/ml Triton X-100 and 1.2 μg/ml of erythromycin (Ery/Tx) was used to determine the MtrR phenotype (13). R, resistant; S, susceptible.
[c] The genes sequenced show the Leu-Pro substitution at amino acids associated with β-lactam resistance (29).
[d] GISP, Gonococcal Isolate Surveillance Project.
[e] WT, wild type.

formant, a point mutation that resulted in G→A at position 1444 was found in *penA*. This mutation caused the substitution G483S. Direct sequencing of the amplified *penA* gene of SPN284 3-1 confirmed the presence of this mutation (Fig. 1A). To directly demonstrate that this mutation resulted in increased resistance in SPN284 3-1, the *penA* gene was amplified with specific primers (Table 3) by PCR, and the amplified DNA was used to transform 3502 to resistance to 4.5 μg/ml of cefpodoxime. The transformants, obtained at a frequency of 5×10^{-3} transformants/recipient CFU, were phenotypically indistinguishable from SPN284 3-1 and the initial transformants constructed with native genomic DNA.

A similar approach was used to characterize the mutation in SPL4 3-4. Transformants from the cross SPL4 3-4 × 3502 were selected for resistance to 3.4 μg/ml of cefpodoxime. The MIC of four transformants was found to be identical to that of the donor strain. DNA was extracted and subjected to genome sequencing and the data to partial analysis in the Centers for Disease Control and Prevention (CDC) Genomics Sequencing Laboratory. The results showed an identical 2-bp insertion in the *pilQ* gene of each of the transformants (Fig. 1B). Similar to the previous experiment, the *pilQ* gene was amplified (Table 3) from the DNAs of both SPL4 3-4 and 3502L3-1. Amplified DNA was used to transform 3502 as described above. The *pilQ* genes from three transformants from the SPL4 3-4 donor and two transformants from the 3502L3-1 donor, all selected on 3.4 μg/ml of cefpodoxime, were sequenced. In all cases, the same 2-bp insertion was present. All of the 3502 transformants derived from SPL4 3-4 and 3502L3-1 donors and a Pil^{+} 3502 recipient were phenotypically Pil^{-}, not competent, and did not revert to Pil^{+} at a detectible frequency regardless of the source of the DNA.

The results show that it was possible to isolate *in vitro* a mutation in the *penA* gene of *N. gonorrhoeae* that caused elevated resistance to at least three different cephalosporins. This previously unrecognized mutation in SPN284 3-1 gave a phenotype similar to those of two strains isolated from clinical sources and was located in the same region as mutations found in H041 and F89 (5, 6, 30). Additionally, mutations in genes other than *penA* can increase MICs to cephalosporins, as demonstrated by the insertion

TABLE 2 MICs of mutants selected on cefpodoxime gradient plates

Cpd concn (μg/ml)[a]	Strain	No. of isolates tested	MIC (μg/ml)[b]				
			Pen	Tet	Cro	Cfx	Cpd
4.0	SPL4	5	32.0–64.0	4.0–8.0	0.25–0.5	0.5	≥8.0
	SPN284	4	16.0	4.0	0.25–0.5	0.5	8.0
6.0	SPL4	8	32.0–64.0	4.0–8.0	0.25–0.5	0.5–1.0	≥8.0
	SPN284	3	32.0	4.0	0.25–0.5	1.0	8.0

[a] Concentration of cefpodoxime (Cpd) in the gradient plate substratum used for *in vitro* selection of mutants with elevated MICs to cefpodoxime.
[b] Pen, penicillin; Tet, tetracycline; Cro, ceftriaxone; Cfx, cefixime.

A.
SPN284
464 474 484 494 504
VIKASTAKKVRELMVSVTEAGGTGTAGAVDGFDVGAKTGTARKL

SPN284 3-1
VIKASTAKKVRELMVSVTEAGSTGTAGAVDGFDVGAKTGTARKL

B.
pilQ*
427 437 447 457 467 477
GCCGCGCCTGCCG—CACCGGCAAAACAACAGGCTGCCGCACCGTTTACCGAGTCCGTAG

SPL4 3-4 *pilQ*
GCCGCGCCTGCCGcaCACCGGCAAAACAACAGGCTGCCGCACCGTTTACCGAGTCCGTAG

FIG 1 (A) The G→S substitution at position 483 and the flanking amino acid sequence for the protein coded for by the *penA* gene of SPN284 3-1. The coordinate system used is from reference 20. (B) The 2-bp insertion in the sequence of the *pilQ* gene of SPL4 3-4 with immediate flanking nucleotide sequence. The alignment was with *N. gonorrhoeae pilQ** (accession no. U40596). The highlighted nucleotides denote the termination codon created by the insertion.

TABLE 3 Primers for amplification of *penA* and *pilQ* for transformation and sequencing

Primer	Sequence
ppPNAmut3	5′-CGGGCAATACCTTTATGGTGG-3′
ppPNAmut4	5′-AGCCAAAGGGCTTAACTTGC-3′
ppPilQ1	5′-GGTGTCGGCAACTATTTGGG-3′
ppPilQ4	5′-CGGAATGACGGCATTTCGG-3′

mutation in *pilQ* present in SPL4 3-4. Alteration of *pilQ* was previously shown to increase resistance to penicillin by alteration of the permeability of the outer membrane (31, 32). These results demonstrate that this mutation in *pilQ* had a similar effect on cephalosporin resistance and increased the MIC to ceftriaxone almost 10-fold. Because mutations in *pilQ* commonly result in a Pil$^-$ phenotype, it is not evident that such mutations will be of consequence, as piliation is required for pathogenesis (33, 34).Since mutations in *pilQ* can result in complex phenotypes (35), it may be possible to isolate mutations in *pilQ* that allow for both pathogenesis and for increased cephalosporin resistance.

Finally, it is apparent that caution must be used in selecting alternative cephalosporins and possibly other β-lactam antibiotics for treatment of gonococcal infections. While mutants resistant to slightly elevated concentrations of ceftriaxone or cefixime were difficult to isolate (data not shown), it proved relatively easy to select mutants resistant to moderate concentrations of cefpodoxime that also showed significantly increased resistance to both of the other antibiotics, suggesting that the use of cefpodoxime could adversely affect the value of the other cephalosporins.

Nucleotide sequence accession numbers. The complete sequences of the *penA* genes of SPN284 and SPN284 3-1 and the *pilQ* gene of SPL4 3-4 have been deposited in GenBank under accession no. KM403400, KM403401, and KM452733, respectively.

ACKNOWLEDGMENTS

Y.H.G was supported by grant 1-K08-AL104767 from NIAID, and M.L. was supported by grant U54GM088558 from NIGMS.

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the official position of the Centers for Disease Control and Prevention, the Agency for Toxic Substances and Disease Registry, NIGMS, or NIH.

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