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Genome Sequencing of a *Neisseria gonorrhoeae* Isolate of a Successful International Clone with Decreased Susceptibility and Resistance to Extended-Spectrum Cephalosporins

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The recent emergence of *Neisseria gonorrhoeae* strains with decreased susceptibility to extended-spectrum cephalosporins is a major concern globally. We sequenced the genome of an *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) ST1407 isolate (SM-3) with decreased susceptibility and resistance to oral extended-spectrum cephalosporins. The isolate was cultured in 2008 in San Francisco, CA, and possessed mosaic *penA* allele XXXIV, which is associated with an international clone that possesses decreased susceptibility as well as resistance to oral extended-spectrum cephalosporins globally. The genome sequence of strain NCCP11945 was used as a scaffold, and our assembly resulted in 91 contigs covering 2,029,064 bp (91%; >150× coverage) of the genome. Numerous instances of suspected horizontal genetic transfer events with other *Neisseria* species were identified, and two genes, *opa* and *txf*, acquired from nongonococcal *Neisseria* species, were identified. Strains possessing mosaic *penA* alleles ($n = 108$) and nonmosaic *penA* alleles ($n = 169$) from the United States and Europe (15 countries), cultured in 2002 to 2009, were screened for the presence of these genes. The *opa* gene was detected in most (82%) *penA* mosaic-containing isolates (mainly from 2007 to 2009) but not in any *penA* nonmosaic isolates. The *txf* gene was found in all strains containing *opa* but also in several (18%) *penA* nonmosaic strains. Using *opa* and *txf* as genetic markers, we identified a strain that possesses mosaic *penA* allele XXXIV, but the majority of its genome is not genetically related to strain SM-3. This implies that *penA* mosaic allele XXXIV was transferred horizontally. Such isolates also possessed decreased susceptibility and resistance to oral extended-spectrum cephalosporins. These findings support that genetic screening for particular *penA* mosaic alleles can be a valuable method for tracking strains with decreased susceptibility as well as resistance to oral extended-spectrum cephalosporins worldwide and that screening using only NG-MAST may not be sufficient.

Infections with *Neisseria gonorrhoeae* are the second most commonly reported notifiable diseases in the United States (8), and an estimated 88 million new cases of gonorrhea occur each year worldwide (47). *N. gonorrhoeae* has developed resistance to all antimicrobials previously used as first-line treatments, such as sulfanilamides, penicillins, tetracyclines, macrolides, and fluoroquinolones (4, 6, 20, 21, 36, 44). Extended-spectrum cephalosporins are the only first-line agents remaining for the treatment of gonorrhea in many settings worldwide (4, 5, 36, 45), and in the United States (46) and the United Kingdom (5), two-drug treatment with extended-spectrum cephalosporins combined with azithromycin or doxycycline is recommended. However, isolates with decreased susceptibility and resistance to extended-spectrum cephalosporins have been identified in Asia, Australia, Europe, Canada, and the United States (1, 3, 4, 6, 10, 11, 16, 19, 20, 23, 27, 28, 30, 33, 36, 48). Furthermore, treatment failures using oral extended-spectrum cephalosporins have been reported in Japan, Hong Kong, and Europe (3, 11, 18, 24, 30, 38, 39, 41–43, 48, 49). Recent gonorrhea treatment failures with cefixime in Norway (43), Austria (42), France (40), and the United Kingdom (18) were caused by *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) ST1407 or sequence types (STs) evolving from ST1407. NG-MAST ST1407 is highly prevalent in Europe and has been spreading worldwide (16, 39). Recently, the first two extensively drug-resistant gonococcal strains, which were both highly resistant to the most potent extended-spectrum cephalosporin, ceftriaxone, were isolated in Japan (30) and France (39). The strain from France also belonged to NG-MAST ST1407. If these strains that are resistant to extended-spectrum cephalosporins start to spread globally, gonorrhea will become untreatable in certain circumstances and especially in some settings (6, 30, 36, 45). Thus, it is crucial to investigate in detail all gonococcal strains suspected to be resistant to the recommended extended-spectrum cephalosporins in order to confirm resistance, predict the possibility of treatment failure and the further spread of the strains, and elucidate their phenotypic and genetic characteristics, in particular their resistance mechanisms (30, 36, 39).

Previous reports have linked decreased susceptibility and resistance to extended-spectrum cephalosporins to different "mosaic" forms of penicillin binding protein 2 (the lethal target for these antimicrobials) encoded by mosaic *penA* alleles (3, 4, 6, 16, 19, 20, 23, 30, 31, 33, 36–39, 41, 42, 49). We previously reported five *N.*

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gonorrhoeae isolates in San Francisco, CA, with related variants of the mosaic *penA* allele and decreased susceptibility to oral cephalosporins (33). However, these identified mosaic *penA* alleles were not sufficient to achieve high-level resistance to extended-spectrum cephalosporins. Resistance determinants such as *mtrR* (causing the overexpression of the MtrCDE efflux pump, which increases the efflux of the extended-spectrum cephalosporins) and *penB* (resulting in the decreased intake of the extended-spectrum cephalosporins through the PorB1b porin) are also contributing to the enhanced MICs of extended-spectrum cephalosporins (16, 23, 30, 36, 39, 49). However, *in vitro* transformation experiments indicated that another, currently unknown, resistance determinant is likely playing a role (16, 30, 39, 49).

In order to more fully characterize the genomic alterations among *N. gonorrhoeae* strains containing mosaic *penA* allele XXXIV and decreased susceptibility to extended-spectrum cephalosporins in San Francisco, we subjected NG-MAST ST1407 isolate SM-3 (33), with decreased susceptibility and resistance to oral extended-spectrum cephalosporins (cefpodoxime MIC = 1.0 μg/ml; cefixime MIC = 0.25 μg/ml), to genome sequencing. On the basis of the genome sequence, we designed and constructed molecular tests for several novel genetic markers from the sequenced strain (SM-3 [33]). We used these molecular tests to screen for these markers in a panel of both *penA* mosaic and *penA* nonmosaic strains from the United States and Europe (collected in 15 countries). These data revealed a pair of interesting strains whose genomes had a mixture of markers that matched strain SM-3 (33) and markers that matched strains with full susceptibility to extended-spectrum cephalosporins. A focused analysis of these strains showed that *penA* mosaic allele XXXIV in SM-3 (33) is the primary determinant of decreased susceptibility and resistance to oral extended-spectrum cephalosporins. Furthermore, we identified a strain that appeared to have obtained *penA* mosaic allele XXXIV found in SM-3 (33) via horizontal genetic transfer, which conferred decreased susceptibility as well as resistance to oral extended-spectrum cephalosporins to the strain.

MATERIALS AND METHODS

Bacterial collection (California). Approximately 20 isolates of urogenital *N. gonorrhoeae* were collected monthly from symptomatic males at San Francisco City Clinic, the municipal sexually transmitted disease (STD) clinic. Two additional isolates were obtained from the Public Health Laboratory in San Diego, CA. Isolates for this study were collected in 2008 and 2009. Among these isolates, 30 were positive for the *penA* mosaic, and 57 did not contain the *penA* mosaic. The *penA* mosaic isolates included isolates with *penA* allele XXXIV as well as other *penA* alleles. All cultures were initially isolated on modified Thayer Martin selective agar (Becton Dickinson, MD) and immediately incubated for 24 h in a 10% CO_2-enriched atmosphere at 35°C. Plates were transferred to the San Francisco Public Health Laboratory, and isolates were identified via microscopy after Gram staining and oxidase testing. Cultures were purified on chocolate agar and incubated for 24 h in a 10% CO_2-enriched atmosphere at 35°C. Pure cultures were frozen as paired samples in tryptic soy broth (TSB) with 1% glycerol. One set of samples was shipped to a Centers for Disease Control and Prevention (CDC)-sponsored Gonococcal Isolate Susceptibility Project (GISP) laboratory at the University of Washington, Seattle, and the second set was stored at −30°C.

Bacterial collection (Europe). Previously examined (16) *penA* mosaic isolates ($n = 78$) and *penA* nonmosaic isolates ($n = 112$) originating from 15 countries in Europe (cultured in 2002 to 2009) were screened for the novel genetic markers identified from the genome sequence of SM-3 (33). The *penA* mosaic isolates included isolates with *penA* allele XXXIV as well as other *penA* alleles.

GISP. Isolates sent to the University of Washington GISP Laboratory were subjected to antibiotic susceptibility testing using the agar dilution method as outlined by the CDC GISP protocol, which is in accordance with the protocols from the Clinical and Laboratory Standards Institute (http://www.cdc.gov/std/gisp/) as of 19 March 2012. Isolates were tested for MICs of eight antimicrobials: cefixime (for isolates from 2009 only), ceftriaxone, cefpodoxime (for isolates from 2009 only), penicillin, tetracycline, spectinomycin, ciprofloxacin, and azithromycin.

NG-MAST. *N. gonorrhoeae* DNA was obtained by automated DNA extraction using the Total NA kit and protocol on a MagNA Pure instrument (Roche Molecular Diagnostics, Mannheim, Germany). NG-MAST STs were determined by PCR amplification and sequencing of more variable regions of the *porB* and *tbpB* genes, as previously described (26).

Mosaic *penA* allele identification. Screening for the mosaic *penA* allele was performed by using a real-time PCR assay as previously described (29). The confirmation of the mosaic *penA* alleles was performed by the sequencing of *penA* as described previously (33).

Genome sequencing. Five micrograms of DNA (isolated by using the same method as that used for NG-MAST) was concentrated to 50 μl by using a vacuum centrifuge. The DNA was sequenced on a single lane of an Illumina Genome Analyzer II system to generate 7,642,574 single-end, 54-bp reads. To analyze the data, *de novo* contig assembly was performed by using ABySS (35). We filtered these contigs based on size (>300 bp) and coverage (>70×), which yielded 573 contigs with an average coverage of 150×. Using the previously reported NCCP11945 gonococcal genome (9) as a scaffold, we further assembled these 573 contigs into 91 continuous contigs with an average size of 22,500 bp. These 91 larger contigs covered 2,029,064 bp (91.1%) of the NCCP11945 reference genome (9). The vast majority of the gaps between these contigs fell into homopolymeric regions of the *N. gonorrhoeae* genome. These contigs are contained in a single FASTA file (see File S1 in the supplemental material). One of these 91 contigs (ST1407 contig 091) did not match any unique region of the NCCP11945 genome (see "Identification of *opa* and *txf*" below).

SM-3 genetic marker identification. The determinations of *penA* nonmosaic alleles, SM-3 *penA* mosaic allele XXXIV, and other SM-3 genetic markers were performed on DNA extracted from each examined isolate by using the same method as that used for NG-MAST (see above). All primers used for screening are listed in Table S4 in the supplemental material. The *porB* and *tbpB* alleles were determined by using a previously described NG-MAST protocol (26). *penA* mosaic alleles were identified and confirmed as described above (see "Mosaic *penA* allele identification"). PCRs to amplify the *opa* and *txf* alleles found in SM-3 were performed by using SM-3 DNA as a positive control, with primers opa primerA/opa primer B and txf primerA/txf primerB, respectively. High-mutation-density region I (HM01), HM02, HM04, and HM10 alleles were determined by PCR amplification using the appropriate PA and PB primers (i.e., for HM01, primers HM01PA and HM01PB were used). Sanger sequencing was then performed by Sequetech (Mountain View, CA), using the Applied Biosystems 3730xl DNA analyzer on the PCR amplicons by using a nested set of sequencing primers (SA and SB). Sequences from all analyzed strains were aligned and compared by using CLUSTAL-W alignment with BioEdit 7.0.5.3 (17). Finally, for genomic rearrangement region 10 (GR10), two sets of PCRs were performed by using primers GR10A/GR10B and GR10A/GR10C. For the wild-type allele of GR10, primer pair GR10A/GR10B gave rise to a 500-bp product, but primer pair GR10A/GR10C yielded no PCR product. For the SM-3 allele, primer pair GR10A/GR10B did not yield a PCR product, but primer pair GR10A/GR10C yielded a 500-bp PCR product.

***mtrR* allele identification.** Each of the major strain types used in this study had the *mtrR* promoter and coding gene region sequenced by PCR amplification and subsequent Sanger sequencing (Sequetech, Mountain View, CA) (using the Applied Biosystems 3730xl DNA analyzer). The

TABLE 1 Description of each of the 10 major genomic rearrangements detected by our sequencing and their locations with respect to the NCCP11945 genome[a]

Genomic rearrangement	Size of deletion (bp)	Size of insertion (bp)	NCCP11945 genome location (positions)[a]
1	45		590736–590780
2	154		2146961–2147115
3		44	634917
4		213	1805954
5 (*opa*)		738	Unknown
6	318	132	77893–78211
7	159	38	299343–299502
8 (*txf*)	582	1,106	1602296–1603402
9	1,709	1,643	1644876–1646519
10	607	617	1740461–1741068

[a] For rearrangements with both insertions and deletions, the section deleted in NCCP11945 is noted in the genome location column.

TABLE 2 Effect on the coding sequence of each of the 10 major genomic rearrangements detected in our sequencing and their location with respect to the NCCP11945 genome

Genomic rearrangement	Effect[a]
1	In-frame deletion of aa 166–181 of succinate semialdehyde dehydrogenase
2	In-frame deletion of 37 aa of a conserved hypothetical protein
3	Noncoding
4	Inserts 104 aa into a conserved hypothetical protein
5 (*opa*)	Insertion of opacity ORF not previously seen in *N. gonorrhoeae*
6	Deletes *pilS* cassette and start of a signal peptidase II protein
7	Noncoding
8 (*txf*)	Insertion of transcription factor ORF not previously seen in *N. gonorrhoeae*
9	Mutates the last 2 aa of an ATP-dependent helicase and removes 3 hypothetical proteins
10	Introduces an allele of a conserved hypothetical protein likely from *N. meningitidis*

[a] aa, amino acid(s).

primers used for PCR amplification were mtrrPA and mtrrPB, while the primers used for Sanger sequencing were mtrrSA and mtrrSB (see Table S4 in the supplemental material). Sequences for the *mtrR* locus, including 100 bp of the promoter region and the entire coding region, are reported in Table S3 in the supplemental material.

SNP detection. Each of the 91 large contigs was pairwise blasted against their counterpart sequence from NCCP11945 (9), using default settings for NCBI BLAST (2). Single-nucleotide polymorphisms (SNPs) are reported in Table S2 in the supplemental material. In cases of an amino acid coding change with respect to the NCCP11945 genome annotations (9), these were also recorded along with the open reading frame (ORF) annotation of the protein function (see Table S2 in the supplemental material).

Maximum likelihood phylogenetic tree generation. Partial *porB* alleles (from NG-MAST) for all the identified NG-MAST STs were assembled into a standard FASTA file. These sequences were aligned by using a BioEdit 7.0.5.3 standard CLUSTAL-W alignment (17). The aligned sequences were assembled into a relatedness tree by using the DNAml DNA maximum likelihood phylogenetic method (15).

RESULTS

Illumina genome sequencing of gonococcal *penA* mosaic allele XXXIV of strain SM-3. We performed whole-genome sequence analysis on an *N. gonorrhoeae* isolate, SM-3, which was previously reported to contain a mosaic *penA* gene (allele XXXIV [30]) and decreased susceptibility and resistance to oral extended-spectrum cephalosporins (cefpodoxime MIC = 1.0 μg/ml; cefixime MIC = 0.25 μg/ml), although the isolate was sensitive to the injectable extended-spectrum cephalosporin ceftriaxone (MIC = 0.03 μg/ml) (33). SM-3 was obtained from a male patient with symptomatic urethritis seen at the San Francisco municipal STD clinic.

Genome sequencing was performed by using the Illumina Genome Analyzer II platform (one full lane of sequence). We collected 7,642,574 single-end, 54-bp reads. These reads were assembled by using the complete genome sequence of strain NCCP11945 (9) as a scaffold. Strain NCCP11945 possesses chromosomally mediated resistance to both penicillin and tetracycline in addition to high-level resistance to ciprofloxacin (MIC of 16 μg/ml) (9). However, strain NCCP11945 does not have decreased susceptibility to cephalosporins. The sequence reads covered over 99% (2,029,064 bp) of the nonrepetitive sequence in the NCCP11945 genome and 91% of the entire genome (summary of sequence files can be found in Table S1 in the supplemental material, and the sequences of the contigs can be found in File S1 in the supplemental material), with a coverage of over 150×.

Analysis of the SM-3 genome in comparison to the NCCP11945 genome revealed striking similarities. The genome sequences of SM-3 and NCCP11945 showed 99.9% identity, with only 1,829 SNPs and 445 small (less than 10 bp) insertions or deletions in the 2,029,064 bp (see Table S2 in the supplemental material for a summary of SNPs). In addition to these changes, there were 10 regions of larger (greater than 10 bp) genomic rearrangements (Tables 1 and 2 and Fig. 1). Two of these changes were insertions (44 bp and 213 bp), and two were deletions (75 bp and 154 bp). Four of the changes were sequence swaps, where a portion of the NCCP11945 genome was replaced with another sequence. The

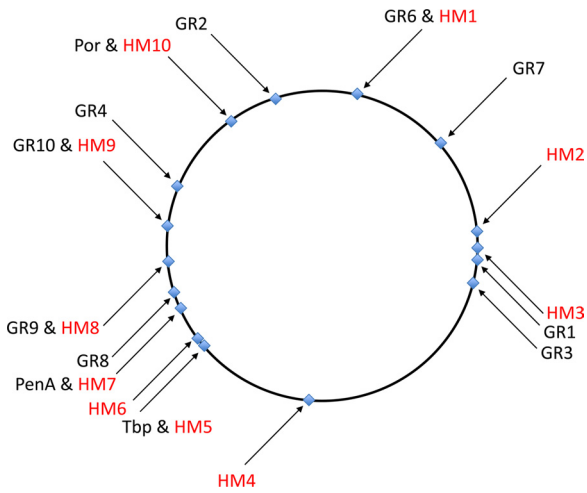

FIG 1 Chromosomal positions of the major features reported in the manuscript. GR stands for genomic rearrangements and shows the locations of the 10 rearrangements reported in Tables 1 and 2. HM stands for high-mutation-density region and shows the locations of these 10 regions, as reported in Table 3. In addition, the location of the *penA* gene is shown, along with the two loci used to determine the NG-MAST sequence type: *porB* and *tbpB*.

TABLE 3 Details regarding the 10 regions that demonstrate elevated mutation rates in the genome sequence of *N. gonorrhoeae* strain SM-3[a]

Region	Start position	Stop position	Length (bp)	No. of SNPs	No. of indels	SNP density
HM01	78237	79649	1,413	49	1	29
HM02	520791	521643	853	42	8	20
HM03	569959	570156	198	49	13	4
HM04	1181692	1183313	1,622	40	12	41
HM05	1467757	1471900	4,144	265	68	16
HM06	1481866	1487570	5,705	102	8	56
HM07	1522374	1525855	3,482	265	6	13
HM08	1646709	1650103	3,395	107	0	32
HM09	1738998	1742393	3,396	140	0	24
HM10	2049316	2050842	1,527	74	10	21

[a] See reference 33. Each region is listed with its chromosomal location and total numbers of single-nucleotide polymorphisms (SNPs) and insertions and/or deletions. The SNP density is calculated by dividing the length of the region by the total number of SNPs in the region.

largest example of this was a 1,709-bp sequence replaced by a 1,643-bp sequence. Finally, we detected two instances of large whole-gene insertions not previously seen in other sequenced *N. gonorrhoeae* isolates. Those sequences which encoded entire genes, and not just gene fragments (*opa* and *txf*), showed substantial similarity to genes from other sequenced *Neisseria* species and are discussed below. In addition to *penA* mosaic allele XXXIV, SM-3 contained the *mtrR* (see below) and *penB* resistance determinants (16, 23, 30, 36, 39, 49).

Identification of *opa* and *txf*. The identification of *opa* arose from an analysis of ST1407 contig 091, which did not map to any region on the NCCP11945 genome (9), nor did it map to any region in the genome of the first genome-sequenced gonococcal strain, that is, FA1090 (12). When this 738-bp sequence was searched against the nonredundant database of sequences using NCBI BLAST, the closest matches were from nongonococcal species of *Neisseria*, with the highest identity (90% identity; 674/745 bp; 7 bp in gaps between the sequences) to an *opa* gene, encoding an opacity protein, from the *Neisseria lactamica* 020-06 genome (GenBank accession no. FN995097.1). The presence of the *opa* gene was determined by PCR amplification and Sanger sequencing of the PCR product. *txf* was detected as a sequence inserted into ST1407 contig 061 and is referred to as GR8 in Tables 1 and 2. This inserted sequence deleted 1,106 bp of sequence from the reference NCCP11945 genome (9). Similar to the *opa* query, this inserted sequence did not map to any region in the NCCP11945 (9) or FA1090 (12) genome. A BLAST analysis of the 582-bp sequence that did not map to NCCP11945 (9) against the nonredundant database also revealed that the closest matches were nongonococcal species of *Neisseria* (81% identity; 451/555 bp; 26 bp in gaps between the sequences), with the highest identity to several *Neisseria meningitidis* genome sequences (GenBank accession no. CP002424.1). PCR amplification and Sanger sequencing of the PCR product confirmed the presence of the *txf* sequence in SM-3. These two genes were analyzed further (see below).

The majority of the genomic divergences between SM-3 and NCCP11945 was located in a small portion of the genome. In addition to the genomic rearrangements mentioned above, 10 regions of the genome demonstrated extremely elevated levels of SNPs (Table 3 and Fig. 1). These 10 regions of the genome totaled only 25,735 bp in length (1.3% of the total sequence obtained) yet contained half of the detected SNPs (1,133 SNPs; 49.8%). These regions showed an SNP density of 1 SNP per 23 bp. This density is substantially higher than the SNP density for the rest of the genome (1 SNP per 1,756 bp). Furthermore, these 10 regions are spread throughout the genome and are separated by large genetic distances and thus must have arisen from multiple events that reshaped the genome of isolate SM-3.

These regions with a high mutation density, along with the genomic rearrangements, serve as important genetic markers in the SM-3 genome. Analyses of these markers and their correlation with elevated MICs of oral extended-spectrum cephalosporins allowed us to narrow the regions of the *N. gonorrhoeae* chromosome that contain sequences associated with a decreased susceptibility to oral extended-spectrum cephalosporins. Furthermore, we can use these genetic markers to identify other isolates that are related to isolate SM-3. In order to identify candidate regions of the chromosome that might contain an unknown determinant of elevated MICs of extended-spectrum cephalosporins, we tested *N. gonorrhoeae* isolates for these markers. These isolates had known MICs of extended-spectrum cephalosporins and a known presence or absence of the mosaic *penA* alleles.

The *opa* gene is present only in specific *penA* mosaic allele strains. The nongonococcal *opa* gene identified by using genome sequencing as a sequence inserted into the SM-3 genome encodes a full-length opacity protein, which is found in the outer membrane of the gonococcal cell. Using PCR for this *opa* gene (Tables 1 and 2), we tested 108 gonococcal isolates with a mosaic *penA* allele and elevated MICs of cefpodoxime (MIC = 0.5 to 2.0 μg/ml) and 169 isolates with nonmosaic *penA* alleles collected during 2008 to 2009 on the west coast of the United States and during 2002 to 2009 in 15 countries of Europe (Table 4). The *opa* gene was not detected in any *penA* nonmosaic isolates. However, it was detected in 88 of the 108 (82% of which were mainly from 2007 to 2009) *penA* mosaic-containing isolates. Furthermore, 84 (95%) of those 88 isolates were assigned as NG-MAST ST1407 or closely related STs (see below) and thus are likely to be descendant from a common strain. *N. gonorrhoeae* isolates of ST1407 containing *penA* mosaic allele XXXIV have been detected throughout the world and are associated with decreased susceptibility and resistance to extended-spectrum cephalosporins and multidrug resistance (16, 39). Additionally, all 88 isolates containing the *penA* mosaic and the *opa* gene also contained the transcription factor (*txf*) sequence acquired by horizontal gene transfer (see below).

The chromosomal location of the *opa* gene in strain SM-3 is not yet known. The contig containing the ORF for this gene ends in the repeated sequence that flanks the *opa* genes in other *Neisseria* species. Because of that, we suspect that this nongonococcal

TABLE 4 Two of the genes in the genome sequence of *N. gonorrhoeae* strain SM-3[a] that have been acquired from other *Neisseria* species[b]

Isolate type	Presence of *txf* gene	No. of isolates		Total no. of isolates with gene acquired from other *Neisseria* spp.
		With *opa*	Without *opa*	
penA mosaic ($n = 108$)	+	88	4	92
	−	0	16	16
Total		88	20	108
penA nonmosaic isolates ($n = 169$)	+	0	31	31
	−	0	138	138
Total		0	169	169

[a] See reference 33.
[b] The *opa* and *txf* genes were examined for their presence or absence in 108 *penA* mosaic and 169 nonmosaic isolates. The *opa* gene was detected in only a subset of *penA* mosaic isolates and always co-occurred with the *txf* gene.

TABLE 5 NG-MAST STs of all the *penA* mosaic isolates that were positive for the *opa* gene[a]

NG-MAST ST	*porB* allele	*tbpB* allele	No. of isolates	bp similarity with:	
				porB allele 908	*tbpB* allele 110
1407	908	110	48	490/490	390/390
3378	2043	110	8	489/490	390/390
3431	2078	110	5	489/490	390/390
4269	2623	110	3	488/490	390/390
4275	2622	110	3	489/490	390/390
2212	1388	110	2	489/490	390/390
3158	1914	110	2	488/490	390/390
4268	2620	110	2	483/490[b]	390/390
4274	3	110	2	449/490	390/390
1513	971	110	1	489/490	390/390
3128	1900	110	1	487/490	390/390
3149	1903	110	1	489/490	390/390
3294	1994	110	1	488/490	390/390
3499	2115	110	1	484/490[b]	390/390
3709	2237	110	1	489/490	390/390
3787	2292	110	1	488/490	390/390
4265	2621	110	1	488/490	390/390
4266	2624	110	1	488/490	390/390
4533	2763	110	1	489/490	390/390
4267	908	901	1	490/490	389/390
3501	908	782	1	490/490	358/390
4276	2622	4	1	489/490	313/390

[a] $n = 88$ (6 strains were not genotyped). The number of isolates of each ST identified is listed. Additionally, the relatedness to each component of NG-MAST ST1407 is reported by indicating the number of exact nucleotide matches in *porB* and *tbpB* (e.g., 489/490 indicates a 489 out of 490 nucleotide match in *porB*). Most of these isolates show close relatedness to ST1407 by NG-MAST.
[b] Contains an in-frame 6-bp deletion with respect to *porB* allele 908.

opa gene integrated into the genome at one of the 12 *opa* loci in the *N. gonorrhoeae* genome.

***txf* is seen in both *penA* mosaic and *penA* nonmosaic strains.** By using PCR, all the strains screened for *opa* were also screened for the transcription factor (*txf*) (Tables 1 and 2) identified by whole-genome sequencing (Table 4). All 88 of the mosaic *penA* isolates containing the *opa* gene described above also contained *txf*. However, we did detect *txf* in 4 out of the 20 (20%) *penA* mosaic isolates that did not possess the *opa* gene. Furthermore, we detected *txf* in 31 out of the 169 *penA* nonmosaic isolates (18%). These *penA* nonmosaic isolates that were positive for *txf* did not have decreased susceptibility to extended-spectrum cephalosporins. The fact that *txf* was detected in 100% of *penA* mosaic isolates that were positive for the *opa* gene further suggests that these isolates share a lineage. This is also supported by their common NG-MAST genotype, as described below.

NG-MAST STs of *penA* mosaic and *opa*-positive gonococcal isolates. We have determined the NG-MAST STs for the 88 *penA* mosaic and *opa*-positive isolates (Tables 4 and 5). Of these 88 isolates, 48 (55%) had NG-MAST ST1407 (*tbpB* allele 110 and *porB* allele 908), and another 36 isolates had an ST that had a ≤6-bp combined difference in the *tbpB* and *porB* genes of ST1407 (Table 5 and Fig. 2). The ≤6-bp cutoff value was based on a previously reported analysis of a large number of NG-MAST strain types (7). Eighty-three of these 84 isolates had *tbpB* allele 110. The remaining isolate had *tbpB* allele 901, which differs by a single nucleotide (T296G) from *tbpB* allele 110.

While there was more diversity among the *porB* alleles in our isolates, almost all of them were closely related. A phylogenetic tree using maximum likelihood methods was generated by using the *porB* alleles identified from isolates in this study. The *porB* allele of ST4274 was an extreme outlier, and it was removed from the analysis (Fig. 2). The tree is highly related and rooted with *porB* allele 908 from ST1407. This analysis strongly supports our hypothesis that *penA* mosaic isolates with the *opa* gene form a closely genetically related family of *N. gonorrhoeae* strains.

Identification of discordant isolates with partial genetic matches to strain SM-3. During the course of our investigation, we identified several isolates that were a close match to SM-3 but that differed at 1 or more of the 10 genetic markers tested. *penA* mosaic allele XXXIV isolates with NG-MAST ST4274, ST3501, ST4276, and ST3431, with decreased susceptibility to oral extended-spectrum cephalosporins (cefpodoxime MIC = 0.5 to 2.0 μg/ml), were further examined for the genetic markers and showed suspected recombination events that caused the loss of 1 or 2 markers found in the SM-3 genome (*porB* allele 908, *porB* allele 908 and GR10, *tbpB* allele 110, and GR10, respectively). As all of these isolates still demonstrated decreased susceptibility to extended-spectrum cephalosporins, this finding clearly shows that these genetic markers are not necessary for decreased susceptibility to oral extended-spectrum cephalosporins (data not shown).

Genetic maps of two discordant strain types revealed possible recombination events with cephalosporin-susceptible strains. In addition to the isolates described above, isolates of two other NG-MAST strain types (ST4252 and ST5895) were further investigated. Unlike the isolates described above, isolates of ST4252 and ST5895 differed from SM-3 at more than just one or two genetic markers. These isolates were intriguing because they shared only a partial set of the genetic markers with the sequenced strain SM-3.

For each of these strain types, the 10 generic markers used in this study (Fig. 1 and 3) were assayed to determine if those loci matched strain SM-3 or cephalosporin-susceptible strains. As a control, these 10 genetic markers were also sequenced for isolates with the five most common NG-MAST STs in the 2009 San Francisco GISP collection (7). All of the control isolates were susceptible to extended-spectrum cephalosporins. The

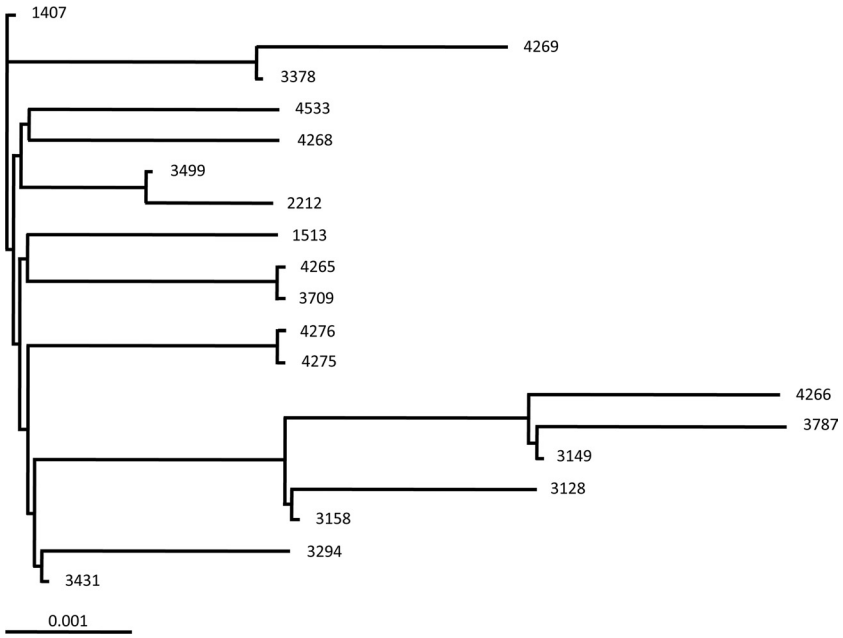

FIG 2 Phylogenetic trees based on the *porB* allele of *penA* mosaic, opacity factor-positive strains ($n = 83$). DNAml was used to generate maximum likelihood trees.

data are summarized in Fig. 3 and in Table S5 in the supplemental material.

NG-MAST ST4252 matched SM-3 at 8 of 10 alleles but did not display decreased susceptibility to oral extended-spectrum cephalosporins. The only two markers that were discordant were the *penA* gene, which did not have a mosaic allele, and *tpbB* (allele 892), which did not match allele 110 present in strain SM-3. These data suggest that strain SM-3 contains a nucleic acid sequence necessary for decreased susceptibility to oral extended-spectrum cephalosporins and that the necessary sequence exists between the *txf* marker and HM4, which clearly appears to be *penA* mosaic allele XXXIV.

Two isolates of NG-MAST ST5895 with decreased susceptibility to oral extended-spectrum cephalosporins (cefpodoxime MIC = 0.5 and 1.0 μg/ml) were tested for all 10 genetic markers and showed identical results. ST5895 matched strain SM-3 at four markers (GR10, *txf*, *penA*, and *tbpB*). At all other markers, these strains had alleles that matched cephalosporin-susceptible strains. The most parsimonious explanation to explain such a genetic map is a recombination event between an SM-3-like strain and a susceptible *N. gonorrhoeae* strain. Interestingly, these two isolates of ST5895 have acquired the SM-3 trait of decreased oral extended-spectrum cephalosporin susceptibility but do not possess the ciprofloxacin resistance seen for strain SM-3 and other ST1407 isolates. This finding is in agreement with our genetic map, as the primary determinant for ciprofloxacin resistance is located in the *gyrA* gene, which is found near the genetic marker HM4. These isolates may represent the transfer of decreased extended-spectrum cephalosporin susceptibility out of the NG-MAST ST1407 background into another genetic background. When the genetic maps from NG-MAST ST4254 and ST5895 are taken together, they clearly show that *penA* mosaic allele XXXIV can be a neces-

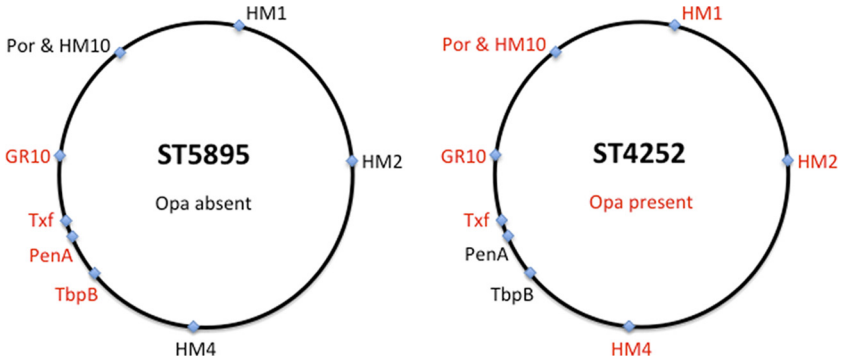

FIG 3 Graphical representations of genetic maps of the two discordant strain types investigated in detail in our study, ST5895 and ST4252. The genetic markers examined are displayed on the circular bacterial chromosome with relative positioning (see also Table S5 in the supplemental material). Genetic markers in red have alleles that match strain SM-3, while genetic markers in black have alleles that match cephalosporin-susceptible strains. Since the genomic location of the opacity factor is not known, its presence or absence is denoted underneath the relevant strain type.

sary and sufficient determinant for decreased susceptibility to oral extended-spectrum cephalosporins at the levels observed for isolate SM-3 (cefpodoxime MIC = 1.0 μg/ml; cefixime MIC = 0.25 μg/ml).

***mtrR* promoter and coding sequence of the major NG-MAST strain types examined in this study.** The *mtrR* locus has been associated with decreased susceptibility and resistance to several antibiotics in *penA* mosaic gonococcal strains (16, 23, 30, 36, 39, 49). As such, we wanted to examine the *mtrR* locus in the sequenced strain SM-3 (see Tables S3 and S6 in the supplemental material). The *mtrR* locus harbored two mutations previously associated with decreased susceptibility to antibiotics: the deletion of an A at position −57 in the promoter and a coding mutation resulting in an H105Y amino acid change (22, 32, 49). Interestingly, several of the strain types examined as described above had exact *mtrR* allele matches to SM-3 (see Tables S3 and S6 in the supplemental material). These included ST4252, ST2992, ST3935, ST730, ST3501, and the reference genome of NCCP11945 (9). ST4254 and ST28 had both the −57A deletion and the H105Y mutation but also had additional *mtrR* coding changes not previously associated with decreased susceptibility to antibiotics (see Table S6 in the supplemental material). Interestingly, the likely recombinant isolate (ST5895) that acquired *penA* mosaic allele XXXIV had a very different *mtrR* allele. This allele of *mtrR* did not possess either the −57A deletion or the H105Y mutation. Instead, it contained one mutation previously associated with decreased antibiotic susceptibility, the G45D amino acid change, and three nucleotide changes that did not result in coding changes (A345G, C354T, and G360A) (13, 34). The presence of *penA* mosaic allele XXXIV with a very different *mtrR* allele further supports our hypothesis that ST5895 acquired the *penA* mosaic via horizontal gene transfer.

DISCUSSION

In the present study, the genome of an *N. gonorrhoeae* NG-MAST ST1407 isolate (SM-3 [33]) with *penA* mosaic allele XXXIV was sequenced. This *N. gonorrhoeae* clone and its closely related subtypes appear to represent the spread and evolution of a very successful gonococcal clone. These isolates have been detected throughout the world and account for a substantial proportion of the decreased susceptibility and resistance to extended-spectrum cephalosporins and multidrug resistance (16, 39). Furthermore, one of the two extensively drug-resistant gonococcal strains identified in France (39), which was highly resistant to all oral extended-spectrum cephalosporins as well as ceftriaxone, was also assigned to ST1407. This isolate contained *penA* mosaic allele XXXIV with only one additional amino acid alteration (A501P), which resulted in high-level resistance to all extended-spectrum cephalosporins (39). ST1407 and its variants have accordingly appeared to be clonally related, but additional knowledge regarding this important clone is imperative. A critical finding of the present study was the identification of two isolates with NG-MAST ST5895. ST5895 does not appear to be a direct descendant of the ST1407 strain, or vice versa. Almost all *penA* mosaic allele XXXIV isolates in this study shared most, if not all, of the genetic markers observed for the genome sequence of strain SM-3. These genetic markers were not found in other common isolates of *N. gonorrhoeae* found in San Francisco. In contrast to this, the ST5895 isolates shared only four genetic markers with SM-3 (*penA* mosaic XXXIV, *tbpB* allele 110, and two additional genetic markers). These four markers are closely linked on the gonococcal genome. All other SM-3 genetic markers tested demonstrated alleles found in extended-spectrum cephalosporin-susceptible strains of *N. gonorrhoeae*. However, phenotypically, these two ST5895 isolates possessed decreased susceptibilities to cefpodoxime, with MICs of 0.5 and 1.0 μg/ml, which are mainly identical to that for SM-3 (MIC of 1.0 μg/ml). The most likely explanation for these results is a single recombination event that transferred a segment of DNA, including *penA* allele XXXIV and at least the three flanking genetic markers, to an extended-spectrum cephalosporin-susceptible strain. This transfer of DNA then conferred the phenotype of decreased susceptibility to oral extended-spectrum cephalosporins. The transfer of a *penA* mosaic allele was demonstrated previously *in vitro* (31), but this is the first report of such a genetic transfer event apparently *in vivo*, involving *penA* mosaic allele XXXIV from the successful ST1407 clone spreading worldwide (16, 39). To date, all isolates of ST1407 and its variants have appeared to be clonally related. However, in the present study, we have described two ST5895 isolates that have the potent *penA* mosaic allele XXXIV but that do not appear to be clonally related to ST1407 and instead arose through recombination. This has implications for the use of NG-MAST as a screening tool for public health investigations, as such efforts would have to screen for more than just specific STs, such as ST1407, to detect isolates with decreased susceptibility to extended-spectrum cephalosporins. These data, however, also confirm that screening for the mosaic *penA* alleles (or other resistance determinants) is a very useful method for identifying isolates with decreased susceptibility to oral extended-spectrum cephalosporins.

It is striking that nearly half of the SNPs detected by our genome sequencing are clustering in just 1.3% of the genome. The two most likely explanations for these regions are recombination with other *Neisseria* species that are more divergent from NCCP11945 (9) than SM-3 (33) or error-prone DNA damage repair, such as lesion bypass synthesis. As *Neisseria* species are known to exchange repetitive regions of their genomes (14, 25), we favor recombination as the explanation for these regions. Intriguingly, our examination of the SM-3 (33) genome focused on nonrepetitive regions of the genome, suggesting that recombination in *Neisseria* is not limited to repetitive regions but extends to the unique coding regions that make up the bulk of the genome. An understanding of the rates and extent of genomic recombination may be important for properly tracking and controlling multidrug-resistant strains of *N. gonorrhoeae*.

One goal of our genome sequencing of SM-3 (33) was to determine if there were other necessary large-sequence genetic factors for decreased susceptibility and resistance to extended-spectrum cephalosporins in *penA* mosaic allele XXXIV strains. The two discordant and recombinant strains in this study eliminated all of the high-mutation areas and the majority of rare SNPs as necessary factors for decreased susceptibility to oral extended-spectrum cephalosporins. Our data clearly show that *penA* mosaic allele XXXIV is a necessary factor for the decreased susceptibility to extended-spectrum cephalosporins in strain SM-3 (33). It remains possible that other mutations tightly linked to *penA* may also be necessary, but distinguishing these two models is not experimentally practical. Furthermore, the transfer of *penA* mosaic allele XXXIV to isolates with an ST5895 background which gained the decreased susceptibility to oral extended-spectrum cephalosporins strongly suggests that this *penA* allele may be sufficient for

the phenotype in at least some *N. gonorrhoeae* strain backgrounds, e.g., the ones already containing *mtrR* and *penB* alterations (16, 23, 30, 36, 39, 49). These findings support the assertion that screening for particular *penA* mosaic alleles by molecular techniques may be a valuable method for the identification of isolates with decreased susceptibility to oral extended-spectrum cephalosporins and that screening using only NG-MAST may not be sufficient. The vast majority of clinical and public health screenings in many settings are performed by using nucleic acid amplification tests where no culture-based antimicrobial susceptibility testing can be done. Thus, the strategy of molecular testing will be important for improving the early identification and detection of the further spread of isolates likely to fail treatment with extended-spectrum cephalosporins. Ongoing investigations of the genomic sequences of *N. gonorrhoeae* isolates with resistance phenotypes for extended-spectrum cephalosporins may increase the knowledge of genetic contributors to resistance and further aid in the development of the ability to identify such resistant strains with molecular tests.

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