Insights on Population Structure and Within-Host Genetic Changes among Meningococcal Carriage Isolates from U.S. Universities

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ABSTRACT In 2015 and 2016, meningococcal carriage evaluations were conducted at two universities in the United States following mass vaccination campaigns in response to *Neisseria meningitidis* serogroup B (NmB) disease outbreaks. A simultaneous carriage evaluation was also conducted at a university near one of the outbreaks, where no NmB cases were reported and no mass vaccination occurred. A total of ten cross-sectional carriage evaluation rounds were conducted, resulting in 1,514 meningococcal carriage isolates collected from 7,001 unique participants; 1,587 individuals were swabbed at multiple time points (repeat participants). All isolates underwent whole-genome sequencing. The most frequently observed clonal complexes (CC) were CC198 (27.3%), followed by CC1157 (17.4%), CC41/44 (9.8%), CC35 (7.4%), and CC32 (5.6%). Phylogenetic analysis identified carriage isolates that were highly similar to the NmB outbreak strains; comparative genomics between these outbreak and carriage isolates revealed genetic changes in virulence genes. Among repeat participants, 348 individuals carried meningococcal bacteria during at least one carriage evaluation round; 50.3% retained *N. meningitidis* carriage of a strain with the same sequence type (ST) and CC across rounds, 44.3% only carried *N. meningitidis* in one round, and 5.4% acquired a new *N. meningitidis* strain between rounds. Recombination, point mutations, deletions, and simple sequence repeats were the most frequent genetic mechanisms found in isolates collected from hosts carrying a strain of the same ST and CC across rounds. Our findings provide insight on the dynamics of meningococcal carriage among a population that is at higher risk for invasive meningococcal disease than the general population.

IMPORTANCE U.S. university students are at a higher risk of invasive meningococcal disease than the general population. The responsible pathogen, *Neisseria meningitidis*, can be carried asymptomatically in the oropharynx; the dynamics of meningococcal carriage and the genetic features that distinguish carriage versus disease states are not completely understood. Through our analyses, we aimed to provide data to address these topics. We whole-genome sequenced 1,514 meningococcal carriage isolates from individuals at three U.S. universities, two of which underwent mass vaccination campaigns following recent meningococcal outbreaks. We describe the within-host genetic changes among individuals carrying a strain with the same molecular type over time, the primary strains being carried in this population, and the genetic differences between closely related outbreak and carriage strains. Our results provide detailed information on the dynamics of meningococcal carriage and

the genetic differences in carriage and outbreak strains, which can inform future efforts to reduce the incidence of invasive meningococcal disease.

KEYWORDS *Neisseria meningitidis*, United States, WGS, carriage, comparative genomics, meningococcal, university, whole-genome sequencing

Invasive meningococcal disease (IMD) is a severe bacterial infection. Although overall incidence of IMD has declined in the United States to historically low levels (1), college students are at increased risk for serogroup B meningococcal disease compared to that of other adolescents (2). Between 2013 and 2018, 11 university-associated outbreaks of IMD were reported (3) (CDC, unpublished data). The responsible organism, *Neisseria meningitidis*, can colonize the human oropharynx asymptomatically. Approximately 8% to 25% of the healthy human population are asymptomatic carriers of *N. meningitidis* (4), with higher prevalence of carriage in teenagers and young adults (5). The highest carriage prevalence has been reported in populations in semiclosed settings or with extensive close contact, such as universities (6–8) and military barracks (9, 10). Studies have shown that meningococcal carriage is highly dynamic and may be chronic, intermittent, or transient (11–15).

The meningococcal polysaccharide capsule is a major virulence factor that protects the bacteria against the host's immune system. Six encapsulated serogroups (A, B, C, W, X, and Y) are responsible for the majority of IMD worldwide, while nonencapsulated meningococci are mostly associated with carriage and rarely cause IMD (11). Most of the IMD cases in the United States are caused by strains belonging to a few hyperinvasive lineages, namely, clonal complex (CC) 32, CC41/44, CC11, and CC23 (16–18). These hyperinvasive lineages are rarely found among healthy carriers, and carriage isolates are primarily associated with sequence types (STs) or CCs that seldom cause disease (12, 19, 20).

In response to two separate *N. meningitidis* serogroup B (NmB) outbreaks at two U.S. universities in Oregon (OR) and Rhode Island (RI-1) between January and May 2015, mass vaccination campaigns with serogroup B meningococcal vaccines (predominantly MenB-FHbp [Trumenba] at both universities and some MenB-4C [Bexsero] at OR) were launched (7, 8). Meningococcal carriage evaluations were conducted at both universities during 2015 and 2016 to evaluate meningococcal carriage before and after completion of the vaccination campaigns (6, 21); in both evaluations, carriage rates remained stable, suggesting the vaccination did not rapidly reduce meningococcal carriage or prevent acquisition. Additional work is under way to analyze the possible impact of mass vaccination on the carriage and acquisition of strains potentially covered by the vaccine, and this work will be published at a later date. A third carriage evaluation was conducted at another university in Rhode Island (RI-2), where no IMD outbreak or cases were reported (22) and no mass vaccination occurred. We sequenced isolates from these three carriage evaluations in order to describe within-host genetic changes in individuals carrying *N. meningitidis* at multiple time points, elucidate the meningococcal carriage population structure, and compare closely related carriage and outbreak isolates.

RESULTS

Population structure of *N. meningitidis* carriage isolates. A total of 1,514 meningococcal carriage isolates from the three universities were sequenced and analyzed. Sequencing information, multilocus sequence typing (MLST), and serogroup information about these isolates are provided in Table S1 in the supplemental material. The vast majority of these isolates were nongroupable (92.1% [1,395/1,514]), followed by serogroup B (4.8% [74/1,514]) and serogroup E (3.0% [45/1,514]). Fewer than five isolates belonging to serogroups Y, X, W, C, and Z were identified. Phylogenetic analysis revealed that these isolates clustered into CC-specific phylogenetic clades with 12 major clades (≥20 isolates per clade) and 28 minor clades (<20 isolates per clade) across the three universities (Fig. 1). The most frequent CCs were CC198 (414/1,514
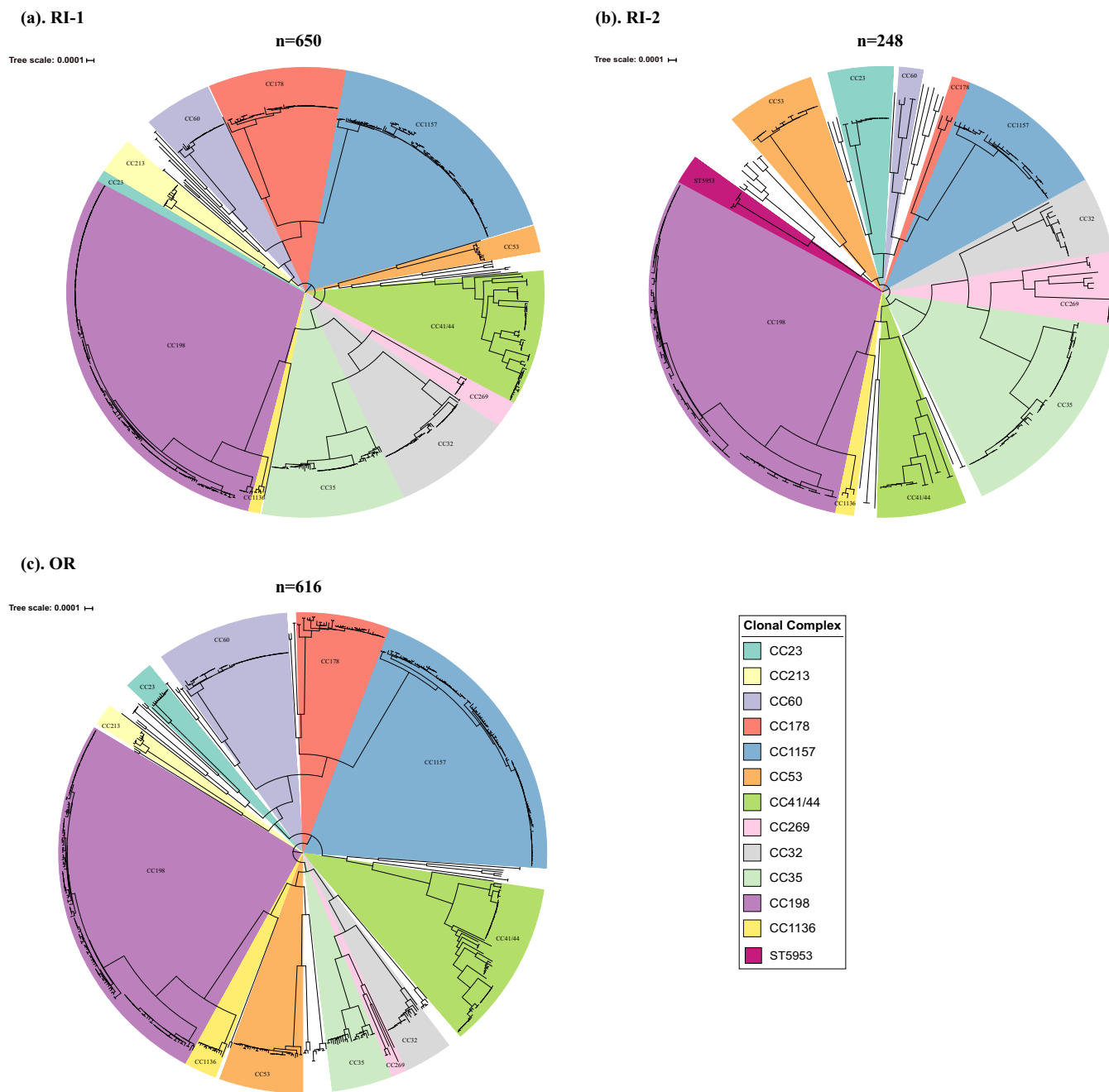
(a). RI-1

n=650

Tree scale: 0.0001 ⊢⊣

(b). RI-2

n=248

Tree scale: 0.0001 ⊢⊣

(c). OR

n=616

Tree scale: 0.0001 ⊢⊣

Clonal Complex
CC23
CC213
CC60
CC178
CC1157
CC53
CC41/44
CC269
CC32
CC35
CC198
CC1136
ST5953

FIG 1 Whole-genome phylogenetic relationship of carriage isolates from 3 U.S. universities. (a) The phylogenetic relationship among carriage isolates identified at RI-1. (b) The phylogenetic relationship among carriage isolates identified at RI-2. (c) The phylogenetic relationship among carriage isolates identified at OR. CC/ST lineages are highlighted and named according to the corresponding CC or ST.

[27.3%]), followed by CC1157 (264/1,514 [17.4%]), CC41/44 (148/1,514 [9.8%]), CC35 (112/1,514 [7.4%]), and CC32 (85/1,514 [5.6%]). A total of 20 STs were found in all three universities, while the majority ($n = 69/89$ [77.5%]) of STs were only detected in a single university (Table 1).

Stability of population structure across rounds. The stability of the most frequently observed CCs at each university was assessed across rounds (see Table S2). Overall, the observed counts of these CCs at each university were not significantly different across rounds, indicating that they remained stable throughout the course of our study within each respective university. Specific CCs, such as CC198, CC1157, and CC41/44 were among the most frequently observed CCs across each university.

TABLE 1 Sequence types identified within the 12 major CCs across all the 3 universities[a]

Clonal complex	ST			
	OR, RI-1, RI-2	OR	RI-1	RI-2
CC41/44	136, 2578	44, 414, 1489, 5881, 10982, 11737, 11857, 12632, 12918, 12920	43, 437, 2578, 4682, 4971, 7574, 11525, 11541, 11737, 13079	8052
CC32	32, 1130, 11395		2506	
CC35	35, 278	12908	11735, 11740	1679, 3085, 11530
CC178	178	11550, 12933, 13038	11733	12927
CC1157	1157, 1649, 12475		11580	
CC23	23	2533, 3582		5733, 6800
CC60	60, 11739	12912		2476, 13042
CC213		3496, 11852	3123, 11528	9413
CC269	467	3091, 11294, 12910, 12919	269	4221, 5329, 12753
CC53	53	2647, 12909		
CC198	198, 823, 2384	2236, 10148, 12916, 12710	12716, 12755	10042, 12754, 12756, 12937, 12932
CC1136	1136	11459, 12917		

[a]STs identified uniquely in each of the three universities and those identified at all three universities are shown.

Genomic comparison between the meningococcal outbreak strain and carriage isolates. Whole-genome sequences of 7 CC32/ST-32 IMD isolates from the 2015 meningococcal outbreak at OR that precipitated the carriage study (21) were compared with those of 86 CC32 carriage isolates from the three universities (Fig. 2A). Phylogenetic analysis demonstrated that these 93 CC32 carriage and outbreak isolates clustered into five main clades, each associated with a specific ST. Clades 1 to 4 contained only carriage isolates, while clade 5 (ST-32) contained a subclade that included the seven outbreak isolates and one carriage isolate collected from OR during round three (Fig. 2A).

ST-9069 caused the outbreak at RI-1 in 2015, and whole-genome sequences of the two outbreak isolates were used for comparison against those from carriage isolates. Three ST-9069 carriage isolates were identified, with two obtained from a single participant at RI-1 during rounds two and three and one from a participant at OR in round four. The two RI-1 ST-9069 carriage isolates were more closely related to the two RI-1 outbreak isolates than to the OR carriage isolate (Fig. 2B).
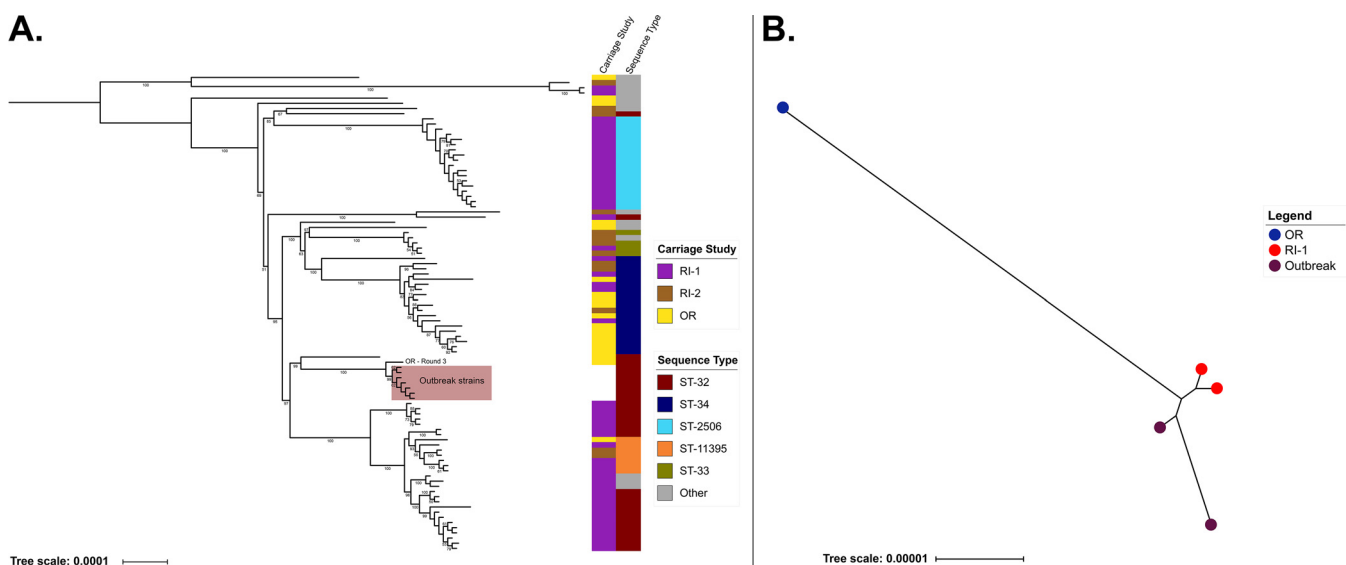

FIG 2 Whole-genome phylogenetic relationships for CC32 and ST-9069 carriage and outbreak isolates. (A) The phylogenetic relationship among CC32 carriage isolates and outbreak strains collected from OR. Major carriage clades and STs are indicated in the figure. The OR carriage isolate from round 3 that was most closely related to the outbreak isolates is noted. Bootstrap values of >50 are included in the phylogeny. The "other" ST category consists of those STs with less than or equal to 3 isolates included in the phylogeny and consisted of ST-1130, ST-11527, ST-12936, ST-12914, ST-11397, ST-11278, ST-8758, ST-12924, ST-10875, and ST-12750. (B) The phylogenetic relationship among ST-9069 carriage isolates and outbreak isolates from RI-1.

Comparative genomic analysis revealed genetic changes in 30 genes between the round three ST-32 OR carriage isolate and the most closely related outbreak isolate and in 72 genes between the ST-9069 RI-1 carriage isolates and the outbreak strain. Genetic changes were found in known *N. meningitidis* virulence genes such as those involved in adherence to the human epithelium and tissue tropism (type IV pili genes *pilC*, *pilF*, and *pilU*), host invasion (opacity protein genes such as *opa*), *csb* (capsular polymerase gene), *lgtA*, *lgtG* (*N. meningitidis* adhesion genes), and *modB* (DNA methylation) as well as in other genes not known to be associated with *N. meningitidis* virulence (see Table S3).

N. meningitidis carriage dynamics among repeat participants. Among 1,587 individuals who participated in multiple rounds of carriage evaluations (repeat participants), 348 were identified to be *N. meningitidis* carriers in at least one of the rounds and 194 were carriers of *N. meningitidis* in two or more rounds (repeat carriers) (Table 2). RI-1 had the highest number of repeat participants ($n = 615$), followed by OR ($n = 613$) and RI-2 ($n = 359$). Among the 348 repeat participants with *N. meningitidis* carriage in at least one round, 50.3% (175/348) maintained carriage of *N. meningitidis* with the same ST and CC across rounds (see Table S4), 44.3% (154/348) carried *N. meningitidis* in only one round of the study, and 5.4% (19/348) acquired a new *N. meningitidis* strain between rounds.

The numbers of repeat participants who carried *N. meningitidis* in only one round of the study were 70/615 (11.4%), 15/359 (4.2%), and 69/613 (11.3%) for RI-1, RI-2, and OR, respectively. The number of repeat carriers among repeat participants was 111/615 (18.0%) at RI-1, 30/359 (8.3%) at RI-2, and 53/613 (8.6%) at OR. Among the repeat carriers from the three universities, 174/194 (89.7%) were carriers in two rounds (Table 2). Excluding repeat carriers from RI-2 (as it contained only two carriage rounds), 20/164 (12.2%) were carriers in three rounds; no repeat carriers were identified to be carriers in all four rounds. Eight repeat carriers tested negative for *N. meningitidis* carriage after testing positive in a previous round, and regained *N. meningitidis* carriage in a subsequent round; two regained carriage of a strain with the same CC and ST, and six gained carriage of a new strain.

TABLE 2 Number of repeat carriers that were positive for *N. meningitidis* carriage in each combination of rounds

Round	No. of carriers[a]			
	RI-1	RI-2	OR	Total
Two carriage rounds				
1 + 2	36	29	19	84
1 + 3	9	0	1	10
1 + 4	10	0	2	12
2 + 3	19	0	8	27
2 + 4	3	0	4	7
3 + 4	9	0	9	18
1, 2 different ST	1	1	3	5
2, 3 different ST	3	0	0	3
1, 4 different ST	2	0	0	2
1, 3 different ST	0	0	0	0
2, 4 different ST	2	0	1	3
3, 4 different ST	1	0	2	3
Total	95	30	49	174
Three carriage rounds				
1 + 2 + 3	8	0	2	10
1 + 2 + 4	3	0	0	3
1 + 3 + 4	1	0	2	3
2 + 3 + 4	1	0	0	1
1 + 2, 4 different ST	3	0	0	3
Total	16	0	4	20

[a]The elapsed days from round 1 to subsequent rounds for each university were as follows. RI-1: round 2 (56 days), round 3 (185 days), round 4 (380 days). RI-2: round 2 (41 days); OR, round 2 (69 days), round 3 (218 days), round 4 (350 days).

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TABLE 3 Within-host new strain (with a new CC/ST) acquisition events during the carriage evaluation time period in all three U.S. universities[a]

| Evaluation | CC (ST) or outcome | | | |
	Round 1	Round 2	Round 3	Round 4
RI-1				
A	CC32 (ST-11527)	CC32 (ST-11527)	Not swabbed	CC35 (ST-35)
B	CC41/44 (ST-409)	Not swabbed	Not swabbed	CC198 (ST-823)
C	CC198 (ST-2384)	CC198 (ST-2384)	Not swabbed	CC269 (ST-269)
E	CC1136 (ST-1136)	CC1136 (ST-1136)	Not swabbed	CC35 (ST-11740)
F	Swabbed (neg[b])	CC32 (ST-1130)	CC198 (ST-6321)	Not swabbed
G	Not swabbed	CC198 (ST-823)	Swabbed (neg)	CC41/44 (ST-7574)
H	Not swabbed	CC1157 (ST-1157)	CC60 (ST-60)	Not swabbed
I	CC1157 (ST-1157)	CC198 (ST-823)	Swabbed (neg)	Not swabbed
J	Swabbed (neg)	CC60 (ST-11739)	CC35 (ST-11740)	Not swabbed
K	Swabbed (neg)	CC60 (ST- 60)	Swabbed (neg)	CC32 (ST-2506)
L	CC32 (ST-34)	Not swabbed	Not swabbed	CC41/44 (ST-409)
M	Not swabbed	Not swabbed	CC41/44 (ST-409)	CC60 (ST-60)
RI-2				
A	CC53 (ST-53)	CC35 (ST-3085)	Not swabbed	Not swabbed
OR				
A	Not swabbed	Not swabbed	CC178 (ST-178)	CC178 (ST-11550)
B	Not swabbed	CC35 (ST-35)	Not swabbed	CC53 (ST-12930)
C	CC60 (ST-60)	CC1157 (ST-1649)	Not swabbed	Not swabbed
D	CC198 (ST 823)	CC41/44 (ST 12632)	Not swabbed	Not swabbed
E	CC60 (ST 60)	CC35 (ST 278)	Not swabbed	Not swabbed
F	Not swabbed	Not swabbed	CC 1157 (ST 1649)	CC53 (ST 53)

[a]A new strain was defined as identification of a strain with a different CC or ST than was previously identified for a given carrier.
[b]neg, negative for *N. meningitidis*.

Acquisition of new *N. meningitidis* strains in repeat carriers. The 19 new strain acquisition events which were observed (Table 3) among repeat carriers involved the carrier either acquiring a different ST within the same CC or acquiring an entirely different CC. The only instance of new strain acquisition from a different ST within the same CC was detected in an individual from OR who was carrying an ST-178 (CC178) strain during round three and a ST-11550 (CC178) strain in round four. We did not observe any patterns or trends of certain STs or CCs being preferentially gained between any of the carriage evaluation rounds.

Within-host genetic diversity and variations of *N. meningitidis* isolates over time. To assess if the 175 repeat carriers who maintained carriage of *N. meningitidis* with the same ST and CC across rounds were each carrying the same strain, the single nucleotide polymorphism (SNP) differences among these isolates, along with the SNP differences from the carried isolates among nonrepeat participants of the same CC, were compared (see Table S5). Within each of the CCs, the median SNP difference between isolates carried by repeat carriers was significantly different than the median SNP difference for the rest of the carried population, indicating that these repeat carriers were likely carrying the same strain across rounds. The pairwise SNP differences between isolates collected from these repeat carriers were compared against the elapsed time between rounds. In general, a weak correlation (Pearson's $r = 0.21$) (Fig. S2) was observed between SNPs and days among the isolates collected from these repeat carriers.

Among the isolates carried by these 175 repeat carriers, genetic variations were detected in 782 genes over time (see Table S6), with 16 of these genes being common among the strains harbored by >20% (30/175) of the repeat carriers. The most frequently observed gene with genetic variations was *pilC1*, which had undergone genetic variations in strains harbored by 72.6% (127/175) of the repeat carriers. The next most frequently changed genes were *pilE* (54.3%), *pilC2* (53.14%), *hpuA* (39.4%), and *pglA* (37.7%).

Recombination was the most common mechanism behind the genetic differences observed between isolates obtained from repeat carriers with the same ST and CC and presented as multiple nucleotide differences along with insertions or deletions in the gene. Point mutations, deletions, and short sequence repeats (SSRs) were the next most frequently observed mechanisms responsible for genetic variations in isolates obtained from these repeat carriers. The genes that most frequently sustained SSRs in the isolates were NEIS1156 (unknown function), NEIS1750 (S-adenosylmethionine-dependent methyltransferase), *lgtG* (O-3 linked glucosyltransferase), NEIS3112 (unknown function), *pglI* (O-acetyltransferase), and *pilC1* (pilus-associated protein). Previously characterized as phase variations induced by SSR, $(CTTCT)_n$ and poly(G/C) (23), were also found in *opaA* and *pilC2* genes. Two SSRs, $(CCTTGG)_3$ in the NEIS1987 gene and $(CTTCAG)_7$ in the *lbpB* gene, were only identified in CC198 isolates.

DISCUSSION

To better understand the dynamics of U.S. meningococcal carriage in university and outbreak settings, we analyzed whole-genome sequencing (WGS) data for 1,514 *N. meningitidis* carriage isolates collected from ten cross-sectional carriage evaluation rounds conducted at three U.S. universities. While the predominant CCs were the same at the three universities, these three student populations were largely differentiated by the carried meningococcal ST distributions found at each, as many STs were observed only within one university during the evaluation time period. Carriage of *N. meningitidis* belonging to ST-32/CC32 and ST-9069, responsible for outbreaks at OR and RI-1, respectively, were detected. *N. meningitidis* carriage among U.S. university students appeared to be dynamic, and new strain acquisition events among repeat carriers primarily consisted of acquisition of a CC or ST which was also observed among other carriers within the same university population. Recombination, point mutations, and simple sequence repeats were the most frequent mechanisms for the genetic variation observed among carriage isolates that were detected within the hosts who maintained carriage of a strain with the same ST and CC for multiple rounds.

In this evaluation, CC198, CC1157, CC41/44, CC32, and CC35 were observed to be the five predominantly carried CCs among study participants from all three universities. Similarly, a previous carriage evaluation study among U.S. high school students in Georgia and Maryland during the academic year 2006 to 2007 also identified CC198 as the predominant CC, followed by CC23 and CC41/44, while carriage isolates associated within CC1157 and CC35 were the 5th and 6th most common CCs detected (24). In general, the most observed CCs identified at each university remained stable across evaluation rounds. CC198, CC1157, and CC41/44 were frequently observed in all 3 universities across all rounds, suggesting that strains belonging to these CCs were commonly carried in these populations.

Phylogenetic comparison indicated that the two ST-9069 carriage isolates from RI-1 were genetically similar to the RI-1 outbreak isolates, while the carriage ST-9069 isolate detected from OR was genetically different. ST-9069 is newly identified in the United States, with the only previous case reported before the RI-1 outbreak being a single carriage isolate from Ireland in 2009. As such, it is likely that the full diversity of this virulent lineage has not yet been captured. While ST-32 (CC32), which caused the outbreak in OR, is common among invasive disease cases in the United States, only two carriage isolates from this ST were detected at OR. One of these two isolates was highly similar to the outbreak strains. Overall, the low number of carriage isolates which were similar to the outbreak strains suggests that these strains were not commonly carried in the student population.

N. meningitidis carriage among U.S. university students appeared to be highly dynamic; nearly half of repeat participants with *N. meningitidis* carriage in at least one round retained carriage of a strain with the same ST and CC across rounds, and the other 44.3% carried *N. meningitidis* in only one round, and 5.4% acquired a new *N. meningitidis* strain. In a previous meningococcal carriage study among European teenagers, researchers found that among repeat participants who carried *N. meningitidis* in

at least one round, 54.4% (147/270) retained the same strain across rounds, 37.4% (101/270) carried *N. meningitidis* in only one round, and 8.1% (22/270) acquired a new strain at some point over a 23-week time period (25). While these results were similar to ours, more data are needed to be able to produce generalized conclusions about carriage dynamics and strain acquisition events among the general population.

Given the extent of recombination and horizontal gene transfer observed in meningococci, it is likely that simultaneous carriage of multiple meningococcal strains occurs (26). Since only a single colony was selected for sequencing from the culture plates, this study does not consider whether strain heterogeneity was present within an individual or even on the collected swab. However, it was previously shown that colonization of the throat by multiple meningococcal clones is rare (27) and that only a small minority of swabs yield more than one simultaneous phenotypically distinct meningococcal isolate (28). Nevertheless, it is not possible to conclude from this study whether the new strain acquisition events observed were actual replacement of the existing strain or due to mixed carriage in which one strain was sampled during one round and the other in a subsequent round. As culturing several isolates from a single throat swab is labor intensive, metagenomics approaches for answering these questions should be considered for future carriage studies, such as sequencing bacteria directly from swabs. While expensive, this methodology would provide higher resolution and confidence in identifying novel strain acquisition events.

Recent studies investigating within-host meningococcal evolution by comparing genome sequences of throat-blood strain pairs from a limited number of patients with IMD (29), and within-host genetic changes in paired carriage isolates (30), identified genetic changes predominantly affecting the meningococcal type IV pilus genes. These genes are involved in the adherence to the human epithelium and tissue tropism. Similarly, our analyses comparing within-host genetic changes across rounds and outbreak isolates to their closest carriage isolate also found genetic changes in the genes involved in *N. meningitidis* adherence and virulence, as well as other genes associated with *N. meningitidis* invasion, escape from host immune defenses, iron uptake, and proteases. These results can inform future studies focused on identifying the significant genetic variations between carried and invasive meningococci.

Exposure to meningococcal bacteria can lead to either asymptomatic carriage, or less commonly, to IMD. Asymptomatic carriage may be an immunizing event, thereby helping to protect an individual against future IMD. In cases where IMD occurs, the time between exposure to the bacteria and development of IMD may be anywhere from 1 day to approximately 2 weeks after the acquisition of the bacterium (4, 31). The pathophysiological factors that lead to meningococci invading the bloodstream are not fully known. It has been hypothesized that this fast-track from exposure to the invasive phenotype is related to the short time required by SSRs to modulate gene expression and promote phenotypic variation (32). During this process, a random reassortment of proteins relevant to the host-pathogen interaction may produce a pathogenic variant capable of crossing the nasopharyngeal epithelium, resulting in IMD (33). The data collected during this carriage evaluation were not sufficient to confirm this hypothesis mainly because, thankfully, no participant developed IMD during this evaluation. Additionally, no preceding carriage isolates were available for the patients who developed meningococcal disease during the outbreaks. Nevertheless, future carriage studies will help to address questions about carriage acquisition, transmission, risk factors, and duration of carriage. Knowledge gained from such studies will be important for future research efforts working toward disrupting the transmission of meningococcal carriage isolates in the population, thereby potentially preventing future cases of IMD.

MATERIALS AND METHODS

Data collection. From February 2015 to March 2016, a total of ten rounds of cross-sectional carriage surveys were conducted at three U.S. universities (RI-1, RI-2, an OR) using standardized methods, as previously described (6, 21, 22). A total of 1,514 *N. meningitidis* carriage isolates were obtained from 8,905 swabs collected from 7,001 unique participants. A total of 1,587 individuals participated in multiple survey rounds (repeat participants). *N. meningitidis* carriage isolates included 650 from four RI-1 carriage

rounds (February 2015, April 2015, September 2015, and March 2016), 616 from four OR rounds (March 2015, May 2015, October 2015, and February 2016), and 248 from two RI-2 rounds (March 2015 and April 2015) (see Fig. S1 in the supplemental material). The serogroup for all isolates was determined using slide agglutination (SASG).

Whole-genome sequencing and molecular typing. All *N. meningitidis* carriage isolates were characterized with whole-genome sequencing (WGS). Genomic DNA was prepared for sequencing using the 5Prime ArchivePure DNA Purification kit (5Prime, Gaithersburg, MD) or Gentra Puregene yeast/bacteria DNA extraction kit (Qiagen, Germantown, MD) as previously described (34) for sequencing on Pacific Biosystems (PacBio, Menlo Park, CA, USA) and/or Illumina (HiSeq2500 or MiSeq; Illumina, San Diego, CA, USA) platforms; genomic DNA was further prepared for Illumina sequencing with the dual-index NEBNext Ultra DNA library preparation kits (New England BioLabs Inc., Ipswich, MA). Genomes generated using the PacBio platform were processed as previously described (35). The Illumina reads were trimmed with Cutadapt (36), and *de novo* short read assembly was carried out using SPAdes 3.7.0 (37) with the "careful" option. Assemblies were compared with BLAST (38) against the PubMLST *Neisseria* allele collection (39) to identify MLST alleles. The MLST genes were compared against the PubMLST MLST scheme collection to identify the *N. meningitidis* ST and CC.

Phylogenetic and comparative analyses. The phylogenetic trees were constructed on carriage isolates from each university using a whole-genome alignment (WGA) produced by Parsnp (Harvest suite, version 1.2) (40). WGA was used to infer the maximum likelihood (ML) phylogeny using RAxML with a general time-reversible nucleotide model (41). The recombination corrected phylogenetic tree was generated using ClonalFrameML (42). The final phylogenetic tree was annotated in Interactive Tree of Life (43).

For the CC32 and ST-9069 phylogenetic analysis and comparative genomic analysis of carriage and outbreak isolates and the calculations of SNP differences between repeat and nonrepeat participants, trimmed Illumina sequencing reads were aligned to a completed reference genome of each CC/ST generated using PacBio sequencing and curated Illumina reads. Whole-genome alignments were generated using Snippy (https://github.com/tseemann/snippy), which identifies variants using Freebayes v1.0.2 (44) with a minimum 10× read coverage and 90% read concordance at a locus for each SNP. Putative regions of recombination were predicted and masked using Gubbins (45). The resulting SNP alignment was used to calculate the SNP differences among repeat and nonrepeat participants, and for CC32 and ST-9069, the alignment was used as input for RAxML in order to infer the ML phylogenetic tree using the general time reversible (GTR) model and 100 bootstrap replicates. The genomes involved in the CC32 and ST-9069 comparative genome analysis were annotated using the PubMLST *Neisseria* allele collection using custom scripts with BLAST (38). The genes harboring genetic changes between the carriage and invasive isolates were identified by comparing the alleles present for each locus. Pairwise SNP differences between isolates obtained from repeat carriers harboring a strain with the same ST and CC across rounds were obtained by generating a recombination corrected whole-genome alignment using Snippy and Gubbins, as described above, against a same-ST reference.

To identify genes with frequent genetic variations, as well as the types of variations, among carried isolates from repeat carriers with the same ST and CC across rounds, genetic variations were identified through mapping sequencing reads of carriage isolates against a common reference genome of the same CC to identify variant locations. Variation type was defined as point mutation if only a single nucleotide difference was detected in a gene, homologous recombination if multiple nucleotide variations (SNPs, multinucleotide polymorphisms [MNPs], and complex) and insertions were observed in the same gene, simple sequence repeats if repeated nucleotides with various length were inserted into a gene, and indels if random sequences were inserted or deleted in the gene. These variant locations were mapped against gene locations determined by annotating each reference genome using the PubMLST *Neisseria* allele database, through the use of custom python scripts, to identify the genes harboring these variations. The significance of the median SNP differences between repeat and nonrepeat participants was calculated using the Wilcoxon's rank sum test (46). The stability of the most frequently observed CCs per university across each round was assessed with a chi-square statistical analysis using SAS 9.4.

Data availability. Sequence reads for all isolates used in this analysis are available in BioProject PRJNA533315.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.1 MB.
FIG S2, DOCX file, 0.02 MB.
TABLE S1, XLSX file, 0.1 MB.
TABLE S2, DOCX file, 0.1 MB.
TABLE S3, XLSX file, 0.1 MB.
TABLE S4, XLSX file, 0.1 MB.
TABLE S5, DOCX file, 0.1 MB.
TABLE S6, XLSX file, 0.3 MB.

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