

Loss of Genomic Diversity in a *Neisseria meningitidis* Clone Through a Colonization Bottleneck

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Abstract

Neisseria meningitidis is the leading cause of epidemic meningitis in the “meningitis belt” of Africa, where clonal waves of colonization and disease are observed. Point mutations and horizontal gene exchange lead to constant diversification of meningococcal populations during clonal spread. Maintaining a high genomic diversity may be an evolutionary strategy of meningococci that increases chances of fixing occasionally new highly successful “fit genotypes”. We have performed a longitudinal study of meningococcal carriage and disease in northern Ghana by analyzing cerebrospinal fluid samples from all suspected meningitis cases and monitoring carriage of meningococci by twice yearly colonization surveys. In the framework of this study, we observed complete replacement of an A: sequence types (ST)-2859 clone by a W: ST-2881 clone. However, after a gap of 1 year, A: ST-2859 meningococci re-emerged both as colonizer and meningitis causing agent. Our whole genome sequencing analyses compared the A population isolated prior to the W colonization and disease wave with the re-emerging A meningococci. This analysis revealed expansion of one clone differing in only one nonsynonymous SNP from several isolates already present in the original A: ST-2859 population. The colonization bottleneck caused by the competing W meningococci thus resulted in a profound reduction in genomic diversity of the A meningococcal population.

Key words: meningococcal meningitis, *Neisseria meningitidis*, genomic diversity, clonal expansion, whole genome sequencing, nasopharyngeal colonization.

Introduction

Neisseria meningitidis, a Gram-negative human-specific bacterium, is the major cause of epidemic meningitis in children and adults, particularly across the so-called “African meningitis belt”. The epidemiology of

N. meningitidis in Africa is characterized by a succession of epidemics, which occur during the dry season (December–May), stop abruptly at the onset of rain and may flare up again in the next dry season. In recent years, the periodicity of epidemic cycles has varied considerably

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from country to country and their dynamics are still not well understood.

The human nasopharynx is the only habitat of *N. meningitidis*, but although this bacterium inhabits such a restricted ecological niche, it shows considerable genetic diversity. In Europe complex and temporally relatively stable colonizing meningococcal populations have been observed, which primarily consist of isolates belonging to lineages that have a low virulence (Yazdankhah et al. 2004; Caugant et al. 2007). In contrast, in the African meningitis belt meningococcal colonization is unstable and clonal waves of colonization and disease have been observed (Leimkugel et al. 2007). When the dominating colonizing population belongs to a hypervirulent lineage, these waves are accompanied by outbreaks of invasive meningococcal disease. Lack of a temporally stable and genetically diverse pharyngeal microbiota of meningococci may contribute to the susceptibility of the population of the African meningitis belt to meningococcal disease epidemics (Leimkugel et al. 2007; Chow et al. 2016). Point mutations and horizontal gene exchange lead to constant diversification of meningococcal lineages during clonal spread (Lamelas et al. 2014). In particular the potential for wide variability in cell surface structures allows *N. meningitidis* lineages to evade immune surveillance.

Meningococci are classified into 12 serogroups based on the composition of their polysaccharide capsule, but only 6 (A, B, C, W, X, and Y) produce frequently invasive disease. Although serogroup A meningococci have been responsible for most epidemics in the meningitis belt in the last 100 years, other serogroups such as C (Chow et al. 2016), W (Taha et al. 2000; Lamelas et al. 2017), and X (Boisier et al. 2006) have also caused outbreaks and are dominating after the introduction of the monovalent serogroup A capsule polysaccharide conjugate vaccine MenAfriVac (Tiffay et al. 2015) in the countries of the meningitis belt (Trotter et al. 2017).

In the framework of a longitudinal meningococcal carriage and disease study in the Kassena-Nankana District (KND) of northern Ghana and an analysis of meningococcal isolates by multilocus sequence typing (MLST), we have observed between 1998 and 2009 three sequential waves of colonization with serogroup A meningococci with the sequence types (ST) 5, 7, and 2859 (Leimkugel et al. 2007). These three closely related lineages have caused meningitis outbreaks in many countries of the meningitis belt (Nicolas, Norheim, et al. 2005). A comparative whole genome sequencing study has revealed that the A: ST-2859 meningococci share a common ancestor with the ST-7 bacteria and differ in multiple homologous recombination events involving incorporation of DNA sequences from meningococci and other *Neisseria* species (Lamelas et al. 2014). Analysis of the hot spots of recombination indicated that the complex changes in bacterial cell surface structures associated with the recombination events has enabled the A: ST-2859 clone to multiply in host populations that have developed immunity against the A: ST-7 bacteria. A

large proportion of individuals belonging to local populations in the meningitis belt may become transiently colonized by dominating clones (Leimkugel et al. 2007) and develop mucosal immunity. When a large enough percentage of the population has become immune to colonization, individuals who are not immune are indirectly protected (herd immunity), leading to the local disappearance of meningococcal clones after a colonization and disease wave (Leimkugel et al. 2007; Lamelas et al. 2014).

Owing to the natural competence of meningococci for transformation and homologous recombination (Rotman and Seifert 2014) the genomic diversity of epidemic *N. meningitidis* lineages is extensive (Lamelas et al. 2017). Occasionally, individual immune escape variants may expand from these clonal complexes (CCs), as exemplified by the emergence of the A: ST-2859 clone after multiple recombination events from the genomically diversified A: ST-7 population. Here, we demonstrate that also a colonization bottleneck associated with competition for mucosal colonization can cause a massive decrease in genomic diversity in a meningococcal population by expansion of one of the pre-existing haplotypes.

Materials and Methods

Study Area

The study was conducted in the KND of the Upper-East Region of Ghana, located within the guinea Savannah woodland. The area has two major seasons; a short wet season from June to October and a long dry season for the rest of the year. The district-population is about 140,000, most of them rural, except for the 20,000 inhabitants of Navrongo town. In the KND, people live in compounds with an average of 10 inhabitants (http://www.indepth-network.org/dss_site_profiles/navrongo.pdf). Between 2007 and 2011, vaccination campaigns with meningococcal serogroup A/C polysaccharide vaccine targeted the whole district population. Ethical clearance for this study was obtained from the responsible institutional and national review boards.

Colonization Isolates

Thirty-seven residential compounds were randomly selected from a complete listing of the district population using the Navrongo Demographic Surveillance System and throat swabs were taken from about 300 individuals per survey. Starting in March 1998 throat swabs were taken twice per year (in April and November) from all inhabitants of the 37 compounds present at the time of the visit who agreed to participate. Study participants in the consecutive carriage surveys thus were at least in part, the same individuals. Here, we present the data of surveys 19 (April 2007) to 27 (April 2011); in each of them between 280 and 364 study participants have been swabbed (table 1).

Table 1*Neisseria meningitidis* Carriage Rates

| Survey No. (time) | Number of Swabs Analyzed | % Carriage Rate for Different Serogroups (Number of Isolates) | | | |
|-------------------|--------------------------|---|------------|----------|----------|
| | | A ST-2859 | W ST-2881 | Y ST-767 | NG |
| 19 (April 2007) | 306 | 6.2% (19) | 0% | 0% | 1.0% (3) |
| 20 (Nov 2007) | 334 | 12.3% (41) | 0% | 0.6% (2) | 0.6% (2) |
| 21 (April 2008) | 312 | 1.3% (4) | 0% | 0% | 0.6% (2) |
| 22 (Nov 2008) | 307 | 7.8% (24) | 0% | 0% | 0% |
| 23 (April 2009) | 343 | 0.3% (1) | 0% | 0% | 0% |
| 24 (Nov 2009) | 364 | 0% | 6.6% (22) | 0% | 0.8% (3) |
| 25 (April 2010) | 309 | 0% | 15.2% (47) | 0% | 0% |
| 26 (Nov 2010) | 338 | 3.5% (12) | 16.7% (56) | 0% | 0.9% (3) |
| 27 (April 2011) | 280 | 3.9% (11) | 11.4% (32) | 0% | 1.1% (3) |

NG=nongroupable.

Throat swabs taken from the study participants were directly inoculated on Thayer-Martin agar plates. Colonies with *Neisseria* morphology were subcultured. *Neisseria meningitidis* colonies were identified by standard bacteriological methods as previously described (Leimkugel et al. 2007).

Disease Isolates

Suspected meningitis patients presenting at the War Memorial Hospital (WMH), Navrongo, or one of the four Health Centers of the KND were recruited throughout the study period. A suspected meningitis patient was defined by sudden onset of fever and stiff neck, or fever and stiff neck and altered mental status, in accordance with WHO guidelines. A lumbar puncture was performed before treatment from all suspected meningitis patients, and the cerebrospinal fluid specimen was analyzed by latex agglutination and culture (Leimkugel et al. 2007).

Characterization of Bacterial Isolates

Meningococcal isolates were serogrouped by Multiplex PCR (Taha et al. 2000), and selected isolates were analyzed by MLST. DNA extraction was done using a Promega DNA isolation kit and PCR was performed in accordance to standard protocols available on the MLST homepage (<http://pubmlst.org/neisseria/>). PCR products were sequenced at Macrogen Inc., Europe (Amsterdam, The Netherlands). Allelic profiles were analyzed using applications available on the MLST homepage.

Preparation and Sequencing of Genomic DNA

N. meningitidis isolates (supplementary table S1, Supplementary Material online) were grown in liquid Brain Heart Infusion (BactoTM) medium and chromosomal DNA was prepared as described (Lamelas et al. 2014). Briefly, bacterial pellets were resuspended in 0.5 ml of TES buffer (50 mM Tris, 20 mM EDTA, 50 mM NaCl [pH 8]). Two microliters of

RNase A (Qiagen 100 mg/ml) and 20% SDS were added to a final concentration of 1% and cells were lysed for 5 min at 42 °C. After two phenol:chloroform:isoamyl alcohol (25:24:1, Sigma) extractions and one chloroform:isoamyl alcohol (24:1, Sigma) extraction, DNA was precipitated in two volumes of isopropanol followed by suspension in TE buffer (10 mM Tris, 1 mM EDTA [pH 8]). Ammonium acetate was added to a final concentration of 2.5 M, and the DNA was precipitated in two volumes of ethanol. After two 70% ethanol wash steps and subsequent drying, the DNA was diluted in TE buffer (10 mM Tris, 0.1 mM EDTA [pH 8]).

Multiplexed genomic DNA libraries were prepared with an insert size of 200 using 24 unique index tags. Libraries were combined into pools of 24 and sequenced on an Illumina HiSeq for 75 cycles from each end to produce paired-end reads plus an 8-base index sequence read. Downstream analysis used the index tags to assign reads to individual samples.

Read Alignment and SNP Detection

Variation in the form of single nucleotide polymorphisms (SNPs) was detected using a mapping approach essentially as described (Harris et al. 2010). The paired-end Illumina reads were mapped against the *N. meningitidis* genome of the A: ST-5 isolate WU2594 (accession numbers FR774048) as reference with an insert size between 50 and 400 bp using SMALT v0.7.4 (<http://www.sanger.ac.uk/resources/software/smalt/>), giving, on average, a depth of coverage of 177x. SNPs were identified using SAMtools v1.1. The minimum base call quality to call an SNP was 50; the minimum root mean square of the mapping quality was 30 and the minimum number of reads matching SNP was 4 and per strand 2. SNPs were filtered to remove those at sites with an SNP quality score below 50. SNPs called in phage sequences and repetitive regions of the *N. meningitidis* reference genome were excluded. Repetitive regions were defined as exact repetitive sequences of ≥ 50 bp in length, identified using the repeat-match function of the MUMmer package (Kurtz et al. 2004).

SNPs at sites with heterogeneous mappings (indetermination) were filtered out if the SNP was present in <90% of the reads at that site.

Identification of Recombination Events

To identify recombination events based on high SNP density, we applied a moving window approach using the Gubbins software as described (Croucher et al. 2015).

Phylogenetic Analyses

Maximum-likelihood phylogenetic trees were performed by RAxML v7.0.4 (Stamatakis 2006) using a general time-reversible substitution model with γ correction for among-site rate variation. Support for nodes on the trees was assessed using 100 bootstrap replicates.

SNP Pairwise Distance Analysis

To compute the pairwise distance based on the genome wide SNP count between the isolates, we used MEGA6 (Tamura et al. 2013). Two-tailed Mann–Whitney tests were performed using Stata (version 12). The pairwise SNPs distances within the lineages were plotted using software package R (<http://www.r-project.org/>).

Results

Consecutive Meningococcal Colonization and Disease Waves in the KND

Within the framework of a longitudinal study, which started in 1998 (Gagneux et al. 2000; Leimkugel et al. 2007), we have monitored the dynamics of meningococcal meningitis and of pharyngeal carriage of *N. meningitidis* in the KND of northern Ghana. After a A: ST-5 meningococcal meningitis epidemic in 1997/1998, A: ST-7 cases were observed in the meningitis seasons of 2001–2005 (Leimkugel et al. 2007). A third A meningococcal colonization and disease wave caused by A: ST-2859 meningococci started in 2007 (fig. 1A). Not a single ST-5 or ST-7 isolate was found after 2005 among the colonization or disease isolates. We attribute complete disappearance of these clones in the KND to the development of lineage-specific immunity and have provided evidence that the A: ST-2859 meningococci have evaded mucosal immune mediated colonization resistance by alterations in several non-capsular cell surface components through multiple homologous recombination events (Lamelas et al. 2014).

During the nine colonization surveys between April 2007 and April 2011 we have collected 2,893 pharyngeal swabs; 287/2,893 (9.9%) of these yielded growth of *N. meningitidis*. Point prevalences of the different serogroups at the eight individual surveys are given in table 1. Altogether we isolated 112A: ST-2859 and 157W: ST-2881 colonization isolates. Only 18 isolates not belonging to these two clones, two

serogroup Y: ST-767 and 15 nongroupable (ST-129 or ST-823) isolates were obtained in addition (table 1). Whole genome sequencing has revealed that the W: ST-2881 meningococci are unrelated to W: ST-11 lineages (Lamelas et al. 2017), which have been only sporadically isolated in the KND between 1998 and 2004 (fig. 1A) (Forgor et al. 2005), but have caused major epidemics elsewhere (Mustapha et al. 2016).

Disappearance and Re-Emergence of A: ST-2859 Meningococci

Overall, we observed a very close temporal correlation between the colonizing and the meningitis causing meningococcal populations. Colonization with A: ST-2859 meningococci was first detected in spring 2007 and the highest point prevalence of carriage (12.3%) was observed in the fall of 2007. Carriage prevalence subsequently decreased to 0.3% in spring 2009 (table 1 and fig. 1A). Although not a single A carrier was found in the fall 2009 and spring 2010 colonization surveys, A: ST-2859 carriage rates of 3.5% and 3.9% were again observed in fall 2010 and spring 2011, respectively. Cases of A: ST-2859 meningitis were found in the meningitis seasons of 2007 and 2008 and then again in 2010 and 2011 (fig. 1A). In contrast, none of the disease isolates from the meningitis seasons of 2009 belonged to the A: ST-2859 clone. Compared with A: ST-7, the incidence of A: ST-2859 meningitis was relatively low (fig. 1A). This may be related to repeated vaccination campaigns in the KND with a serogroup A carbohydrate vaccine, which has no effect on colonization, but protects from invasive disease. In general, it has been found that A: ST-2859 meningococci are hypervirulent and can cause major outbreaks, such as the A: ST-5 and A: ST-7 lineages (Sié et al. 2008).

A first three W: ST-2881(cc175) meningitis cases were identified in spring of 2009 (fig. 1B). At that time the W colonization rate was still below our detection limit defined by pharyngeal sampling of about 300 individuals per survey. In the following four colonization surveys from fall 2009 to spring 2011, W ST-2881 meningococcal colonization rates between 6.6% and 16.7% were observed (table 1 and fig. 1B). Although all meningococcal meningitis cases in the meningitis seasons of 2009 were caused by the W: ST-2881 meningococci, both A: ST-2859 and W: ST-2881 cases were found in the meningitis seasons of 2010 and 2011 (fig. 1).

Phylogenetic Analysis of Serogroup A ST-2859 *N. meningitidis* Isolates

To clarify the phylogenetic relationship of the A: ST-2859 meningococci re-emerging after an apparent colonization bottleneck in 2010, we sequenced the genomes of a collection of 27A: ST-2859 colonization and disease isolates isolated in 2010 and 2011. Using previously analyzed whole genome

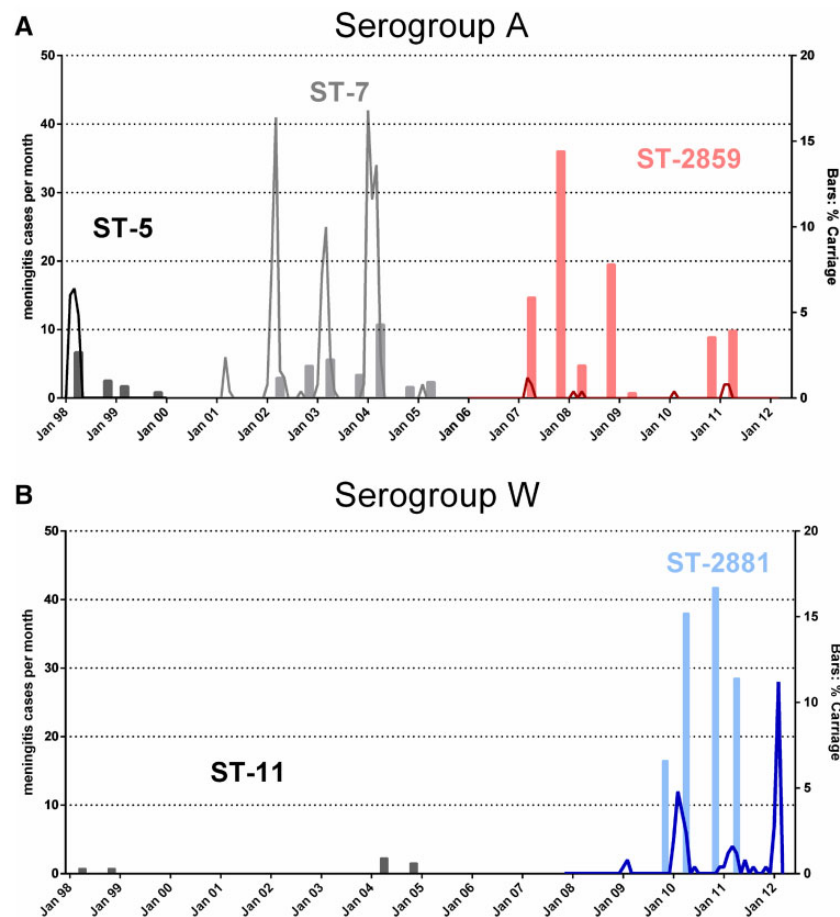


Fig. 1.—Dynamics of colonization and invasive disease by A: ST-2859 and W: ST-2881 meningococci in a longitudinal study in the KND of Ghana. Carriage rates recorded during twice yearly colonization surveys (April and November each year) and monthly numbers of meningitis cases caused by (A) serogroup A (ST-5, ST-7 and ST-2859) meningococci and (B) serogroup W (ST-11 and ST-2881) meningococci.

sequences from 16A: ST-2859 and 49A: ST-7 Ghanaian strains isolated before the replacement of the A: ST-2859 meningococci by the W: ST-2881 clone (Lamelas et al. 2014), we reconstructed the phylogenetic relationship between the original and the re-emerging A: ST-2859 populations. We found 5,647 variable nucleotide positions (SNPs) in total, without taking repetitive regions into account. Totally, 4,975 of these SNPs were located in recombination blocks and 672 were putative point mutations. Based on the 672 SNPs not associated with recombination events a maximum likelihood phylogenetic tree was obtained, which was structured in two major lineages, perfectly correlating with the MLST-based differentiation into A: ST-7 and A: ST-2859 isolates (supplementary fig. S1, Supplementary Material online). For the A: ST-2859 isolates a clear division into two subgroups was observed, one comprising the isolates isolated from November 2007 to May 2009 and the other one comprising the re-emerging A: ST-2859 meningococci isolated in 2010 and 2011 (fig. 2A).

The genomes from the 27 isolates belonging to the second A: ST-2859 colonization and disease wave were unusually homogenous with no new recombinations found. Only four isolates differed from the overall population in either two SNPs (isolates 3338 and 3319) or one SNP (isolates 3340 and 3322) that represent most likely point mutations. The genomes of 23/27 isolates, including all seven disease isolates from 2010 to 2011, were thus identical, providing evidence for clonal expansion after the colonization bottleneck in 2009. In contrast, the genomic diversity of the 16 sequenced isolates from 2007 to 2009 was significantly higher (P -value < 0.01) than that of the 27 isolates from 2010 to 2011 (fig. 2B). The genome data are indicative for the expansion of one clone that differs in only a single SNP at position 1655554 from five isolates (2808, 2809, 2810, 2811, and 2934) belonging to the original A: ST-2859 population (fig. 2A). This SNP produces an R to G amino acid substitution in the conserved hypothetical integral membrane protein NMAA_141. All isolates belonging to the second A: ST-2859 wave shared the recombination

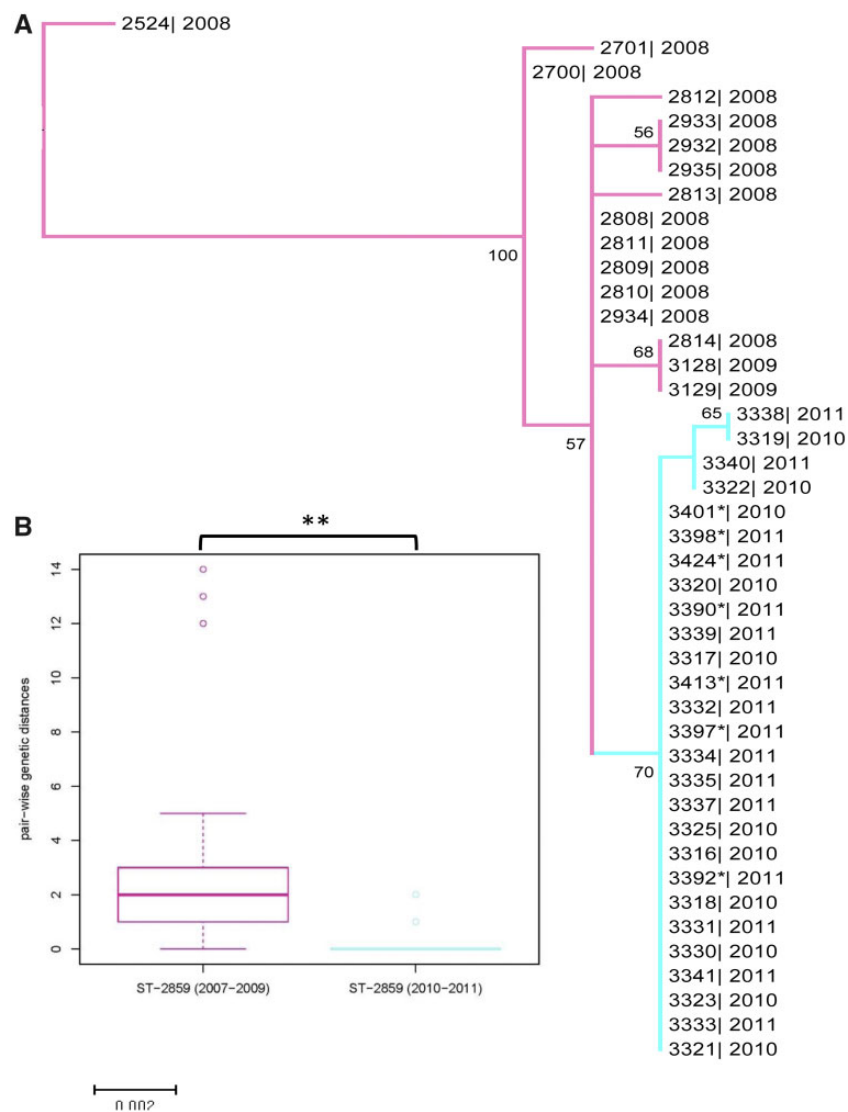


Fig. 2.—(A) Maximum-likelihood tree of *N. meningitidis* A: ST-2859 isolates from the KND. Phylogenetic analysis was performed using the whole-genome sequence of 43 A: ST-2859 isolates after removal of SNPs associated with the predicted recombination events. Asterisks indicate cerebrospinal fluid isolates. The IDs of the isolates is provided together with the year of isolation. The tree was rooted in the “outlier” A: ST2859 isolate 2524. Branches are colored according to the isolation dates (pink: A: ST-2859 meningococci isolated between spring 2007 and spring 2009; light blue: A: ST-2859 meningococci isolated in 2010 and in 2011). The bootstrap values higher than 50% are labeled by numbers along the branch. (B) SNPs pairwise distance between isolates from the first (2007–2009) and second (2010–2011) A: ST-2859 colonization and disease wave. The variation in pairwise distance for each group of isolates is shown in a box plot, with circles representing outlier sequence pairs. Although the distribution of 120 pairwise genomic comparisons of the A: ST-2859 meningococci isolated in 2007–2009 is represented in pink, the corresponding distribution for 351 pairwise genomic comparisons of the A: ST-2859 meningococci isolated in 2010–2011 is represented in blue. The results were plotted using the software package R (<http://www.r-project.org/>). Two-tailed Mann–Whitney test, *flags disease isolates, ** P -value < 0.01 .

blocks previously identified in the common ancestor of all A: ST-2859 isolates (Lamelas et al. 2014) and no additional recombination event was identified. Overall, results thus show that after the colonization bottleneck the second A: ST-2859 colonization and disease wave was caused by the spread of one haplotype from the more heterogeneous population causing the first A: ST-2859 colonization and disease wave.

Discussion

Maintaining a high genomic diversity may be an evolutionary strategy of meningococci that increases chances of fixing occasionally new highly successful “fit genotypes” (Zhu et al. 2001) or immune escape variants out of an immensely diverse repertoire of genomic variants present in spreading clones. In particular, the high competence for transformation and

homologous recombination of meningococci (Rotman and Seifert 2014) would normally foster the maintenance of this diversity, because it will allow beneficial mutations to be transmitted through the population without the fitter genotype replacing the entire population of genotypes. However, recombination may also enable the combination of multiple beneficial variants creating a highly fit haplotype which could rapidly sweep to fixation. Based on these observations, an epidemic clonality model has been proposed, which postulates that meningococcal populations undergo occasional rounds of clonal propagation in an otherwise recombining population structure (Smith et al. 1993). More recently it has been argued that a predominant clonal evolution model fits best to the population genetics of *N. meningitidis* (Tibayrenc and Ayala 2015).

Here, we present results of the second phase (2007–2011) of the first longitudinal meningococcal colonization and disease study in the meningitis belt of sub-Saharan Africa. Our sampling strategy allowed us to describe the dynamics of meningococcal carriage and disease in the KND over a 13 year study period that included both meningococcal outbreaks and interepidemic periods. In the course of this longitudinal study, we observed in 2009 for the first time an overlap and a transient replacement of one hypervirulent clone (A: ST-2859) by another one (W: ST-2881). The W: ST-2881 meningococci have emerged by capsule switching from a hypervirulent Y: ST-175 clone belonging to the CC 175 (Lamelas et al. 2017). They have been first isolated in Niger in 2002, where they caused in 2003 sporadic cases (Nicolas, Djibo, et al. 2005; Nicolas, Norheim, et al. 2005; Boisier et al. 2006). In 2003, W: ST-2881 meningitis cases have also been reported from Benin, Nigeria (Nicolas, Djibo, et al. 2005) and Burkina Faso (Mueller et al. 2007) and outbreaks have been reported from Cameroon (Massenet et al. 2013) and Ghana (Lamelas et al. 2017).

Interestingly, the A: ST-2859 meningococci re-emerged in the KND after a gap of 2 years, when their colonization rate was for most of the time below our detection limit of about 0.3%. Our previous comparative whole genome sequencing analyses have indicated that the A: ST-2859 clone represents a new adaptive genotype that has emerged from A: ST-7 meningococci by multiple recombination events. The A: ST-2859 bacteria apparently evaded A: ST-7 lineage-specific mucosal immunity through multiple changes in noncapsular cell surface components (Lamelas et al. 2014). Based on these findings, we had hypothesized that the re-emerging A: ST-2859 bacteria studied here would belong to a new “fit genotype” or immune escape variant that has accumulated additional recombination blocks. However, we observed a completely different scenario: there is no evidence, that the A: ST-2859 colonization and disease isolates re-emerging in 2011 contain a recombination block not present in the A: ST-2859 isolates from 2007 to 2009. Also at the level of point mutations no accumulation of genomic changes was

observed and 23 of the 27 “new” isolates differed in only one SNP from five of the “old” isolates. The genomes of the four other “new” isolates differed from the other 23 in only 1 or 2 additional SNPs. The second A: ST-2859 colonization wave was thus unusually homogenous, providing a striking example on how genomic diversity of an epidemic clone can collapse through the spread of a particular haplotype. In the scenario described here, the elimination of genomic variation appears to be related to a colonization bottleneck caused by the W: ST-2881 meningococci, but that does not exclude that the clone that emerged in 2010 is a particular fit haplotype.

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Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

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