Genomic Content of *Neisseria* Species[▽]

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The physical properties of most bacterial genomes are largely unexplored. We have previously demonstrated that the strict human pathogen *Neisseria gonorrhoeae* is polyploid, carrying an average of three chromosome copies per cell and only maintaining one pair of replication forks per chromosome (D. M. Tobiason and H. S. Seifert, PLoS Biol. 4:1069–1078, 2006). We are following up this initial report to test several predictions of the polyploidy model of gonococcal chromosome organization. We demonstrate that the *N. gonorrhoeae* chromosomes exist solely as monomers and not covalently linked dimers, and in agreement with the monomer status, we show that distinct nucleoid regions can be detected by electron microscopy. Two different approaches to isolate heterozygous *N. gonorrhoeae* resulted in the formation of merodiploids, showing that even with more than one chromosome copy, these bacteria are genetically haploid. We show that the closely related bacterium *Neisseria meningitidis* is also polyploid, while the commensal organism *Neisseria lactamica* maintains chromosomes in single copy. We conclude that the pathogenic *Neisseria* strains are homozygous diploids.

Bacteria are unicellular organisms that exhibit a multitude of shapes and sizes and exist in a wide range of environments. Despite the extreme diversity of capabilities and physiology evidenced by different bacterial species, most bacteria are assumed to conform to the enteric model of genomic organization, chromosomal replication, and genomic segregation during cell division exemplified by *Escherichia coli*. In contradiction to this limited view of bacterial genome biology, some bacterial species have their genome divided between multiple DNA elements (10), and some possess linear chromosomes (2, 19). A few bacterial species have been reported to carry multiple genome copies per cell (members of the genera *Azotobacter*, *Borrelia*, *Buchnera*, *Deinococcus*, *Neisseria*, and *Epulopiscium*), with copy number estimates ranging from two copies to thousands of copies per cell (1, 7, 17, 23, 25, 26, 34, 35, 39, 46). The exact number of genomes per cell has not been determined for most of these organisms, and the mechanisms for organizing polyploid genomes and segregating them during cell division remain to be determined. An exception is *Deinococcus radiodurans*, which has been shown to possess four complete chromosomes during exponential growth and up to 16 genomes within the stationary phase. The polyploid genomes of *D. radiodurans* have been proposed to assemble into a toroidal mass in the cell (29), but the validity of this finding has been questioned (11, 13, 49). There are few obvious commonalities between these polyploid organisms, except that some *Neisseria*, *Deinococcus*, and *Borrelia* species utilize homologous recombination to mediate specialized processes essential for the survival of these species. In addition, members of the *Azotobacter*, *Buchnera*, and *Epulopiscium* genera are obligate symbionts that do not possess a free-living stage, but

the reasons why obligate symbionts would possess polyploid chromosomes are unknown.

Neisseria gonorrhoeae and *Neisseria meningitidis* are the two pathogenic members of the *Neisseria* genus. *N. gonorrhoeae* is the sole causative agent of the disease gonorrhea, and *N. meningitidis* is the most common cause of bacterial meningitis in adolescents and young adults. One attribute that these human-specific pathogens use to coexist and evolve within humans lies in their capacity to antigenically vary and phase vary several outer membrane structures, including pili, Opa proteins, and the lipooligosaccharide (LOS) (12, 21). Variation of the Opa and LOS antigens is mediated by polynucleotide repeat variation that modulates expression of biosynthetic genes (40, 48). These changes in polynucleotide repeat sequences are mediated through slipped-strand mispairing that occurs during normal DNA replication and therefore would not obviously benefit from polyploidy. In contrast, pilin antigenic variation is a RecA-mediated gene conversion event (27), which could be aided by having two copies of all the recombining pilin loci within a single cell to facilitate the nonreciprocal transfer of pilin sequences. Therefore, we postulated that the presence of multiple genome copies per gonococcal cell may be required to facilitate these high-frequency gene conversion events. Analysis of gonococcal genome content by flow cytometry and fluorescent microscopy indicated that there existed greater than one genome equivalent of gonococcal DNA content per cell (46). Additionally, quantitative real-time PCR and genome microarray analysis measured a marker frequency pinpointing a single DNA replication event per round of cell division. On average, each coccal unit had three genome copies per cell, and a population of cells with a single genome equivalent per cell was never observed, even under conditions of slower growth. These observations predicted a model for gonococcal replication (Fig. 1) in which each coccal unit has a minimum of two chromosomes that replicate in unison to produce four chromosomes prior to cell division and the conclusion that this species is diploid.

Though the analysis of the genomic content has only been reported for the gonococcus, the genus *Neisseria* encompasses

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FIG. 1. Model of gonococcal DNA replication and chromosome segregation in a monococcus. The gonococcal chromosome is indicated by a dotted line. An antibiotic resistance (Ab^R) marker recombined into a gonococcal chromosome (solid line). At time zero minutes, DNA replication begins, and after 35 min, DNA replication is complete. Gonococcal cell division occurs after 60 min. In scenario I, homozygous chromosomes are segregated together. In scenario II, heterozygous bacteria are produced.

a number of pathogenic and commensal bacteria. *N. meningitidis* is the leading cause of bacterial meningitis worldwide and is asymptotically carried in the human nasopharynx (47). Most of the *Neisseria* species are commensal organisms that inhabit the nasopharynx and rarely cause disease (24). The most extensively studied commensal *Neisseria* species, *N. lactamica*, shares extensive homology with the pathogenic *Neisseria* species and also predominately resides in the human nasopharynx (31). Only the pathogenic neisseriae, *N. gonorrhoeae* and *N. meningitidis*, have been shown to undergo pilin antigenic variation (43). Since the polyploid nature of *N. gonorrhoeae* has been proposed to be required for pilin antigenic variation, *N. meningitidis* may also have multiple genome copies per cell. The genomic copy number of other *Neisseria* species and the putative relationship between genomic content and pathogenesis remain to be determined.

In this work, we tested several predictions or models resulting from the observation that the gonococcus is polyploid. We confirmed that the chromosomes exist as separate molecules and show that the gonococcal nucleoids reside in discrete cellular regions. We confirm that these bacteria are genetically haploid, suggesting that chromosomal segregation mechanisms ensure a homozygous population. Finally, we show that the other pathogenic *Neisseria* species, *N. meningitidis*, is also polyploid, while a commensal *Neisseria* species, *N. lactamica*, is not. These studies show that polyploidy is correlated with *Neisseria* pathogenesis and suggest that this property has evolved to allow diploid chromosomes while maintaining the haploid status of these obligate human pathogens.

MATERIALS AND METHODS

Strains and plasmids. The gonococcal strains used in these studies were FA1090, FA1090 Δ *pilE*, and FA1090*recA6*, which has an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible *recA* gene. Meningococcal serogroup A strains Z2491 and ATCC 13077, along with *N. lactamica* strain ATCC 23970, were also used in these studies.

The plasmids pGCC5 and pGCC2 were transformed into FA1090 to create the strains FA1090*cat* and FA1090*erm*, respectively. pMB25 (kindly provided by M. Bos) contains a kanamycin resistance (Kan^r) marker interrupting the meningococcal *imp* gene (4, 5).

Media and growth conditions. All *Neisseria* strains were grown in GC liquid medium (GCL; 1.5% protease peptone no. 3 [Difco], 0.4% K_2HPO_4 , 0.1% KH_2PO_4 , 0.1% NaCl) amended with Kellogg supplements (22) and 0.042% sodium bicarbonate at 37°C with shaking or on GC medium plates (GCB; Difco) plus Kellogg supplements at 37°C with 5% CO_2 .

For growth curves in liquid medium, bacteria were swabbed from a 14-h-old plate and resuspended in amended GCL to an optical density at 550 nm (OD_{550})

of ≈ 0.3 . Cultures were incubated at 37°C with rotation for 2 h for *N. meningitidis* and 3 h for *N. gonorrhoeae* and *N. lactamica* and then diluted in amended GCL to an OD_{550} of 0.05 to 0.1 and grown as described above. At an OD_{550} of 0.4 to 0.5, 5 ml of culture was transferred to a 15-ml conical tube, tetracycline (Tet; 2 μ g/ml [Sigma]) was added (18), and the culture was incubated for 60 (*N. meningitidis*) or 90 min (*N. gonorrhoeae*, and *N. lactamica*) at 37°C with rotation. At each time point, the following steps occurred. (i) The OD_{550} was recorded. (ii) One milliliter of culture was transferred to an Eppendorf tube and centrifuged at 10,000 rpm for 5 min, and pelleted cells were washed with 500 μ l Tris-EDTA (TE), resuspended in 100 μ l ice-cold TE, and added to 900 μ l ice-cold 70% ethanol (EtOH) to fix. (iii) Finally, 1 ml of culture was pelleted as described above and used to extract chromosomal DNA using the QIAamp DNA minikit (Qiagen). Fixed cells were stored at 4°C. Chromosomal DNA was stored at $-20^\circ C$.

Pulsed-field gels. Plugs for the pulsed-field gels were made by swabbing 18 h old bacterial cells from a GCB plate into phosphate-buffered saline to an OD_{550} of 0.5, and the cell count was determined using a hemocytometer. Cells were spun down (4,200 rpm, 5 min) and resuspended in 37°C agarose (1% SeaPlaque GTG in water; Lonza) to a concentration of 2×10^9 cells/ml. Plugs were made with a Bio-Rad plug maker using 100 μ l resuspended cells per plug. Once solidified, plugs were incubated in 30 ml lysis buffer (6 mM Tris-Cl [pH 7.6], 100 mM EDTA, 1% Sarkosyl, 20 μ g/ml DNase-free RNase [Promega]) at 37°C for 18 h. Plugs were transferred to 2.5 ml ESP (1% Sarkosyl, 1 mg/ml proteinase K, 500 mM EDTA [pH 8.0]) and incubated at 50°C for 48 h. Plugs were then incubated twice in TE plus 1 mM phenylmethylsulfonyl fluoride (PMSF) for 2 h, 25°C. Plugs were then incubated 3 times in TE for 1 h at 25°C. Plugs were stored in TE at 4°C.

For partial digests, plugs were digested in a 500- μ l final volume of 1 $\times$ buffer with I-CeuI (NEB) undiluted or at dilutions of 1:100, 1:200, 1:500, or 1:1000 as well as a control with no enzyme added. After digestion, plugs were immediately loaded onto a 0.8% pulsed-field certified agarose (Bio-Rad) gel in 1 $\times$ TAE (Tris-acetate-EDTA) at 14°C and run on a CHEF DR III (Bio-Rad) under conditions adapted from Okada et al. (37): block 1, 1,200- to 1,800-s pulse ramp, 72 h, 2 V/cm, and 106° angle; and block 2, 40.9- to 146-s pulse ramp, 8 h 40 min, 6 V/cm, and 120° angle.

For complete digestion of genomic DNA, plugs were digested in a 250- μ l final volume of 1 $\times$ buffer plus appropriate restriction enzyme (NEB) at 37°C for 20 to 24 h. Digested plugs were melted at 65°C prior to loading onto a 1% pulsed-field certified agarose gel in 0.5 $\times$ Tris-borate-EDTA (TBE) at 14°C and run on a CHEF DR III under the following conditions: block 1, 6- to 13-s pulse ramp, 16 h, 5 V/cm, and 120° angle; and block 2, 13- to 30-s pulse ramp, 8 h, 5 V/cm, and 120° angle.

Southern blot analysis was performed by transfer of DNA to a Magnagraph nylon membrane (Osmonics) and hybridization with digoxigenin (DIG)-labeled dUTP probes and chemiluminescent substrate from the DIG system (Roche Applied Science), according to the manufacturer's instructions.

Thin-section EM. For thin-section electron microscopy (EM), gonococcal cells were grown to mid-log phase (OD_{550} of 0.5) and either processed immediately or treated with tetracycline (2 μ g/ml) as described and then processed. The modified Ryter-Kellenberger (RK) fixation procedure was adapted from Hobot et al. (20). Cells in culture medium were prefixed in 0.1% OsO_4 for 30 min and then centrifuged (2,000 rpm, 5 min). Pelleted cells were scraped out of the tube and put into a thin layer of agar (2% Difco agar and 1% tryptone in RK Veronal-acetate buffer, pH 6.0 (0.14 M sodium Veronal [barbitone sodium], 0.14 M sodium acetate [hydrated], 0.58 M sodium chloride, 10 mM calcium chloride) (14) prior to solidifying. Agar blocks (<1 mm 3) containing cells were fixed in 1% OsO_4 in RK Veronal-acetate buffer at room temperature for 20 h. Blocks were washed with RK Veronal-acetate buffer, postfixed with 0.5% uranyl acetate in RK Veronal-acetate buffer for 2 h, washed again, dehydrated with a series of ethanol solutions, and embedded in Epon.

Thin sections were cut with a Leica Ultracut microtome and stained with uranyl acetate and lead citrate. Sections were examined by transmission EM (TEM) using a JEOL JEM-1220 transmission electron microscope.

Transformation assays. For dual-antibiotic assays, chromosomal DNA from FA1090*cat* and FA1090*erm* were used to transform strains FA1090*erm* and FA1090*cat*, respectively. Liquid gonococcal transformations were performed as described by Long et al. (30). Transformants were selected on 0.5 μ g/ml chloramphenicol and 0.5 μ g/ml erythromycin, either singly or combined. Transformants from dual-antibiotic plates were passaged twice onto dual-antibiotic selection, and stably-maintained transformants were stored at $-80^\circ C$.

For *imp* assays, pMB25 was used to transform FA1090, and transformants were selected on 40 μ g/ml kanamycin. Transformants were passaged twice on

kanamycin-containing plates, and stably maintained transformants were stored at -80°C .

Flow cytometry and fluorescent microscopy. Flow cytometry and microscopy were performed exactly as described by Tobiasson and Seifert (46), except that gonococcal, meningococcal, and *N. lactamica* cells were used.

DNA microarray analysis. Microarrays and subsequent analysis were performed as described by Tobiasson and Seifert (46), except that meningococcal strain Z2491 was used to provide chromosomal DNA from exponentially growing and antibiotic-treated cultures to label for genomic microarray analysis.

RESULTS

Gonococcal chromosomes exist as monomers in separate nucleoid regions. Our previous work demonstrated that the gonococcus has more than one complete chromosome copy, and the data fit best with the model that there is a minimum of two complete chromosomes per coccal cell unit that replicate in unison. These data could be consistent with there being separate diploid chromosomes or with the two chromosomes being linked together as a dimer (46). To determine whether the gonococcal chromosome exists as two monomers or a dimer, pulsed-field gel electrophoresis (PFGE) was used to measure the size of DNA molecules within the gonococcal cell. Although several groups have claimed that circular, bacterial chromosomal DNA is resolved by PFGE (37, 42), there was no evidence that the band observed by PFGE in these studies was circular DNA and not linear. Moreover, Lartigue et al. have shown that separation of supercoiled, circular chromosomal DNA is not possible by PFGE, and uncut circular chromosomes remain in the agarose plug (28). *N. gonorrhoeae* cells were embedded in agarose plugs and gently lysed. A high-molecular-weight band that was approximately the size of the linear chromosome was always observed in these bacterial samples even with no other treatment. To test whether this band was the circular or linear DNA, the agarose plugs were treated with plasmid-safe ATP-dependent DNase (PS-DNase), which only digests linear DNA and will not digest circular or nicked DNA. The linear-sized band was digestible by PS-DNase, showing it carries a double-strand break and represents a broken, linear chromosome (Fig. 2).

A gonococcal chromosome dimer would be 4.6 Mb, and large linear DNA molecules up to 6 Mb in length are readily separated by PFGE. However, cutting a circular dimer with an enzyme that cuts once in the genome would result in two monomers and would not allow us to determine whether the *N. gonorrhoeae* chromosomes exist as a dimer. Therefore, a partial digest was performed using I-CeuI, a restriction enzyme that specifically cleaves at rRNA sequences to produce four fragments from the gonococcal genome (Fig. 2A). Partial I-CeuI digestion of agarose plugs containing *N. gonorrhoeae* chromosomal DNA revealed that there was no 4.6-Mb band corresponding to a dimer, even when weak signals were enhanced through Southern blot analysis, proving that the chromosomes only exist as 2.3-Mb monomers (Fig. 2B).

To directly observe how the chromosomes were arranged within the cell, thin-section electron microscopy was employed to observe nucleoids. Both exponentially growing *N. gonorrhoeae* and tetracycline-treated *N. gonorrhoeae*, in which the initiation of chromosomal replication is inhibited and completely replicated chromosomes result (46), were examined by thin-section transmission electron microscopy (TEM) after fix-

FIG. 2. Analysis of I-CeuI partial digestion of gonococcal chromosomal DNA. (A) Diagram of I-CeuI cleavage sites on the FA1090 chromosome; (B) pulsed-field gel and corresponding Southern blot of I-CeuI digests. The Southern blot probe specifically hybridizes to the largest I-CeuI digestion fragment. Lane 1, *Schizosaccharomyces pombe* marker; lane 2, *Hansenula wingei* marker; lane 3, plasmid-safe DNase digestion of uncut chromosomal DNA; lane 4, complete I-CeuI digestion of chromosomal DNA; lanes 5 to 8, partial I-CeuI digestion; lane 9, undigested chromosomal DNA; lane 10, chromosomal DNA plug stored exclusively in TE. Transfer of large, possibly uncut DNA fragments from the wells onto the blot membrane varied as these large pieces of DNA often remained trapped in the agarose during the blotting process.

ing with osmium tetroxide and uranyl acetate to allow visualization of the nucleoids. Thin-section EM showed that exponentially growing FA1090 contained multiple nucleoid regions with an average of three regions per coccal unit (3.007 nucleoids/coccus; $n = 272$ cells) (Fig. 3A). Diplococcal cells had a membrane clearly separating the coccal units (Fig. 3A). Tet-treated cells had one large nucleoid region (1.265 nucleoids/coccus; $n = 155$ cells) and no separation between coccal units in diplococcal cells (Fig. 3B). Similar results were obtained for strain MS11 (data not shown). These findings suggest that the monomeric chromosomes could reside in separate nucleoid regions within the gonococcal cell, and that after Tet treatment and inhibition of protein synthesis, these separate nucleoids collapse into a single nucleoid region.

Gonococci are homozygous. The polyploid nature of the gonococcus leads to the question of whether different alleles of a gene can exist in the same cell producing heterozygous bacteria. Two independent genetic strategies were used to test the possibility that heterozygous populations of *N. gonorrhoeae* exist. In the first approach, we attempted to force two different antibiotic resistance cassettes in the same locus using dual selection. To accomplish this goal, we employed the *Neisseria* intergenic complementation site (*nics*) (32), which has extensively been used for the ectopic complementation of mutants. Two different plasmid clones containing the *nics* site and its flanking genes (*lctP* and *aspC*) with different antibiotic resistance markers at the identical DNA location were used to

FIG. 3. Thin-section electron microscopy of exponentially grown (A) and tetracycline-treated (B) gonococcal cells. Representative images of monococci, dividing monococci, and diplococci are shown. The scale bar shown is the same for all images. All samples were treated and stained in an identical manner, and unstained portions of each cell contained DNA located in condensed nucleoids.

construct single isogenic gonococcal strains with each of the antibiotic markers at the same chromosomal locus. Transformation of chromosomal DNA from each strain into the other resulted in double-antibiotic-resistant transformants that arose at a frequency that was 1 to 2 logs lower than that of resulting transformants grown on single selection and produced small colonies (data not shown). A majority of these double-antibiotic-resistant transformants would not passage onto solid media containing both antibiotics. However, a few stable double-antibiotic-resistant transformants were obtained, using either of the donor DNAs. Southern blot analysis of PFGE gels revealed that all stable double transformants had insertions of the donor antibiotic resistance marker into a chromosomal locus that was distinct from the *nics* locus (Fig. 4). However, the precise site used for insertion into this locus was not determined. To test whether homologous recombination between chromosomes might influence the ability of *N. gonorrhoeae* to maintain heterozygous chromosomes, the RecA recombinase was transiently expressed during transformation using a strain containing an inducible *recA* gene, and then expression was either maintained or repressed during selection for transformants. No difference in stable transformation efficiencies was observed between parental and *recA*-inducible strains, showing that the ability to perform homologous recombination after transformation does not alter the frequency that heterozygous alleles can be maintained (Fig. 4). Each antibiotic resistance marker being transformed into the cell consistently ended up at a second site on the chromosome, which

does not have extensive homology to the *nics* locus or the flanking DNA sequences (not shown). Therefore, two different antibiotic resistance genes cannot be maintained in the *nics* locus using antibiotic selection.

An independent genetic test for the possibility of heterozygous bacteria was performed using a loss-of-function mutation of an essential *N. gonorrhoeae* gene. The *imp* gene encodes an

FIG. 4. Southern blot analysis of PFGE of dual-antibiotic-resistant transformants. The blot was probed with the erythromycin resistance gene (*erm*) probe, stripped, and probed with a chloramphenicol acetyltransferase (*cat*) probe. Lane 1, FA1090; lane 2, FA1090*erm-cat* control; lane 3, FA1090*erm*; lane 4, FA1090*cat*; lane 5, FA1090*erm* DNA transformed into FA1090*cat*; lane 6, FA1090*cat* DNA transformed into FA1090*erm*; lane 7, FA1090*recA6erm* DNA transformed into FA1090*recA6cat*; lane 8, FA1090*recA6cat* DNA transformed into FA1090*recA6erm*.

FIG. 5. Southern blot analysis of PFGE of *imp::kan* transformants. The blot was probed with an *imp* gene probe. Lanes 1 to 4, BglII-digested chromosomal DNA; lanes 5 to 8, ClaI-digested chromosomal DNA. FA1090 (lanes 1 and 5), FA1090 *imp::kan* transformants (lanes 2 to 4 and 6 to 8).

outer membrane protein important for lipopolysaccharide (LPS) transport, and attempts to inactivate *imp* in *N. gonorrhoeae* have resulted in a cell carrying a wild-type copy and mutated copy of *imp* (4). It was possible that these transformants either were heterozygotes with a wild-type copy of *imp* on one chromosome and a mutated copy on the other or that these transformants represent merodiploids in which the wild-type and mutant forms of *imp* are present on each chromosome. Transformation of *imp::kan* into *N. gonorrhoeae* resulted in many unstable kanamycin-resistant (Kan^r) transformants that would not produce progeny colonies upon passage on Kan. Three stable Kan-resistant mutants were obtained, and each stable transformant carried both mutant and parental versions of *imp*, as assessed by PCR (data not shown). Southern blot analysis of PFGE-separated chromosomal fragments revealed that the *imp::kan* insertion had recombined into a chromosomal locus that was not near the *imp* gene (Fig. 5). Therefore, these transformants are *imp* merodiploids that were produced by a rare recombination event and were not the result of there being two *imp* loci on different chromosomes within the same cell. These genetic tests show that gonococci cannot be forced to be heterozygous but exist exclusively as haploid, homozygous populations.

***N. meningitidis* is polyploid.** It is unknown why some bacteria have evolved polyploidy, but we can assume that the selective pressures to maintain polyploidy must be important for the organism's continued existence. We have proposed that *N. gonorrhoeae* polyploidy may be important for pilin antigenic variation and also may contribute to other aspects of gonococcal pathogenicity. To test whether polyploidy is correlated with antigenic variation and pathogenicity, the DNA content of two close relatives of *N. gonorrhoeae*, the pathogenic *N. meningitidis* and the commensal *N. lactamica*, were examined. All three species have similar size genomes and % GC content. The three *Neisseria* species examined had different growth rates in liquid medium, with *N. meningitidis* showing the fastest growth rate (40-min doubling time), *N. gonorrhoeae* showing the intermediate growth rate (60-min doubling time, as previously observed) (46), and *N. lactamica* showing the slowest growth

rate (80-min doubling time). Since all *Neisseria* species can exist as monococcal and diplococcal cells, microscopic analysis was performed to determine whether there are differences in the percentages of monococcal and diplococcal cells between these three species. There were no significant differences between the percentages of monococci and diplococci between these species (Table 1).

Total DNA content was analyzed using flow cytometry of Hoechst-stained cells (both mono- and diplococci) of the three *Neisseria* species, with DNA content being determined by measuring the fluorescence of each cell and calculating the DNA content by comparing the fluorescence to that of *E. coli* standards with intact chromosomes and adjusting for the difference in genome sizes between *E. coli* and the *Neisseria* spp. (46). Analysis of two different strains of *N. meningitidis* representing the A and B serogroups showed that each population of exponentially growing *N. meningitidis* cells had a greater amount of DNA per cell than *N. gonorrhoeae*, and this level of DNA was observed throughout the growth phase of *N. meningitidis* (Fig. 6B) (data not shown). While the exponentially growing *N. gonorrhoeae* cells carried from two to four genome equivalents, a majority of *N. meningitidis* cells showed three to five genome equivalents. In contrast, the commensal *N. lactamica* carried less DNA per cell than either *N. gonorrhoeae* or *N. meningitidis*, with the majority of exponential cells showing between one and four genome equivalents and with a distinct 2N population being readily apparent.

Treatment with sublethal levels of Tet inhibits new rounds of replication but allows replication to continue to produce fully replicated chromosomes (46). Tet treatment of *N. gonorrhoeae* resulted in a majority 4N population, with a smaller 2N population and a very small 8N population (Fig. 6B and Table 2). The 2N *N. gonorrhoeae* population is most likely newly divided monococci that have not initiated replication. The 4N population would represent two distinct types of cells: diplococci that have not initiated replication and monococci in which the two chromosomes had initiated replication and completed that round of replication to produce the 4N DNA content. Tet treatment of *N. meningitidis* also produced a majority 4N population and a minority 8N population larger than the *N. gonorrhoeae* 8N population (Fig. 6D). The lack of 2N population is consistent with the faster growth rate of *N. meningitidis* producing fewer cells delaying the initiation of replication. In contrast to *N. gonorrhoeae* and *N. meningitidis*, Tet treatment of *N. lactamica* resulted in two relatively equal 2N and 4N populations and also a 1N population (Fig. 6 and Table 2). This pattern of cell content is most consistent with *N. lactamica* being monoploid. The 1N population represents monococci

TABLE 1. Proportion of monococcal and diplococcal forms by microscopic analysis

Species ^a	% monococci	% diplococci ^b
<i>N. gonorrhoeae</i>	60	35
<i>N. meningitidis</i>	45	51
<i>N. lactamica</i>	51	41

^a The following numbers of cells were counted from each species: *N. gonorrhoeae*, 90; *N. meningitidis*, 273; and *N. lactamica*, 134.

^b We cannot differentiate between true diplococci and dividing monococci in these analyses.

FIG. 6. Flow cytometry of Hoechst-stained, exponentially grown and tetracycline-treated *N. gonorrhoeae* (Ng), *N. meningitidis* (Nm), and *N. lactamica* (NI). For each histogram, the x axis represents fluorescence levels, which indicate the amount of DNA per particle counted. Cell counts or particle counts are shown in the y axis. Genome equivalents were determined from stationary phase and rifampin-treated *E. coli* cells (data not shown).

that have not initiated replication, the 2N population represents monococci that had initiated replication and completed it after Tet treatment, and the 4N population represents diplococci with too complete chromosomes in each coccal unit of

the diplococcus. From these analyses, we conclude that *N. lactamica* is not polyploid.

The increased genomic content of *N. meningitidis* could have resulted from multiple initiation events on a single replicon to allow for growth that is faster than a single round of DNA replication can support. To assay initiation at the origin of replication, the marker frequency of genes at the origin and terminus was determined using microarray analysis. Genomic DNAs from both exponentially grown *N. meningitidis* and Tet-treated *N. meningitidis* were fluorescently labeled and hybridized to a pan-*Neisseria* microarray (J. Davies et al., unpublished observations). The results from duplicate hybridizations were averaged, and the relative intensity of the hybridization of DNA from exponentially grown *N. meningitidis* to DNA from Tet-treated *N. meningitidis* was plotted relative to the gene

Species	Analysis of genome copy number by flow cytometry							
	% of binned cells at genome equivalent shown:							
	Exponentially grown				Tetracycline treated			
	1	2	4	>4	1	2	4	>4
<i>N. gonorrhoeae</i>	0	17	65	12	0	10	64	21
<i>N. meningitidis</i>	0	1	54	39	0	2	55	31
<i>N. lactamica</i>	4	32	32	17	6	38	26	16

FIG. 7. Microarray analysis of *N. meningitidis* showing marker frequency. Hybridization signals of labeled untreated and tetracycline-treated exponentially grown *N. meningitidis* were determined, and the $\log_2$ ratio of the medians was plotted versus gene number along the Z2491 chromosome. A polynomial regression trend line was produced with an order of 4.

number on the genomic sequence (Fig. 7). A polynomial regression curve was fitted to the data to represent the average relative gene dosage around the chromosome, and a single peak was observed, indicating that the *N. meningitidis* chromosome has a single origin of replication. The region of the *N. meningitidis* genome where the most replication was observed (i.e., near the origin of replication) coincided with the location where computer prediction programs suggest the origin would occur (data not shown). Moreover, the region of the *N. meningitidis* genome that is predicted to carry the terminus of replication contains the *dif* site, which is associated with other bacterial termini (data not shown). The hybridization ratio of *N. meningitidis* sequences near the origin of replication versus the terminus was 1.4:1, which is identical to that previously measured for *N. gonorrhoeae* using the same technique (46). From this analysis, we conclude that there are not multiple initiation events on a single chromosome and that the increased DNA content of *N. meningitidis* is due to polyploid chromosomes.

DISCUSSION

This work extends our understanding of the polyploid nature of the pathogenic neisseriae. Our previous report about gonococcal polyploidy established that there is more than one chromosome copy per coccal cell unit, and the average measure of DNA at the terminus suggested that the minimal chromosomal copy number was two complete copies that replicated to form four copies before cell division (46). We have firmly established that gonococcal chromosomes exist as unit molecules, which segregate to form homozygous populations of cells. Since most of the replication genes found in other bacteria that do not possess polyploid genomes are found in the *Neisseria* genomes, we predict that the factors that allow multiple copies of the chromosome may not be unique to the pathogenic *Neis-*

seria but rather result from an adaptation of conserved mechanisms to increase the copy number control.

All three *Neisseria* species examined in this work showed a mixture of monococcal and diplococcal cell forms. It has never been defined how a mixture of monococcal and diplococcal forms can be produced from a single progenitor organism. Since neisseriae are known to express adhesins that mediate bacterial to bacterial cell adherence (e.g., pili and Opa proteins), it is possible that every culture is initiated from more than one precursor cell and that the both monococci and diplococci are always present in the precursor population. Alternatively, the mixture of monococci and diplococci could result from asymmetric cell division processes within a culture of cells, and we have observed asynchronous cell division events in gonococcal cultures (unpublished observations). One problem with determining the number of diplococcal cells is the difficulty in differentiating actual diplococci from dividing monococci by optical microscopy or whole-mount electron microscopy. However, the examination of thin sections by TEM clearly allowed us to differentiate true diplococci with a septum from dividing monococci that do not have a detectable septum. A surprising observation made by the thin-section EM was that none of the gonococcal cells had a visible septum after tetracycline treatment, even though this level of antibiotic is sublethal and allows growth after removal of the antibiotic (18; data not shown). A more comprehensive examination of the cellularity of the neisseriae and the growth patterns will be required to fully understand the factors involved in the diplococcal form and its relationship to genome content.

The visualization of nucleoids by thin-section EM showed that most growing coccal units (either monococci or individual halves of a diplococcus) had on average three distinct nucleoid regions. These regions could represent separate nucleoids carrying separate chromosomal copies or alternatively could represent different lobes of a single nucleoid that curves around the inside of the cell but are sectioned in a direction perpendicular to the nucleoid mass. In some thin sections, we did observe curved nucleoids consistent with this interpretation, but not at a high enough frequency to account for all of the separate nucleoid regions observed. Interestingly, these separate nucleoid regions coalesced into a single nucleoid region after sublethal treatment with antibiotics, suggesting that the separate nucleoid regions represent structures maintained only in growing bacteria. Unfortunately, the relatively small size of the *Neisseria* cells (about 0.5 μm in diameter) makes differentiating separate nucleoids in live cells by standard fluorescence microscopy difficult.

The lack of any detectable dimers in the CHEF gel analysis (Fig. 2) demonstrates that there are functions that prevent dimer formation during replication or by way of recombination. *N. gonorrhoeae* possesses the components of the XerCD recombination system that is involved in resolution of chromosome dimers, and these data along with other reports show that this system is functional in *N. gonorrhoeae* (16, 41). We predict that the XerCD system helps to resolve dimers formed during replication. The reason for the absence of dimers formed by recombination is less obvious, but it may reflect the fact that the sister chromosomes reside in distinct nucleoid compartments and thus are unable to undergo recombination during a normal growth and replication program.

Transformation is the main means of genetic exchange within and between *Neisseria* spp. (3). Moreover, hundreds of genes have been successfully mutated in the neisseriae, usually without having a copy of the parental and mutant versions of the gene in the same cell. Only when the mutated gene encodes an essential function have two different copies of the gene been observed in the same cell (6, 45). As modeled in Fig. 1, either random chromosome segregation or directed chromosome segregation can lead to homozygous populations of cells, while only directed segregation similar to that in eukaryotic cells will lead to heterozygosity. The appearance of unstable dual-antibiotic-resistant transformants in our attempt to make heterozygotes strongly supports a random segregation model in which the two resistance genes can coexist for a number of generations but cannot coexist long enough to form a stable heterozygote.

Maintaining heterozygous cells may not be evolutionarily advantageous for *N. gonorrhoeae*. Mutations conferring a benefit for *N. gonorrhoeae* would be masked by the parental version of the gene, and dominant mutations could mask the normal gene copy. In addition, antigenic and phase variation of gonococcal surface molecules would be affected by heterozygosity. For example, during antigenic variation new pilin molecules are formed, and expression of a new pilin protein along with the original pilin may not lead to immune evasion, as the original pilin molecule would still be expressed. Heterozygous *N. gonorrhoeae* may be a viable option if differential silencing of genomic DNA occurred such that only one allele was expressed per cell. However, continual silencing of one genome's activity while the other genome in a polyploid bacterium is transcriptionally active has not been observed in bacterial cells and without the presence of chromatin may not be possible. We suspect that the benefits of a homozygous genome outweigh any advantage that a heterozygous genome would contribute to a unicellular organism.

The hypothesis that polyploid genomes are involved in *Neisseria* pathogenesis can be inferred by our current data, as within the three species examined, only pathogenic *Neisseriae* species are exclusively polyploid. Since *N. lactamica* and *N. meningitidis* both reside in the human nasopharynx, the differences in their genome copy numbers cannot be from selective pressures contributed by the different environmental niches inhabited by these different *Neisseria* spp. As a commensal organism, *N. lactamica* may not be exposed to either the innate or adaptive immune systems. We have proposed that the evolution and maintenance of multiple chromosome copies per gonococcal cell are to allow the gene conversion reactions mediating the high-frequency pilin antigenic variation system (9). Both the gonococcus and meningococcus undergo pilin antigenic variation (15, 36), while the commensal *Neisseria* strains do not carry the silent pilin copies required for this diversity-generating system (43). Additionally, both gonococci and meningococci elicit an innate response consisting mainly of neutrophils (38), which can elaborate antimicrobial responses that can damage DNA, but these oxidative responses are ineffective in killing *Neisseria* (8, 44). It is not known whether the commensal neisseriae elicit an inflammatory response, but the prevailing assumption is that they are not perceived by the innate immune system as being foreign and therefore do not elicit inflammation. Having two copies of a

bacterial chromosome should make the bacteria more resistant to DNA damage and could be a contributing factor to the evolution and maintenance of polyploidy. It is noteworthy that two other polyploid organisms, *Borrelia hermsii* and *Deinococcus radiodurans*, either undergo antigenic variation (*Borrelia*) (33) or have robust DNA repair capabilities (*Deinococcus*) (34), which suggests that each of these selective pressures may be enough to evolve polyploidy in bacteria. It is unknown whether many other bacterial species are also polyploid, and determining the genomic content of diverse bacterial species should provide a framework to understand the reasons behind polyploidy in bacteria.

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