

Neisseria infection of rhesus macaques as a model to study colonization, transmission, persistence, and horizontal gene transfer

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The strict tropism of many pathogens for man hampers the development of animal models that recapitulate important microbe–host interactions. We developed a rhesus macaque model for studying *Neisseria*–host interactions using *Neisseria* species indigenous to the animal. We report that *Neisseria* are common inhabitants of the rhesus macaque. *Neisseria* isolated from the rhesus macaque recolonize animals after laboratory passage, persist in the animals for at least 72 d, and are transmitted between animals. *Neisseria* are naturally competent and acquire genetic markers from each other in vivo, in the absence of selection, within 44 d after colonization. *Neisseria macacae* encodes orthologs of known or presumed virulence factors of human-adapted *Neisseria*, as well as current or candidate vaccine antigens. We conclude that the rhesus macaque model will allow studies of the molecular mechanisms of *Neisseria* colonization, transmission, persistence, and horizontal gene transfer. The model can potentially be developed further for preclinical testing of vaccine candidates.

commensal *Neisseria* | nonhuman primate model | type IV pili

Microbe–host interactions are often refractory to investigation because of the exquisite tropism of many bacteria for the human host. Host-restriction has prevented the development of animal models that recapitulate colonization, transmission, and persistence; hampered studies of horizontal gene transfer (HGT); and prevented preclinical testing of vaccine candidates. One means to circumvent host restrictions is to develop an animal model of infection using a related species indigenous to that animal. For example, infections by human pathogens, such as *Bordetella pertussis* and *Chlamydia trachomatis*, have been effectively modeled in animals with *Bordetella bronchiseptica* and *Chlamydia muridarum*, respectively (1, 2).

The genus *Neisseria* and the rhesus macaque provide us with this opportunity. *Neisseria* are Gram-negative, diplococcal β -Proteobacteria. This genus includes >10 human-adapted species, two of which are pathogens, and an unknown number of species that naturally colonize animals. These species are highly related: >40% of the genes in pathogenic species are also present in commensals, and many of these encode virulence genes and antibiotic resistance alleles. The species are naturally competent and transfer genetic information to each other in vitro (3, 4). HGT is widely assumed to play an important role in the spread of antibiotic resistance among pathogenic *Neisseria*; however, there is little information on the extent to which HGT occurs in vivo.

Pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae* have an exquisite tropism for man. These pathogens do not naturally infect animals; inoculated into nonhuman hosts, the pathogens will colonize only for brief periods (5, 6). *Neisseria* species have been isolated from a number of animals (7–10), suggesting it

may be possible to develop a model for studying *Neisseria*–host interactions in which there are no host-restriction barriers to overcome.

The rhesus macaque (RM) has ~93% DNA sequence identity to man, is similar physiologically and anatomically to man, and expresses orthologs of many human receptors for the pathogenic *Neisseria*. *Neisseria macacae*, isolated from the RM oropharynx, is closely related to human commensals *Neisseria sicca* and *Neisseria mucosa* (11, 12). We explored the feasibility of developing a RM model for studying *Neisseria*–host interactions using species indigenous to this animal.

We report that *N. macacae* encode many orthologs of virulence factors and vaccine antigens of *N. meningitidis* and *N. gonorrhoeae*. At least two species of *Neisseria* inhabit the RM oropharynx. RM *Neisseria* persist for at least 6 y in their monkey hosts; they recolonize animals after laboratory passage and are transmitted between animals. Finally, RM *Neisseria* are naturally competent and transfer genetic markers to each other shortly after inoculation, in the absence of selection. We conclude that the RM model provides an excellent opportunity for studying commensal *Neisseria* colonization, transmission, long-term persistence, and HGT. This model has the potential for allowing a better understanding of asymptomatic carriage by pathogenic *Neisseria* (13, 14). We discuss the promise of this animal model for preclinical testing of vaccine antigens and persistence of antibiotic resistant determinants in a population.

Results

RM *Neisseria* Encodes Orthologs of Virulence Factors and Vaccine Antigens. *N. macacae* was isolated from a RM in 1983 (12). A BLASTP search of its genome revealed genes encoding closely related orthologs of 61 of 70 factors hypothesized or demonstrated to play a role in virulence of human pathogenic *Neisseria*,

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Data deposition: The sequences reported in this paper have been deposited in the National Center of Biotechnology Information GenBank database, www.ncbi.nlm.nih.gov/RefSeq [accession nos. JX549156–JX549186 (abcZ); JX549187–JX549217 (adk); JX549311–JX549341 (aroE); KC422439–KC422440 (fHbp); JX549218–JX549248 (fumC); JX502556–JX502601 (gdh); JX549249–JX549279 (pdhC); JX549280–JX549310 (pgm); KC422436–KC422438 (pilE); JX549153–JX549155 (rpoB); and JX549150–JX549152 (rpsL)].

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including proteins involved in the biogenesis of the type IV pilus (Tfp), an appendage that functions in DNA transformation, colonization, motility, and host cell signaling (Tables S1 and S2). Also present are genes encoding current meningococcal vaccine components such as fHbp, GNA1030, GNA2091, NadA, and Porin P1 (Table S3), and promising vaccine antigens, such as LctP (15). These findings lend additional support to previous reports indicating the genetic relatedness of *N. macacae* to human-adapted *Neisseria*.

Survey Reveals Multiple *Neisseria* Species Colonize RMs. We determined whether *Neisseria* are common inhabitants of RMs. In 2003, we surveyed 23 RMs at the Oregon National Primate Research Center. Seventeen animals were culture-positive for *Neisseria* (SI Materials and Methods). Five culture-negative RMs had been treated previously with enrofloxacin (Baytril) for a Gram-negative infection. Thus, *Neisseria* are a common inhabitant of the RM.

Forty RM-derived *Neisseria* were typed for their housekeeping gene *gdh* (16), an approach used successfully to group *Neisseria* species into monophyletic clusters (17) (Table S4). A comparison of their *gdh* sequences with those from human *Neisseria* species and *N. macacae* allowed us to construct a phylogenetic tree (Fig. 1). The *gdh* alleles of RM *Neisseria* clustered primarily at two locations in the tree, which we term cluster one and cluster two. Cluster one isolates were closely related to *N. macacae* and human commensals *N. sicca* and *N. mucosa*. Cluster two isolates fall between the cluster one strains and *N. elongata*, a human commensal distantly related to human pathogens *N. meningitidis* and *N. gonorrhoeae* (4, 11, 18). These results suggest that multiple species of *Neisseria* colonize RMs.

Different sites in the same animal were colonized with *Neisseria* bearing an identical *gdh* allele (Fig. 1 and Table S5). Six of 16 (37%) RMs were colonized with two *Neisseria* strains bearing distinct *gdh* alleles (Table S5). RMs housed together were frequently colonized with *Neisseria* bearing the same *gdh* alleles (Fig. 1). For example, four animals in housing group A were colonized with strains bearing *gdh* alleles 1 and 2 (cluster one) (Fig. 1). Two of six animals in housing group C were colonized with strains bearing *gdh* allele 5, and three had strains with *gdh* alleles 9 or 11 (cluster one and cluster two; groups A and C RMs) (Fig. 1). Thus, the same *Neisseria* strains can colonize multiple sites in an animal, and multiple strains can simultaneously colonize an animal.

In 2009, the same animals (when possible) were surveyed again. In both surveys group A animals were colonized with *Neisseria* bearing *gdh* alleles 1 and 2. In 2003, group B animals carried *Neisseria* with alleles 7, 10, and 11; however, in 2009 they only carried *Neisseria* with allele 10. Only three group C animals were available for the 2009 survey. Two of three RMs had isolates with different *gdh* alleles in the two studies. Thus, *Neisseria* bearing the same *gdh* alleles can persist in a population of animals for as long as 6 y. Whether they were carried continuously by the same animals or lost and reacquired by the animals over time cannot be distinguished in this study.

The *gdh* gene tree in Fig. 1 suggests the existence of multiple *Neisseria* species in RMs. We performed multilocus sequence typing (MLST) to obtain further evidence for this hypothesis. MLST analysis resolved *Neisseria* into at least two clusters similar to the *gdh* gene tree (Fig. 2). RM *Neisseria* species include those closely related to human commensal species *N. mucosa* and *N. sicca* and at least one other group more distantly related to human *Neisseria* species. This result suggests that at least two species of *Neisseria* colonize RMs. Like humans, RMs are often colonized simultaneously with multiple species of *Neisseria*, and these bacteria can be acquired and lost with time (19–22).

Characterization of RM *Neisseria* Species for Natural Competence.

Human-adapted *Neisseria* are competent for DNA transformation and engage in intra- and interspecies HGT in vitro (3, 4,

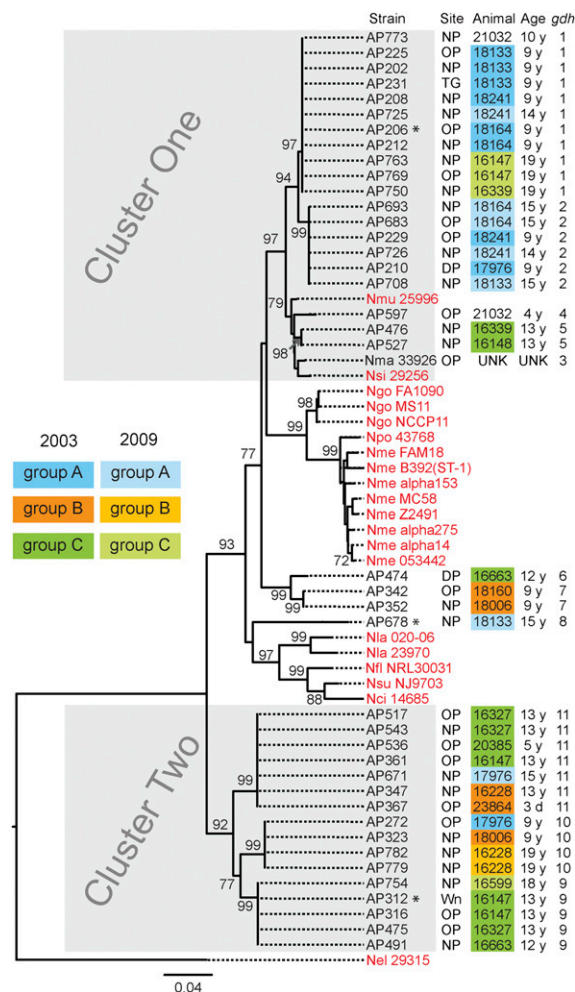
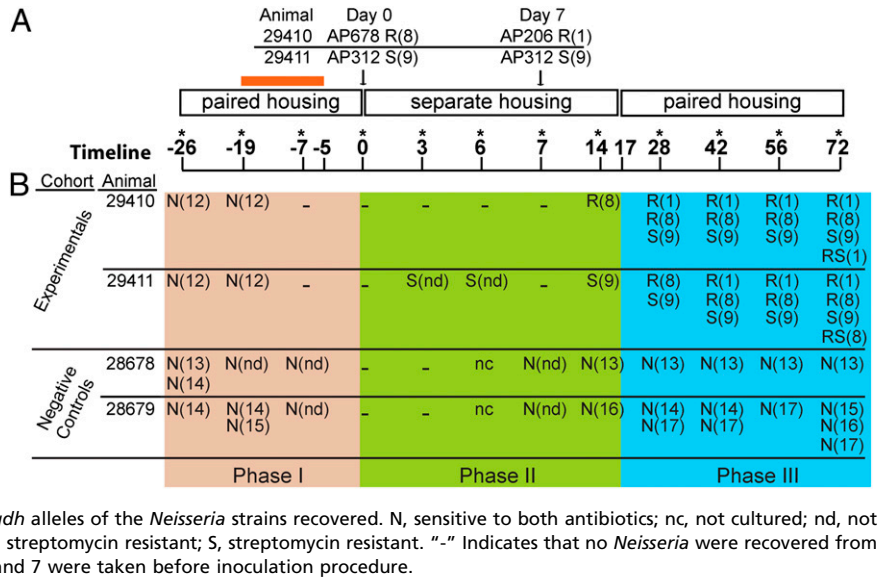


Fig. 1. *gdh* sequences resolve RM *Neisseria* into two clusters. A rooted neighbor-joining tree constructed with *gdh* sequences from 40 RM *Neisseria* isolates (present study), *N. macacae* (Nma; ATCC 33926), and 20 human-adapted *Neisseria* (in red). Shown for each RM *Neisseria* strain are its strain number, its site of isolation, the animal from which it was cultured, the age of the animal, and its *gdh* allele number. Cluster one and cluster two RM *Neisseria* strains are in gray boxes. Housing groups of the animals are color coded: blue, group A; orange, group B; and green, group C. *Neisseria* strains cultured from animals in the same housing group is identified by the housing group color. Darker shade of a color indicates the 2003 survey; lighter shade indicates the 2009 survey. Three strains marked with an asterisk were inoculated into animals during this study. DP, dental plaque; Nci, *N. cinerea* (ATCC 14685); Nel, *N. elongata* (ATCC 29315); Nfl, *N. flavescens* (NRL30031); Ngo, *N. gonorrhoeae* (FA1090, MS11 and NCCP11); Nla, *N. lactamica* (20-060, ATCC 23970); Nme, *N. meningitidis* (FAM18, B392, α 153, MC58, Z2491, α 275, α 14, and 053422); Nmu, *N. mucosa* (Nmu ATCC 25996); NP, nasopharynx; Npo, *N. polysaccharaea* (Npo ATCC 43768); Nsi, *N. sicca* (ATCC 29256); Nsu, *N. subflava* (NJ9703); OP, oropharynx; TG, tongue; Wn, wound. Bootstrap values >70% are indicated (2,000 replications).

23). We determined whether RM *Neisseria* strains from different parts of the MLST tree are also naturally competent. Derivatives of AP206, AP312, and AP678 that spontaneously developed resistance in vitro to rifampicin (Rif^R) or streptomycin (Sm^R) were isolated. Their *rpoB* and *rpsL* alleles were sequenced to identify the mutations that conferred resistance to Rif or Sm, respectively. When antibiotic-sensitive RM *Neisseria* were incubated with DNA from antibiotic-resistant derivatives, they acquired resistance to the relevant antibiotic; they remained antibiotic-sensitive when incubated with DNA pretreated with DNaseI, or medium alone

Fig. 3. Animal studies of RM *Neisseria* reveal colonization, transmission, persistence, and HGT. Timeline of the experiment is given in days. Asterisk above the day indicates the time animals were cultured for *Neisseria*. (A) Listed are the inoculated animals, the RM *Neisseria* strain inoculated into each animal on days 0 and 7, the antibiotic resistance phenotype of the inoculated strain (see abbreviations below), and the *gdh* allele of the inoculated strain in parenthesis. Orange bar over timeline shows duration of enrofloxacin (Baytril) treatment. The boxed text over the timeline indicate the days when the two monkeys in each cohort were housed together (paired housing) or individually (separate housing). (B) The three phases of the experiment are blocked in different colors. Phase I: reduction of preexisting *Neisseria* from animals with Baytril. Phase II: inoculation and monitoring of colonization of inoculated strains. Phase III: monitoring persistence, transmission, and HGT. Listed below each sampling day and next to each animal are the antibiotic resistance phenotypes and *gdh* alleles of the *Neisseria* strains recovered. N, sensitive to both antibiotics; nc, not cultured; nd, not determined; R, rifampicin resistant; RS, rifampicin and streptomycin resistant; S, streptomycin resistant. “-” Indicates that no *Neisseria* were recovered from swabs collected on that day. Swabs taken on days 0 and 7 were taken before inoculation procedure.



Neither SmR nor RifR *Neisseria* was cultured from the negative control cohort. The *Neisseria* isolated from these animals had *gdh* alleles matching those in the preexisting flora. It should be noted that *Neisseria* bearing two new *gdh* alleles were cultured from the negative control cohort during this time (Fig. 3 and Table S6).

HGT in RM *Neisseria* in Vivo. *Neisseria* resistant to both Sm and Rif were cultured from several anatomical sites of both experimental animals 44 d after colonization by the inoculating strains. All SmR/RifR isolates had the *rpsL* allele from AP312-SmR and the *rpoB* allele from either AP206-RifR or AP678-RifR (Table 2). Numerous strain-specific polymorphisms in sequenced segments of *rpoB*, *rpsL*, and *gdh* of 758, 369, and 501 base pairs, respectively, allowed us to conclude that double-resistant isolates arose via HGT and not spontaneous mutation (Fig. S2). RifR, SmR, or SmR/RifR *Neisseria* were never isolated from the control animals.

Colonization of the RM Nasal Cavity. On the last day of the experiment, all animals were cultured for *Neisseria* at the inoculation sites (nose, tongue, and throat) as well as sites that had not received an inoculum. The latter sites include the epithelia covering the cribriform plate, ethmoturbinates, maxilloturbinates, and nasopharynx above the soft palate. AP312, AP206, and AP678 were cultured from both experimental animals at many of these sites (Table 3 and Table S6). We cannot rule out that the inoculation procedure may have introduced the bacteria to these locations. However, strains naturally transmitted between cage-mates had migrated to uninoculated sites high in the nasal cavity, areas thick with mucous and difficult to access. *Neisseria* was also cultured from all sites in the uninoculated controls; their *gdh* alleles matched those of the preexisting strains (Table S6).

Discussion

We have described an animal model that recapitulates several aspects of *Neisseria*–host interactions that are currently refractory to in vivo experimentation. Our system involves the infection of the rhesus macaque with *Neisseria* species indigenous to this animal. This approach circumvents host-restriction barriers, and will allow the dissection of molecular mechanisms underlying pharyngeal colonization, persistence, transmission, and HGT.

We presented evidence that *Neisseria* are a common inhabitant of the RM nasopharynx. At least two species and multiple strains of *Neisseria* naturally colonize RMs (Figs. 1 and

2). Strains with identical *gdh* alleles can persist in the same animals for extended periods (as long as 6 y) (Fig. 1), and strains can be introduced to and lost from an animal over time. Four different strains can colonize an animal simultaneously (Fig. 3). These observations closely parallel the epidemiological findings for *Neisseria* colonization in humans (19, 20). Simultaneous carriage of multiple species/strains is a prerequisite for the dissemination of antibiotic-resistant loci by HGT (see below).

Survey of RMs for *Neisseria* species found that animals housed together often shared strains bearing the same *gdh* alleles. RMs enrolled in the inoculation studies were grooming pairs. Transmission of *Neisseria* strains between animals housed together is therefore likely the result of direct contact. Transmission is unlikely to be through aerosols, as the inoculated *Neisseria* strains were never detected in the pair of negative control RMs that were housed in the same room as the inoculated animals.

RM *Neisseria* is able to recolonize animals after laboratory passage. Our study suggests there may be an “eclipse” period 7–14 d after inoculation, during which the infecting strain could not be cultured from the animal. Whether this is a bona fide characteristic of RM *Neisseria* infection is unclear, although it recalls a similar behavior of *N. gonorrhoeae* upon human inoculation (25). RM *Neisseria* naturally transmitted from one animal to another were able to colonize areas high in the nasal cavity, such as ethmoid turbinates, maxilloturbinates, the nasal meatus, and the epithelium covering the cribriform plate. The ability of RM *Neisseria* to colonize the latter site is of particular interest. It has

Table 2. *rpoB*, *rpsL*, and *gdh* alleles of RifR/SmR *Neisseria* strains isolated from experimental animals 72 d postinoculation

Strain	<i>rpoB</i> -RifR	<i>rpsL</i> -SmR	<i>gdh</i> *	Animal	Site
AP1498	206	312	206	29410	Tongue
AP1500	206	312	206	29410	Hard palate
AP1496	678	312	678	29411	Oropharynx
AP1497	678	312	678	29411	Esophagus
AP1502	678	312	678	29411	Cheek
AP1503	678	312	678	29411	Tongue
AP1504	678	312	678	29411	Nose

rpoB, *rpsL*, and *gdh* alleles of double-resistant strains matched the indicated alleles from inoculation strains AP206, AP312, and AP678.

*The recipient strain was identified by sequencing the *gdh* allele of each RifR/SmR strain.

Table 3. Sites of inoculation of rhesus macaque *Neisseria* strains at day 0 or 7, and sites from which the strains were cultured 72 d postinoculation

Strain	Animal	Resistance (<i>gdh</i>)*	Site
Rhesus <i>Neisseria</i> strains inoculated on day 0 or 7 [†]			
AP678	29410	R(8)	Nose, oral cavity
AP206	29410	R(1)	Nose, oral cavity
AP312	29411	S(9)	Nose, oral cavity
Nasal cavity isolates isolated on day 72 [‡]			
AP1430	29410	R(8)	Ethmoturbinat
AP1525	29410	S(9)	Ethmoturbinat
AP1506	29410	R(8)	Maxilloturbinat
AP1523	29410	S(9)	Maxilloturbinat
AP1519	29410	S(9)	Nasal meatus
AP1528	29410	R(8)	Nasal meatus
AP1529	29410	R(1)	Nasal meatus
AP1426	29410	R(1)	Cribriform plate
AP1505	29410	R(8)	Cribriform plate
AP1520	29410	S(9)	Cribriform plate
AP1479	29411	R(8)	Ethmoturbinat
AP1515	29411	S(9)	Ethmoturbinat
AP1475	29411	R(1)	Maxilloturbinat
AP1512	29411	S(9)	Maxilloturbinat
AP1513	29411	S(9)	Nasal meatus
AP1526	29411	R(8)	Nasal meatus
AP1527	29411	R(1)	Nasal meatus
AP1473	29411	R(8)	Cribriform plate
AP1511	29411	S(9)	Cribriform plate

*R and S: Resistance to rifampicin and streptomycin, respectively. *gdh* allele number is in parenthesis.

[†]Sites of inoculation at day 0 or 7 are listed.

[‡]Sites of culture 72 d postinoculation are listed.

been proposed that pathogen *N. meningitidis* can gain access to the meninges and the central nervous system via the olfactory nerves that connect the nasal cavity to the brain through perforations in the cribriform plate (26).

Despite its importance to microbial pathogenesis and evolution, there is surprisingly little information about HGT in a natural setting. Our study shows that HGT occurred in two of three inoculating strains, in the absence of selection, and within 44 d after the strains established residency in the animal (Fig. 3 and Table 2). The rapid occurrence of HGT in RM *Neisseria* highlights an important public health problem. Commensals are reservoirs of antibiotic resistance determinants, and HGT likely plays an important role in their spread among the pathogens. Supporting this notion are the observations that a *N. meningitidis* strain responsible for an outbreak in the Midwest appears to have acquired its ciprofloxacin-resistant *gyrA* allele from commensal *N. lactamica* carried in the community (27); and that the cephalosporin resistance of some *N. gonorrhoeae* strains is because of mosaic *penA* alleles of commensal origin (28–30).

RM commensal *Neisseria* persist in their monkey host long-term. This finding is reminiscent of the findings from human inoculation studies with *Neisseria lactamica* (31). That study, moreover, suggests *N. lactamica* may impede *N. meningitidis* carriage by mechanisms distinct from humoral immunity. *N. lactamica* was hypothesized to block *N. meningitidis* carriage through competition or cell-mediated immunity. That study did not detect dual carriage of *N. lactamica* and *N. meningitidis*.

Although the RM model has limitations for *Neisseria* research (e.g., cost and small number of animals), it has several distinct advantages over the human colonization model. The RM model allows simultaneous carriage by multiple *Neisseria* species; it can therefore be used for in vivo HGT studies, particularly of antibiotic resistance determinants. Being a closed system, the RM

model provides a means for studying not only colonization but also strain transmission dynamics between animals. Finally, it provides a means for performing competition experiments to identify important in vivo niches and genetic determinants important for long-term persistence.

The RM model may be adapted to study the molecular mechanisms of *Neisseria* pharyngeal colonization. Human-specific commensal and pathogenic *Neisseria* have 896 genes in common, many of which encode virulence factors (32). *N. macacae* harbor orthologs of *Neisseria* virulence factors, putative and established (Tables S1 and S2); their contribution to colonization, long-term carriage, and transmission can be examined via in vitro experimentation followed by animal testing of defined mutants or recombinant mutant libraries. Moreover, human pathogenic *Neisseria* virulence factors expressed in RM *Neisseria* carriage strains may be examined for virulence traits in the monkey host.

The RM model also holds promise for vaccine development. Rodent models have been used to identify antigens that stimulate a protective immune response against meningococemia. The RM model may be useful for testing the efficacy of vaccine candidates against pharyngeal carriage. Serogroup C vaccines have successfully reduced carriage of *N. meningitidis*; this in turn has resulted in herd immunity, reducing the incidence of serogroup C meningococcal disease in unvaccinated individuals (33, 34).

Another advantage of the RM model is the close evolutionary and physiological relatedness of this animal to man. Vaccination of infant RMs with meningococcal outer membrane vesicle results in the development of broadly protective serum antibodies (35). Testing candidates in the RM should allow a better prediction of human antibody responses than in rodents.

Finally, it may be desirable to develop a vaccine with specificity for *N. meningitidis*. As mentioned, there is evidence that a healthy commensal *Neisseria* microflora could protect humans from colonization by pathogenic species (31). Children colonized by *N. lactamica* early in life are less likely to carry *N. meningitidis* at a later time (36). Our study shows that at least two species of *Neisseria* colonize RMs simultaneously for extended periods. This finding provides a means to test vaccine candidates for their ability to prevent carriage and for their specificity.

Materials and Methods

See *SI Materials and Methods* for additional details describing *Neisseria* culture and identification, PCR, oligonucleotides (Table S7), sequencing, phylogenetic and polymorphism analysis, bacterial strains, transformation assays, electron microscopy, Tfp purification, and proteomics.

Sample Collection and Culturing During *Neisseria* Surveys. Swabs (throat, tongue, nose, wound, and so forth) were cultured the same day or transported at ambient temperature in Amies gel with charcoal for culture the next day. Dental plaque scrapings were harvested with a sterile scalpel blade into GC Medium Base (GCB) and disrupted with a microtube pellet pestle (Kontes) before culture. *Neisseria* were grown at 37 °C, 5% CO₂ with a humidified atmosphere. In general, swabs were used to directly inoculate agar plates. In some cases swabs were vortexed in GCB 20% (vol/vol) glycerol and before cryopreservation 25 μL was used to inoculate plates for culture. *Neisseria* species obtained during culture surveys (Table S4) were isolated on the following media: LBVT.SNR (19), chocolate agar (Difco) and 1% hemoglobin (wt/vol) (Oxoid), Trypticase soy agar (Difco) with 5% sheep blood (vol/vol), LB.SNR (LBVT.SNR without vancomycin and trimethoprim). *Neisseria* species isolated during the colonization study, including necropsy (Table S6), were isolated on GCB agar with Kellogg's supplements (37), chocolate agar, or LB agar (Lennox, Research Products International) with or without the following antibiotics: V, vancomycin, 3 μg/mL; T, trimethoprim, 3 μg/mL; Rif50, rifampicin, 50 μg/mL; Sm100, streptomycin, 100 μg/mL.

Animals, Housing, and Experimental Inoculations. Age-matched female RM (*Macaca mulatta*) were of Indian or Chinese origin. RM ages, pairing, and summary of *Neisseria* flora are listed in Table S8. Nonhuman primate studies were performed in biosafety level 2 biocontainment at the Oregon National Primate Research Center (ONPRC) and the protocol was approved by the

Oregon Health Sciences' ONPRC Animal Care and use Committee and carried out in strict accordance with the recommendations of the National Institutes of Health's (NIH) *Guide for the Care and Use of Laboratory Animals* and the US Animal Welfare Act. The ONPRC is an American Association for Accreditation of Laboratory Animal Care-accredited, NIH-supported nonhuman primate research facility, and has an approved Assurance (#3304-01) for the care and use of animals on file with the Office for Protection from Research Risks at the NIH. We specifically selected grooming pairs for this study. The experimental and negative control cohorts were housed indoors. Animals were full-paired (co housed within the same cage) in cages facing each other 8-feet apart to prevent physical contact. To prevent inadvertent spread of *Neisseria* between animals, each cohort was swabbed by a separate animal technician. RMs were sedated with ketamine hydrochloride (5–10 mg/kg body weight) to collect swab samples. Gloves were changed before sampling each animal.

To reduce the level of preexisting *Neisseria* in RM, animals were given intramuscular injections of enrofloxacin (Baytril), 10 mg/kg, once daily for 2 wk. Before inoculation on day 0, animals were rested for 5 d to allow Baytril to clear from the blood stream and mucosal surfaces.

Regarding inoculations, on day 0 animal 29410 received 2.55×10^8 cfus of strain AP678-RifR and animal 29411 received 2.35×10^9 cfus of strain AP312-SmR. On day 7 animal 29410 received 1.35×10^{10} cfus of strain AP206-RifR and animal 29411 received 3.12×10^{10} cfus of strain AP312-SmR. A total of 2 mL of neisserial suspension in supplemented GCB was introduced into the nose (0.25 mL per nostril) and oral cavity (1.5 mL) using a plastic syringe while animals were in a supine position.

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