Emergence of a new *Neisseria meningitidis* clonal complex 11 lineage 11.2 clade as an effective urogenital pathogen

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Neisseria meningitidis **(Nm) clonal complex 11 (cc11) lineage is a hypervirulent pathogen responsible for outbreaks of invasive meningococcal disease, including among men who have sex with men, and is increasingly associated with urogenital infections. Recently, clusters of Nm urethritis have emerged primarily among heterosexual males in the United States. We determined that nonencapsulated meningococcal isolates from an ongoing Nm urethritis outbreak among epidemiologically unrelated men in Columbus, Ohio, are linked to increased Nm urethritis cases in multiple US cities, including Atlanta and Indianapolis, and that they form a unique clade (the US Nm urethritis clade, US_NmUC). The isolates belonged to the cc11 lineage 11.2/ET-15 with fine type of PorA P1.5–1, 10–8; FetA F3-6; PorB 2–2 and express a unique FHbp allele. A common molecular fingerprint of US_NmUC isolates was an IS1301 element in the intergenic region separating the capsule *ctr-css* operons and adjacent deletion of *cssA/B/C* and a part of *csc*, encoding the serogroup C capsule polymerase. This resulted in the loss of encapsulation and intrinsic lipooligosaccharide sialylation that may promote adherence to mucosal surfaces. Furthermore, we detected an IS1301-mediated inversion of an ∼20-kb sequence near the *cps* locus. Surprisingly, these isolates had acquired by gene conversion the complete gonococcal denitrification *norB-aniA* gene cassette, and strains grow well anaerobically. The cc11 US_NmUC isolates causing urethritis clusters in the United States may have adapted to a urogenital environment by loss of capsule and gene conversion of the *Neisseria gonorrheae norB-aniA* cassette promoting anaerobic growth.**

Neisseria meningitidis | meningococcal urethritis | capsule | IS1301 | denitrification

As an obligate human pathogen that can cause large epidemic outbreaks, *Neisseria meningitidis* remains a leading cause of meningitis and rapidly fatal sepsis in otherwise healthy individuals (1). Although new protein-capsular polysaccharide conjugate and serogroup B outer membrane protein vaccines provide protection against invasive meningococcal disease (IMD), ∼500,000 cases of IMD have occurred worldwide annually, with at least 50,000 deaths and as many survivors suffering neurological sequelae (2). The continued worldwide problem of IMD and the capacity of Nm to evolve quickly are well established.

Nm is asymptomatically carried in the nasopharynx of 5–10% of adults in nonepidemic periods, and transmission usually occurs by direct contact with oral or nasal secretions. Nm is also infrequently recovered from other mucosal sites, specifically the urogenital tract (cervix, vagina, urethra) and the rectum. Certain populations can have significantly higher (>30%) nasopharyngeal carriage (3). In particular, populations of men who have sex with men (MSM) can have >40% pharyngeal Nm carriage rates, urethral Nm carriage of 0.7%, and rectal carriage of up to 2% (4, 5). In addition, *Neisseria gonorrheae* (Ng) and Nm have been corecovered in MSM and other populations (6).

Historically, Nm is not documented as a significant cause of urogenital infections. However, case reports of meningococcal urethritis, cervicitis, vaginitis, proctitis, pelvic inflammatory disease, and postpartum endometritis date back to the 1940s (7–9). Since 2001, there have been several outbreaks (e.g., Toronto, Chicago, New York City, Los Angeles, Berlin, and Paris) of IMD among MSM (10, 11). Orogenital and anogenital contacts are postulated to be the sexual transmission route of invasive meningococcal infections observed in MSM (6). Members of the ST-11 clonal complex (cc11), a hyper-invasive meningococcal lineage (12, 13), have been linked to IMD in MSM.

Since January 2015 in the sexual health clinic of Columbus, Ohio, a significant portion (3–36%/month) of initially presumed Ng-symptomatic urethral infections, primarily among heterosexual men, have been determined to be meningococcal urethritis (now >100 cases) caused by nongroupable cc11 Nm (14). Smaller urethritis clusters, caused by Nm with the same genotype, have also been observed in Oakland County, Michigan (14), and later in multiple other US sites. In this study, we define genotypic properties of an emerging clade, termed the US Nm urethritis clade (US_NmUC), through whole-genome sequencing (WGS) analyses

Significance

Neisseria meningitidis **(Nm) remains a leading cause of meningitis and rapidly fatal sepsis in otherwise healthy individuals. Historically, Nm is not recognized as a significant cause of urogenital infections. Since 2015, a significant increase of meningococcal urethritis primarily among heterosexual men has been reported in multiple US cities. We defined that a unique nonencapsulated Nm clade, which belonged to the cc11/ET-15 hyperinvasive lineage, was linked to these Nm urethritis clusters. The clade isolates causing urethritis clusters in the United States may have adapted to the urogenital environment with two unique molecular fingerprints: the insertion of IS1301 with associated deletion of capsule, enhancing mucosal adherence, and the acquisition of the gonococcal denitrification pathway by gene conversion, promoting anaerobic growth.**

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and biological characterization of key virulence factors that may contribute to Nm's success in becoming a urogenital pathogen. This meningococcal cc11 clade, with novel genetic and phenotypic changes, has become competent for efficient transmission via sexual contact and can effectively colonize the urogenital tract to cause an unprecedented large US meningococcal urethritis clusters.

Results

Genotype and Genome Analyses. Multilocus sequence typing (MLST) extracted from WGS data confirmed that all 56 US_NmUC isolates (52 from Columbus, 2 from Atlanta, and 2 from Indianapolis) belong to the ST-11 clonal complex (cc11). Meningococci of cc11 are further divided into two major sublineages, 11.1 and 11.2, with the ET-15 variant (15) found in lineage 11.2 (12). All 56 urethritis isolates were members of cc11 lineage 11.2/ET-15, contained IS1301, and were of fine-type PorA VR1 5–1, VR2 10–8, VR3 36–2, FetA F3-6, and PorB 2–2. Although the urethritis clusters caused by the US_NmUC isolates represent a clonal expansion event with no variation by conventional genotyping (MLST, fine-typing antigens), continued genomic alteration and evolution were evident within the genomes. For example, multiple genomic regions of the Columbus CNM26 isolate showed SNP differences in short nucleotide segments of a few hundred base pairs (mosaic nucleotide sequence variation) (16) from the corresponding regions of CNM10, which was isolated ∼1 1/2 mo earlier, indicating that homologous recombination is continuing to contribute to the evolution of this clade. Phylogenetic comparison of the US_NmUC isolates to lineage 11 isolates presented by Lucidarme et al. (12) and additional urogenital isolates identified in the PubMLST database using the Genome Comparator tool (17) clearly showed that the clade forms a distinct branch within the lineage 11.2 (Fig. S1).

The IS1301-Mediated Modification of the Capsular Polysaccharide Locus in the US_NmUC Isolates. Characterization of the capsular polysaccharide (*cps*) locus of the US_NmUC isolates by serogrouping PCR (18) detected a product for the serogroup C *csc* gene. Subsequent overlapping PCR assays revealed a shorter-than-expected product size between *csc* and *ctrA* and an otherwise intact meningococcal *cps* locus. Sequencing of the *csc* to *ctrA* PCR product revealed a complete deletion of the sialic acid biosynthesis genes, *cssA*/*B*/*C*, and a deletion of 620 bp of *csc* with the insertion of an IS1301 element in a 3′-5′ orientation (Fig. 1*A*). This explained the nongroupable phenotype of US_NmUC isolates (14) and their corresponding susceptibility to killing by normal human serum in serum bactericidal assays. WGS of all 56 isolates confirmed the absence of *cssA*/*B*/*C* genes and the presence of IS1301, suggesting that this modification can serve as a unique genetic marker for this US Nm urethritis clade.

The 3′ junction of IS1301 insertion within the intergenic region (IGR) has been previously described (but in the opposite orientation) in the hyper-encapsulated serogroup C cc11 invasive isolates from Spain that are highly resistant to conjugate vaccine-induced bactericidal antibodies and complement-mediated killing (19). The IS1301 insertion at this IGR site increases transcription of both *css* and *ctr* operons, resulting in increased capsule production and enhanced serum resistance of these hyper-encapsulated cc11 invasive isolates (19). In contrast, the IS1301 insertion in the *cps* locus of the US_NmUC isolates results in loss of the sialic acid capsule and intrinsic lipooligosaccharide (LOS) sialylation. The IS1301 insertion site within *csc* has not been reported previously, but the site does contain the IS1301 target consensus motif (5′-ATTAG-3′) (20).

Surprisingly, the single-contig CNM10 genome generated by PacBio showed that the IS1301 adjacent to *ctrA* was flanked by *pykA*, encoding a pyruvate kinase, whereas the partial *csc* gene was identified ∼20 kb downstream adjacent to another IS1301 element (Fig. 1*B*). This genome arrangement suggested an inversion of an ∼20-kb sequence between the two IS1301 elements.

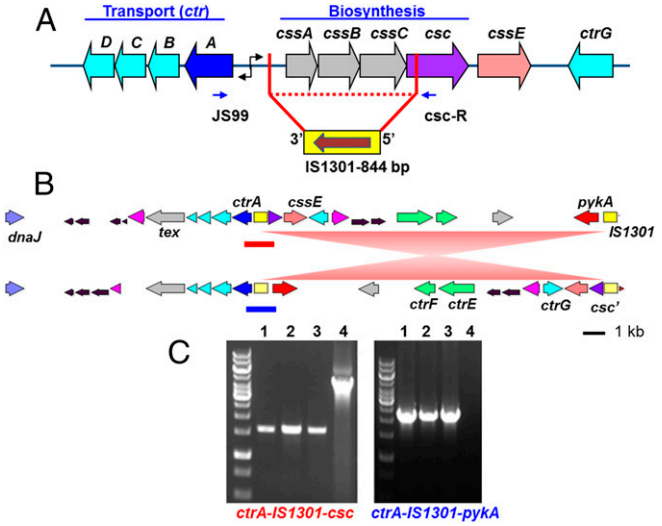

Fig. 1. The *cps* locus of the US_NmUC isolates. (*A*) The IS1301 insertion and deletion of the capsule biosynthesis genes in the US Nm urethritis clade isolates. Based on overlapping PCR products and sequencing of the *cps* locus in clade isolates, an 844-bp insertion element (IS1301) has inserted into the IGR with a simultaneous 3,625-bp deletion of *cssA*/*B*/*C* and a partial *csc* deletion. When amplified across the deleted region using primers located in *ctrA* and *csc* (blue arrows), shorter PCR products (∼1.6 kb) were obtained from clade isolates compared with that (4.4 kb) of the serogroup C WT FAM18 strain (examples shown in *C*). The 1.6-kb PCR product was sequenced to determine the insertion/deletion junctions. (*B*) Genomic segments of ∼38 kb surrounding the *cps* locus of the US_NmUC isolates. A genome configuration between two IS1301 elements corresponding to the *ctrA-IS1301-csc* linkage in the US_NmUC isolates as determined by PCR and sequencing is shown on top, and the genome configuration of the single-contig PacBio genome of CNM10 is shown below. The ∼20-kb genome inversion between two IS1301 elements is depicted by red triangles. Genes are color-coded: *ctrB*/*C*/*D* and *ctrG* in cyan, *ctrA* in dark blue, *ctrE*/*ctrG* in green, truncated *csc* in purple, *tex* in gray, *dnaJ* in light blue, and *pykA* in red. The IS1301 elements are shown as yellow rectangles. (*C*) Gel pictures of the *ctrA* to *csc* (*Left*) and the *ctrA* to *pykA* (*Right*) PCR amplifications. The presence of both *ctrA-IS1301-csc* (red line in *B*) and *ctrA-IS1301-pykA* (blue line in *B*) orientations was determined by PCR amplification across IS1301 and sequencing of the resulting products. Lanes: 1, CNM3; 2, CNM10; 3, CNM26; 4, FAM18. Cell suspensions from single colonies were used as templates. The expected product sizes are 1,614 bp and 2,316 bp for *ctrA-IS1301-csc* and *ctrA-IS1301-pykA*, respectively.

To determine if both the *ctrA-IS1301-csc* and the *ctrA-IS1301-pykA* configurations were present, we designed primers to amplify across IS1301 in both scenarios, using cell suspensions from a single colony as the template in colony PCR assays. Both PCR reactions were positive (Fig. 1*C*) and sequencing of the PCR products confirmed the expected sequences. The presence of two configurations between two IS1301 elements in a single colony population implied that a dynamic inversion process is occurring at the *cps* locus and that the PacBio genome assembly likely captured the dominant genome arrangement.

The Vaccine-Targeted Surface Protein Antigens. Factor H-binding protein (FHbp), a key virulence protein, is the main target of two new multicomponent protein-based meningococcal serogroup B-directed vaccines, 4CMenB and MenB-FHbp (21, 22). The 4CMenB protein vaccine antigens include FHbp, PorA, NHBA, and NadA. The *fHbp* gene of all US_NmUC isolates was allele 1127 and the FHbp peptide was allele 896, placing it in the variant 1 and subfamily B in the original Novartis (now GSK) and Pfizer FHbp classifications, respectively. The FHbp peptide allele, only found in isolates of the US_NmUC, is a hybrid of the subvariants 1.1 and 1.10, but with a unique residue 247 at the C terminus of the mature protein (23). The *fHbp* promoter

sequence of the US_NmUC isolates belonged to the high FHbp-expressing promoter clade I defined by Biagini et al. (24). Preliminary data of flow analyses indicated high FHbp protein expression in US_NmUC isolates. This is in contrast to the urethritis cc11 Nm isolates described by Taha et al., all of which encoded a frame-shift mutation in *fHbp* (25).

The other three 4CMenB protein vaccine antigens, if not inactivated by IS1301 (see below), were conserved among all of the US_NmUC isolates including NadA peptide 2 (variant 2/3), NHBA peptide 20, and PorA P1.5–1, 10–8. An earlier study of 70 ET-15 strains found that 48 isolates (~70%) contain the *nadA::IS1301* insertion (26). Interestingly, the *nadA* gene was intact in 49 of the urethritis Nm isolates from Columbus, but was inactivated by IS1301 in the CNM45, CNM49, and CNM54 isolates (August–September 2015), the Indianapolis NM1 isolate (May 2015), and the Atlanta ATL2 isolate (August 2016), implying a recent movement of IS1301. The IS1301 appears to be shaping this clade.

The US Urethritis Clade Isolates Have Acquired the Gonococcal *aniA* and *norB* Genes by Gene Conversion and Grow Well Anaerobically. *N. gonorrheae* can grow anaerobically in the urogenital tract because it encodes an effective denitrification pathway composed of a nitrite reductase (AniA) and a nitric oxide reductase (NorB) (27) (Fig. 2*A*). In contrast, meningococci less commonly experience anaerobic conditions, and a functional denitrification pathway is not essential for their survival in the nasopharynx (28, 29). Many meningococcal isolates have frame-shift mutations in *aniA* (29) and cannot grow anaerobically using nitrite as the electron acceptor. Even in meningococcal isolates that retain intact *aniA*/*norB*, these alleles appear to be less efficient in supporting anaerobic growth (28).

Both *norB* and *aniA* were in-frame in all 56 US_NmUC isolates. In comparison, only 4 of 33 (12%) urogenital Nm isolates retrieved from the PubMLST database (Table S1) were predicted to encode functional AniA proteins, and the others had a frame-shift mutation. Unexpectedly, the divergently transcribed *norB-aniA* sequence of the US_NmUC isolates aligned almost perfectly with the homologous genes of *N. gonorrheae*, suggesting that the US_NmUC isolates had acquired the gonococcal version of the *norB-aniA* gene cassette, which was also distinct from those of *Neisseria lactamica* (Dataset S1). The phylogenetic tree based on the 3.8-kb *norB-aniA* gene sequence shows that the US_NmUC isolates clustered together with Ng (Fig. 2*B*), whereas they grouped with Nm at the core genome alignment (Fig. S1). The SNP density plot (Fig. 2*C*) demonstrated that the SNP signatures of the US_NmUC and Ng are near identical at this locus. Inspection of the *norB-aniA* locus in additional cc11 Nm urogenital isolates and MSM invasive isolates in the PubMLST database confirmed that all were of meningococcal sequence and that none had such a gene conversion event at this locus, whereas WGS data of all 56 US_NmUC isolates from three cities confirmed the identical gonococcal *norB-aniA* gene conversion event.

To identify the recombination junction, the sequence alignment was extended upstream and downstream of *norB-aniA* (Dataset S1). In meningococci, a 534-bp ORF encoding a glutathione peroxidase (*gpxA*) is 178 bp upstream of the *norB* stop codon (Fig. 2*A* and Dataset S1). The *gpxA* gene and the intergenic sequence between *gpxA* and *norB* were absent in *N. gonorrheae* (a 672-bp deletion), but were present in the US_NmUC isolates. Thus, the recombination event likely occurred very close to the *norB* stop codon. On the other side, a 53-bp gonococcal sequence beginning 103 bp downstream of the *aniA* stop codon was absent in all meningococci including the US_NmUC isolates, indicating that recombination happened upstream of this 53-bp sequence (Fig. 2*A* and Dataset S1). Thus, the *norB-aniA* gene conversion appeared to be a precise transformation/recombination event.

There were 107 SNPs scattered throughout the *norB* coding sequence (2253 bp), and only six SNPs were nonsynonymous between Nm and Ng (and US_NmUC) (Fig. S2). The *aniA* coding sequences of Ng and Nm are 1,179 and 1,146 bp, respectively, and differed by 62 polymorphic sites. The AniA proteins of Ng and US_NmUC were identical. Compared with the meningococcal protein, both have a four-residue extension, one residue change in the N-terminal repeat region, two residue

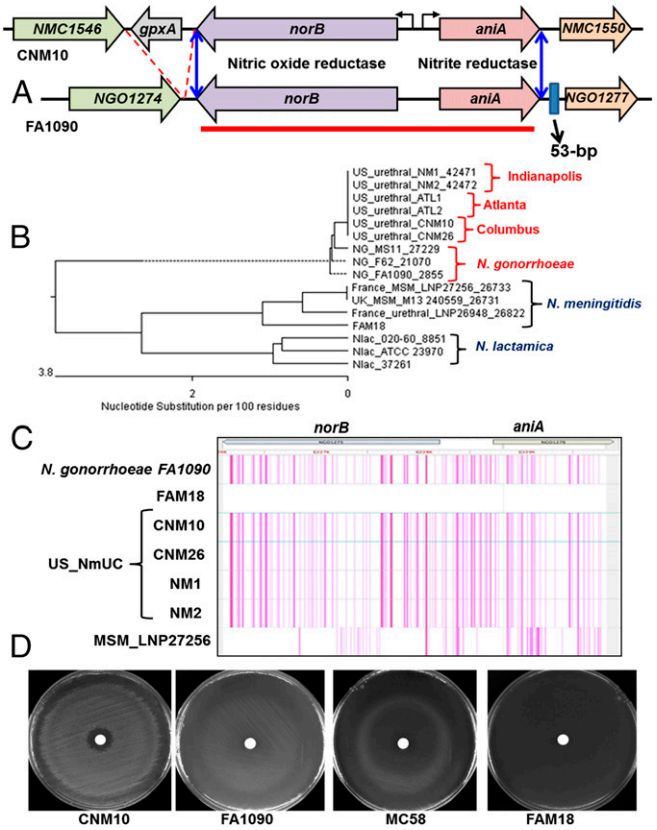

Fig. 2. Acquisition of the gonococcal *norB-aniA* gene cassette in the US Nm urethritis clade. (*A*) Schematics of the *norB-aniA* locus in the US_NmUC isolates and *N. gonorrheae*. Homologous genes are color-coded. A *gpxA* gene encoding a glutathione peroxidase adjacent to *norB* is present in the genomes of the US_NmUC clade isolates and other meningococci, but is absent in *N. gonorrheae*. On the 3′ side, a 53-bp sequence is present downstream of *aniA* in gonococci but absent in the US clade and other meningococci. The presence and the absence of these two genetic features (*gpxA* and the 53-bp element) in the US_NmUC genome indicated the recombination junctions (blue double-arrow lines). (*B*) The sequence between stop codons of *norB* and *aniA* (3808 bp) marked by the red line in *A* was used for the phylogenetic sequence analyses. Two isolates each from Columbus, Indianapolis, and Atlanta, together with three *N. lactamica* strains, two meningococcal urethral isolates, three meningococcal MSM isolates, and the FAM18 reference strain were included in the alignment by Clustal W. The US urethritis clade *norB-aniA* locus clustered with the gonococcal sequences. (*C*) A SNP density plot of the core genome alignment by *Parsnp* at the *norB-aniA* locus with FAM18 set as the reference genome. Each SNP that differed from FAM18 is shown as a single line, and multiple neighboring SNPs appear as thick lines. The sequence identity of four US_NmUC isolates to the gonococcal FA1090 sequence is compared with that of a meningococcal MSM isolate LNP27256 at this locus. The light-gray region indicates that sequence is absent in one or some of the aligned genomes (polymorphisms within the glycosylation motif of *aniA*). (*D*) Anaerobic growth of the US urethritis clade isolates on GC agar plates supplemented with sodium nitrite. The US clade isolate (CNM10) was compared with meningococcal strains with either an intact AniA (MC58) or a frame-shift inactivated AniA (FAM18) and the gonococcal reference strain FA1090. After incubating under anaerobic conditions for 24 h at 37 °C and another 24 h at room temperature, growth on the plates was recorded. Data shown are representative anaerobic growth experiments repeated five times. Additional CNM isolates showed anaerobic growth similar to that of CNM10.

changes in the reductase domain, four amino acid changes, and seven extra residues within the C-terminal glycosylation region (29) (Fig. S3). Finally, *norB* and *aniA* are divergently transcribed and are separated by an IGR of 370 bp in Nm, 373 bp in Ng, and 372 bp in the US_NmUC. The IGR of the US_NmUC was identical to Ng with only one difference: a C_6 vs. a C_5 tract (absent in Nm) within the untranslated region of *aniA* in Ng and the clade, respectively (Fig. S4).

We examined whether the US meningococcal urethritis clade isolates with the gonococcal denitrification pathway differed in nitrite-dependent anaerobic growth compared with the reference strain MC58 with a meningococcal pathway. FAM18 was a negative control (frame-shift in *aniA*) whereas the gonococcal strain FA1090 served as a positive control. No growth was detected for FAM18, consistent with the presence of the frame-shift mutation (Fig. 2*D*). Results of anaerobic nitrite-dependent growth assays correlated with the differences between meningococci and gonococci in the efficiency of the AniA/NorB systems (28). The US_NmUC isolates yielded substantial growth around the nitrite disk similar to that observed for Ng FA1090 (a representative CNM10 shown in Fig. 2*D*), whereas the meningococcal MC58 strain with a less efficient anaerobic system demonstrated scanty growth (Fig. 2*D*).

Discussion

A limited number of virulent clonal complexes cause most of the invasive meningococcal diseases. Encapsulated cc11 strains are hyper-invasive and have caused multiple outbreaks and epidemics, such as epidemics in North America, Europe, and Australia in the 1990s/2000s, Hajj-associated global outbreaks, epidemics in the African meningitis belt, and more recently, endemic disease in South Africa, Europe, and Brazil (12). The serogroup C encapsulated cc11/ET-15 sublineage has been associated with a higher case-fatality rate and a high proportion of sequelae (30). Members of cc11 have been linked to IMD in MSM (10, 11) and now to Nm urethritis in heterosexual men. Our data indicate that a new nonencapsulated meningococcal clade of cc11 lineage 11.2/ET-15 (IS1301 containing), fine-type P1.5–1, 10–8, F3-6, 2–2, which is monophylogenic within cc11 (Fig. S1), is causing unprecedented clusters of urethritis in the United States (14). In addition to the large cluster observed in Columbus, Ohio, other sexually transmitted infection clinics in Oakland County, Michigan, Indianapolis, Atlanta, and other cities have also reported increased cases of cc11 Nm causing urethritis. Since 2015, this clade was the exclusive cause of a significant portion (~20%) of symptomatic urethral infections (with urethral Gram-negative intracellular diplococci and growth of oxidase-positive Gram-negative diplococci) in Columbus (14). In Indianapolis, rates of discordant nongonococcal urethritis were 2.8, 1.4, and 6.9% in 2013, 2014, and 2015, respectively, indicating a significant increase in meningococcal urethritis (31). In addition to the isolates characterized in this study, other isolates from Indianapolis, Atlanta, and other cities also appear to have the same detailed molecular profile and characteristics as the US_NmUC isolates. These data represented a clonal expansion within the cc11 lineage and that the US_NmUC has acquired a capacity to better adapt to the urogenital (e.g., urethra) tract and perhaps to other mucosal environments. The specific signatures of the US_NmUC include (*i*) an IS1301-mediated specific deletion of the sialic acid biosynthesis genes with associated genome inversion near the *cps* locus, (*ii*) expression of a unique fHBP protein, and (*iii*) the acquisition of gonococcal NorB-AniA denitrification apparatus. These unique features differ from the cc11 Nm urethritis isolates described by Taha et al., which express a serogroup C capsule, encode a frame-shifted *fHbp* allele, and have a meningococcal denitrification pathway (25).

The US_NmUC isolates displayed active genomic inversion events mediated by IS1301 elements. Budroni et al. has previously recognized an inversion of ~15 kb localized within the *cps* locus to occur within related lineages, i.e., between cc11 and cc8 and between cc41/44 and cc269, likely mediated by the duplicated regions flanking the *cps* locus (32). We have also detected such an inversion because both cc11 and cc8 orientations of the *cps* locus were identified in the cc11 US_NmUC isolates. Additionally, we discovered a unique and likely independent inversion mediated by IS1301 elements. The inversion may be drifting toward the major configuration detected by PacBio WGS that links the functional capsule transport operon to the intact pyruvate kinase *pykA* instead of the remnant of an inactivated biosynthesis gene. Whether this arrangement achieved any biological benefits awaits further studies. The loss of both capsule and LOS sialylation due to deletion of the sialic acid biosynthesis genes should enhance close adherence at mucosal surfaces (33).

The US_NmUC isolates contain a novel *fHbp* gene coding for a subvariant 1 peptide, not found in other meningococcal isolates. The ability of a strain to bind factor H (fH) is influenced by the expression level of FHbp protein and amino acid differences in the subvariant sequences (34). The fH is present at mucosal surfaces with concentrations expected to be ~10% of serum levels (35). Hence, it is proposed that the different levels of fH binding by subvariants may be more relevant in different niches with low fH levels during colonization or disease, rather than during disease in the blood where fH is sufficiently abundant to saturate FHBP binding (34). An additional role of FHbp is to provide resistance to killing by the antimicrobial peptide LL-37 as the *ΔfHbp* mutants (both subvariants 1.1 and 1.10) have been shown to be more sensitive to LL37 (36). Nm and Ng have adopted different strategies to bind fH to their surface to prevent complement activation. The *fHbp* homolog encoded by Ng is not surface-expressed and does not bind fH; gonococci instead use abundant porin proteins to recruit fH (37). Without capsule, Ng and the nonencapsulated Nm urethritis clade isolates depend on alternative factors to confer complement resistance. The highly expressed, unique fHBP variant in the US_NmUC isolates may afford immuno-protective effects in the urogenital tract that has lower levels of fH. Compared with a related MSM invasive isolate expressing functional fHBP, the Nm urethritis isolates characterized by Taha et al., which has a frame-shifted inactivated *fHbp*, has reduced survival in a transgenic mouse model expressing human factor H (25). Based on the predicted protein sequence, both MenB-FHbp and 4CMenB vaccines might provide protection against this clade, but the effectiveness of MenB vaccines at a mucosal surface has not been demonstrated. The NadA adhesion, another major component of the 4CMenB vaccine that was absent in Ng (21), was inactivated by IS1301 in a few of the later isolates. Thus, IS1301 insertion and possible movement and transformation/homologous recombination continue to shape the pathogenic potential of Nm and provide genetic diversities that facilitate new niche adaptation.

Gene conversion events have been previously documented between meningococcal genomes and are more likely to happen within related lineages defined by MLST (e.g., cc11 and cc8) and to involve longer DNA transformations (3.89 kb vs. 0.68 kb) (32). These differences coincide with the distributions of restriction modification systems (32). In addition, replacement of the capsule biosynthesis genes, in particular the capsule polymerase, by gene conversion results in "capsule switching" and immune escape (38). Ecological separation of Ng and Nm within the human host is proposed as an explanation for the lower frequency of interspecies recombination noted between these species (39). However, a meningococcal vaginal isolate with a gonococcal PIB class porin gene (40) and urethral isolates carrying a 16s rRNA copy from Ng (41) have been reported. The *norB-aniA* exchange of the US_NmUC reported here is a large (3.8 kb), complete, and precise gene conversion event from Ng that may provide an advantageous metabolic function to survive the anaerobic urogenital environment.

AniA catalyzes the conversion of nitrite to nitric oxide (NO), which is subsequently reduced to nitrous oxide by NorB (27). In addition to supporting microaerophilic and anaerobic growth, the denitrification pathway plays a significant role in protection against NO-mediated toxicity. Gonococcal AniA is a glycosylated surface-exposed outer membrane lipoprotein (29), is the major protein induced anaerobically, and has been shown to confer enhanced serum resistance in gonococci (42). Patients with gonococcal infection produced antibodies to AniA, indicating that it is expressed during disease (43). Thus, the denitrification pathway plays a crucial role in gonococcal biology, and gonococcal isolates universally have highly functional AniA and NorB proteins. In contrast, meningococci less commonly experience an anaerobic or oxygen-limited environment at the nasopharynx, and a functional denitrification pathway is not essential for meningococcal survival (28, 29). Many meningococcal isolates have various mutations in *aniA* or completely lack the *aniA* gene (27–29), which seemingly points to a selection against expression of a functional AniA in meningococci. A recent report indicated that the invasive meningococcal MSM isolates and related urethritis isolates, but not other invasive meningococci, maintained a functional meningococcal AniA (25). This trait was proposed to reflect an adaptation and selection, which allows meningococci to colonize at this uncommon etiological site (25). However, even an intact meningococcal AniA/NorB system is less efficient than the gonococcal system. For example, the relative in vitro specific activity of meningococcal AniA is lower than that of the corresponding enzyme from Ng (28). Thus, the acquisition of a gonococcal *norB-aniA* cassette through homologous recombination in the US_NmUC isolates could be a major contributor to the success of this clade in adapting to the urethra, and, like gonococci, causing urethritis clusters. The clade indeed displayed a robust anaerobic growth phenotype over meningococci with an active Nm system. The finding of a gonococcal denitrification system also suggests that US_NmUC evolved in the presence of gonococcal DNA for recombination when in this new niche.

The expression of both meningococcal and gonococcal AniA proteins is under the control of a complex regulatory network involving multiple transcriptional regulators (27), but with subtle differences (44). Expression of *aniA* is induced by fumarate and nitrate reduction (FNR) regulator in response to oxygen limitation, induced by nitrite via the NarQ/NarP two-component system, activated by the iron uptake regulator Fur, and repressed by the NO-sensitive repressor NsrR (45). Meningococcal IGR was quite distinct with ∼30 polymorphic sites (Fig. S4) compared with that of Ng. The critical SNP differences noted in a previous promoter comparison study that distinguished *aniA cis*-regulation of Ng (44) from that of Nm were present in the US_NmUC. Further studies of regulatory network mediated by global regulators are needed to determine if the effects of gonococcal promoter regulation in the US_NmUC genetic background are similar to those observed in Ng (44) and to better delineate the advantage afforded by the gonococcal apparatus in meningococci.

The unprecedented large US meningococcal urethritis clusters suggest that a meningococcal cc11 lineage 11.2 clade, with novel genetic and phenotypic changes, appears to have become competent for efficient transmission via sexual contact and can effectively colonize the urogenital tract. An IS1301 insertion/deletion resulted in the loss of both encapsulation and intrinsic LOS sialylation, which is recognized to facilitate adherence at mucosal surfaces and provide a molecular fingerprint for this clade. A key finding of this study was the discovery of the gonococcal AniA/NorB denitrification system in the US meningococcal urethritis clade that enabled robust anaerobic growth. Considering the uniformity of housekeeping genes, *N. gonorrheae* may have evolved from a subset of meningococci that could colonize the genital tract (39). The further speciation of the gonococcus and meningococcus is suggested to be a consequence of the adaptation to different environments (46). The deletion of capsule genes, the conversion to the gonococcal denitrification pathway, and potentially other phenotypic changes suggest that the emergence of this new cc11 lineage 11.2 meningococcal clade through multiple independent evolutionary events has occurred and been selected for to better assimilate into the same niche first adopted by gonococci. In summary, a *N. meningitidis* cc11 clade causing the US Nm urethritis clusters appears to be newly emergent as a successful urogenital pathogen. Further epidemiologic and laboratory investigations are needed to determine the full extent of spread of this clade within the US population.

Materials and Methods

Bacterial Isolates and Growth Conditions. The collection and demographic characterization of 52 Nm urethritis isolates from nonepidemiologically related cases between January and September 2015 at the sexual health clinic at Columbus Public Health, Columbus, Ohio, has been described earlier (14). The isolate collection was conducted as part of the Gonococcal Isolate Surveillance Project (GISP) that has been determined by the CDC as nonhuman subject research and exempt from Institutional Review Board (IRB) approval. The outbreak investigation, including molecular characterization of the isolates as described in this report, was approved by the Ohio State University IRB (protocol 2015H0388). The isolates were named CNM for Columbus *N. meningitidis*. In addition, two US_NmUC isolates each from Indianapolis (NM1 and NM2) and from Atlanta (ATL1 and ATL2) were included in this study. Gonococcal reference strain FA1090 was kindly provided by W. Shafer, Emory University, Atlanta. The strain M7 is a nonencapsulated meningococcal control derived from the NMB strain (B:2B:P1.2,5:L2, CDC8201085) (47). Meningococcal and gonococcal strains were grown with 5% CO_2 at 37 °C on gonococcal base agar (GC, BD) plates or GC broth as previously described (48).

WGS. WGS of all 52 CNM isolates was performed by Illumina at the Centers for Disease Control and Prevention (CDC), and relevant genotypes were extracted. Two Columbus isolates, CNM10 (April 2015) and CNM26 (May 2015), were sequenced by Pacific Biosciences (PacBio) technology, yielding ∼200× coverage, and assembled with Hierarchical Genome Assembly Process v3 (49), which resulted in a single contig and two contigs, respectively. Nm urethritis isolates from Indianapolis, NM1 (May 2015) and NM2 (February 2016), and isolates from Atlanta, ATL1 and ATL2 (August 2016), were sequenced by MiSeq, yielding ∼600× coverage of paired-end 250-bp reads, assembled using SPAdes (50) and annotated by RAST (51). Draft genome assemblies of urogenital sourced Nm and closed reference genomes of *N. meningitidis*, *N. gonorrheae*, and *N. lactamica* were downloaded from PubMLST (https://pubmlst.org/neisseria/) (52). Genome assemblies were compared using Harvest (v1.1.2); a reference-independent core genome alignment and maximum-likelihood phylogeny were constructed using the Parsnp aligner and then visualized using Gingr (53). The MegAlign program in the DNASTAR Lasergene 13 suite was used for multialignments of short DNA and protein sequences by Clustal W. Allele assignments of MLST, fine-typing antigens, and Bexsero Antigen Sequence Typing (54) were retrieved from the PubMLST database.

Genome assemblies were uploaded to PubMLST, and the Genome Comparator tool was used to calculate allele distances according to the cgMLST scheme (v1.0) (17). A neighbor-joining dendrogram was constructed using SplitsTree v4 (55) and displayed on the interactive Tree of Life website (56). The genomes of the US_NmUC isolates were also compared with ST-11 urethritis and invasive isolates in the PubMLST database (Table S1).

Nitrite-Dependent Anaerobic Growth and Serum Bactericidal Assays. The nitrite-dependent growth assays were performed as previously described with minor modifications (57). GC broth cultures at midlog phase were adjusted to a density of 0.3 at OD_{550}, and 100-μL aliquots of suspension were then spread on GC agar plates. A 6-mm AA disk was placed on the agar, and 10 μL of 20% $NaNO_2$ was spotted onto the disk to provide nitrite. The plates were incubated at 37 °C for 24 h in an anaerobic jar (Oxoid) with the GasPak EZ anaerobe container system with anaerobic indicator (BD) and allowed to grow at room temperature for an additional 24 h before imaging their growth. Serum bactericidal assays were conducted in 96-well microtiter plates with 5% commercial pooled normal human serum (Fisher) as previously described (58) except that the RPMI medium was used for the assays.

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