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NOTES

Distribution of Specific DNA Sequences among Pathogenic and Commensal *Neisseria* Species

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Several traits, including pili and the outer membrane proteins P.II and H.8, have been associated with pathogenic *Neisseria* species. We examined several *Neisseria* species for DNA sequence homology to cloned pilin, P.II, and H.8 genes. Strains of *Neisseria gonorrhoeae* and *N. meningitidis* showed hybridization to all of these genes. Commensal strains showed little hybridization to any of these genes. Strains of *N. lactamica* and *N. cinerea* showed intermediate patterns of hybridization. Generally, organisms that expressed a given trait showed DNA homology to the corresponding cloned gene. However, we observed pili on some commensal strains that did not show hybridization to the cloned gonococcal pilin gene.

The genus *Neisseria* consists of several different species that are commonly categorized as pathogens or commensals. *Neisseria gonorrhoeae* and *N. meningitidis* are well-established primary pathogens of humans, and the other *Neisseria* species are rarely implicated in infection. However, the actual properties that distinguish pathogenic *Neisseria* from commensals are not totally understood. The evolutionary events responsible for generating such pathogenic diversity within this closely related group of organisms are also unclear.

Numerous studies have examined relatedness among *Neisseria* species by measuring overall DNA homology (14, 19, 20). These studies have indicated that, although there is considerable DNA homology between pathogenic and commensal *Neisseria* species, the two pathogenic species are more closely related to one another than to any of the commensal strains. We wished to complement these overall homology studies by examining the distribution of some specific DNA sequences among the *Neisseria* species. We examined sequences encoding proteins that have been associated with the pathogenic *Neisseria* species in an effort to determine if these sequences are pathogen specific or more widely distributed. Examples of pathogen-specific DNA can be found in many other bacterial systems in the form of chromosomal sequences, plasmids, and phage (8, 9, 17). Examining the distribution of such sequences can yield information about virulence traits, as well as the relationships between pathogenic and nonpathogenic members of a genus (17). In several cases the use of pathogen-specific DNA sequences has been proposed as a diagnostic tool (7, 15). Thus, studies examining the distribution of specific DNA sequences among pathogenic and commensal members of a genus may yield practical applications, as well as provide clues to the nature and evolution of pathogens.

Several traits have been associated with one or both of the pathogenic *Neisseria* species. These include pili and the outer membrane proteins P.II and H.8. Pili are a well-established virulence factor in gonococcal infections and may be important in meningococcal virulence as well (4, 25, 36). Pili are involved in the attachment of these organisms to human mucosal surfaces (4, 25, 30, 31, 35). In addition, gonococcal pili undergo phase and antigenic variation, which may play a role in evasion of the host immune response (22). Less is known about meningococcal pili; however, they share morphologic characteristics, antigenicity, and some amino acid sequence homology with gonococcal pili (12, 29, 32). Pili have also been observed on commensal *Neisseria* species (24, 37), but the function of these pili and their relationship to pili found on pathogenic species are unclear.

P.II is a heat-modifiable outer membrane protein found on gonococci (21, 34). This protein has been associated with adherence to host cells (21) and serum resistance (16, 21). P.II is also subject to phase and antigenic variation, and a single gonococcal strain can make several distinct P.II proteins (3, 11, 21, 33, 34). The class 5 proteins of the meningococcus are analogous to the gonococcal P.II proteins in several ways, although the former have not been as well characterized (10).

H.8 is an outer membrane protein expressed by both pathogenic *Neisseria* species but not by most commensal species (5). (Cannon et al. [3, 5] previously reported 1 of 10 tested *N. sicca* strains bound the H.8 monoclonal antibody; in subsequent studies the antibody-binding strain has been reclassified as *N. meningitidis*.) H.8 is immunogenic in patients with disseminated gonococcal and meningococcal infections (1). However, the nature of its possible role in neisserial pathogenesis is still unknown. The H.8 proteins expressed by different *Neisseria* strains and species all share a common epitope, although the apparent molecular weight of the protein varies from strain to strain (5, 13).

In this study we used a dot blot hybridization technique to survey pathogenic and commensal *Neisseria* species for the presence of DNA sequences homologous to cloned structural genes for H.8, P.II, and pilin. The bacterial strains we surveyed are indicated in Table 1. Isolates obtained from the

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TABLE 1. Hybridization of DNA from *Neisseria* species to specific gene probes

Strain	Hybridization to probe[a]			
	H.8 meningococcal (pFLOB802)	H.8 gonococcal (s6H.8)	P.II (pFLOB709)	Pilin (pNG1102)
N. gonorrhoeae	+	+	+	+
FA1089	+	+	+	+
JB1122	+	+	+	+
JB1126	+	+	+	+
JB1131	+	+	+	+
FA1091	+	+	+	+
FA1080	+	+	+	+
NRL 6611	+	+	+	+
NRL 5767	+	+	+	+
FA1090	+	+	+	+
F62	+	+	+	+
N. meningitidis				
NM2	+	+	+	+
JB511	+	+	+	+
NMC	+	+	+	±
NMS-PS	+	+	+	±
6495	+	+	+	+
6771	+	+	+	+
6779	+	+	+	+
NMC-PW6	+	+	+	+
FAM18	+	+	+	+
JB516	+	+	+	+
N. lactamica				
F-5445	+	+	+	−
F-5886	+	+	+	−
NNRL 15 (NRL 24409)	+	+	+	−
NNRL 17 (NRL 36016)	+	+	+	−
NNRL 18 (NRL 36046)	+	+	+	−
NNRL 23 (NRL 37168)	+	+	+	±
NNRL 24 (NRL 37170)	+	+	+	+
NNRL 25 (NRL 37174)	+	+	±	−
NRL 30011	+	+	+	−
F-6865	±	±	−	−
N. cinerea				
NNRL 10 (NRL 32828)	+	+	+	−
NNRL 11 (NRL 33295)	+	+	−	−
NNRL 12 (NRL 33683)	+	+	+	+
NNRL 13 (NRL 33720)	+	+	−	−
NNRL 14 (NRL 33807)	+	+	+	−
NNRL 37 (NRL 32165)	+	+	−	−
NNRL 38 (NRL 32824)	+	+	±	±
NNRL 39 (NRL 35450)	+	+	+	−
ATCC 14685 (NRL 30003)	+	+	±	−
NNRL40 (NRL 36263)	+	+	+	±
NNRL 9 (NRL 300066)	+	+	±	−
Commensals				
N. perflava ATCC 14799	±	±	−	−
N. flavescens NRL 30031	±	±	+	−
N. flava NRL 9993	±	−	−	−
N. flava NRL 30037	±	−	−	−
N. flava NRL 30008	±	−	−	−
N. subflava NRL 9992	±	−	−	−
N. mucosa NRL 9297	±	±	−	−
B. catarrhalis 316[b]	−	−	−	−
N. sicca 272	±	±	−	−

[a] The hybridization reactions indicated for each strain are the results of two to six experiments. There was some variation in signal intensity in individual blots; this table represents a summary of the results of all experiments. +, Strong hybridization to probe; ±, barely detectable hybridization to probe; −, no detectable hybridization to probe.

[b] *B. catarrhalis*, *Branhamella catarrhalis* (negative control).

Neisseria Reference Laboratory, Seattle, Wash. (indicated by NRL or NNRL prefixes), were provided by J. Knapp. Other strains were obtained from the collection of P. F. Sparling. The cloned structural genes we used as DNA probes are also indicated in Table 1. The meningococcal H.8 probe, pFLOB802, is a 0.9-kilobase meningococcal insert from strain FAM18 in the vector pBR322 (T. H. Kawula and J. G. Cannon. Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, D21, p. 58). The gonococcal H.8 probe, s6H.8, is a 14-kilobase gonococcal insert from strain FA1090 in lambda phage (2). s6H.8 contains a much larger fragment of cloned DNA than does pFLOB802 and therefore detects homology in sequences flanking the H.8 gene, as well as the gene itself. The P.II probe, pFLOB709, is a 1.5-kilobase gonococcal insert from strain FA1090 in pBR322 (T. D. Connell and J. G. Cannon, manuscript in preparation; Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, D97, p. 82). Although the P.II cloned gene encodes a specific P.II variant, P.IIa, the gene hybridizes to multiple P.II genes in the gonococcal chromosome. Thus pFLOB709 was used to screen for all P.II homology, not just P.IIa specific homology. The pilin probe, pNG1102, is a 1-kilobase gonococcal insert from strain MS11 in pBR322 (27) and was the generous gift of M. So.

Organisms were grown overnight on GCB medium base agar (Difco Laboratories, Detroit, Mich.) with Kellogg defined supplements (18) in a 5% CO_2 atmosphere at 37°C. Overnight growth was then suspended in GCB broth to a reading of 10 on a Klett meter. With a Minifold apparatus (Schleicher & Schuell, Inc., Keene, N.H.), 200 μl of the suspension was applied to BA85 nitrocellulose paper (Schleicher & Schuell) according to the instructions of the manufacturer. Each dot on the nitrocellulose contained approximately 2×10^7 organisms. DNA was then liberated from the bacteria and bound to the nitrocellulose by the method of Maniatis et al. (23). Nitrocellulose filters were hybridized with individual nick-translated (23) probes for 18 h at 68°C. The filters were then washed two times for 30 min each at room temperature in a solution of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate. This was followed by two 1-h washes at 68°C in 0.1× SSC–0.1% sodium dodecyl sulfate. The filters were allowed to dry and were then autoradiographed. A representative panel of dot blots is shown in Fig. 1. The results of all of the experiments are summarized in Table 1.

All isolates of *N. gonorrhoeae* and *N. meningitidis* hybridized to each of the four probes. Isolates of *N. lactamica* and *N. cinerea* showed intermediate patterns of hybridization: all of these isolates (21/21) hybridized to the H.8 probes, most isolates (17/21) hybridized to the P.II probe, and a few isolates (5/21) hybridized to the pilin probe. Isolates of commensal species (other than *N. lactamica* and *N. cinerea*) showed little or no hybridization to any of these probes.

These experiments were performed under conditions of high stringency, and it is possible that less stringent conditions might reveal homology to these probes that we did not detect. We have reported several results with the notation ± (i.e., hybridization of *N. meningitidis* strains NMC and NMS-PS to pNG1102), indicating a reproducible low level of hybridization. This low-level hybridization may reflect either reduced homology to the probe or fewer homologous copies of a given sequence in the chromosome than those of organisms giving strong positive results. Southern blotting experiments in which pNG1102 was used to probe restriction digests of chromosomal DNA from various *N. meningitidis* strains indicated that in this case variable copy numbers may be the reason for differences in dot blot hybridization intensity (data not shown).

Whereas the pathogenic *Neisseria* spp. uniformly contained DNA sequences that hybridized to all of the DNA probes, these sequences were not absolutely pathogen specific. However, we observed a definite pattern in which *N. lactamica* and *N. cinerea* strains represented an intermediate group with clearly less homology to the P.II probe than the pathogenic strains had and with very little homology to the pilin probe. The rest of the commensals showed a striking lack of homology to both the P.II and pilin probes and greatly reduced homology to the H.8 probes.

In addition to screening for DNA homology, we also did studies to correlate this homology with expression of H.8, P.II, and pili. H.8 expression was assessed by testing the ability of organisms to bind to a monoclonal antibody directed against the H.8 protein in a colony blot radioimmunoassay. Cannon et al. (5) reported binding of the H.8 monoclonal antibody to all pathogenic *Neisseria* strains tested and to a small number of *N. lactamica* and *N. cinerea* strains. The H.8 antibody did not bind to most other commensal strains. We tested 10 additional strains each of *N. lactamica* and *N. cinerea* and found positive H.8 antibody binding to all of them (data not shown). Thus, these species resembled the pathogens in their expression of the H.8 antigen. Others have also found *N. lactamica* and *N. cinerea* to be more closely related than are other commensals to *N. gonorrhoeae* and *N. meningitidis* by a variety of criteria (6,

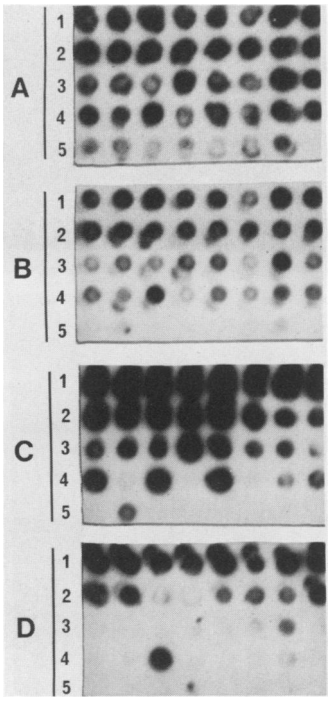

FIG. 1. Each dot represents a different strain of bacteria, and the arrays of strains are identical for all four panels. Rows: 1, *N. gonorrhoeae* strains; 2, *N. meningitidis* strains; 3, *N. lactamica* strains; 4, *N. cinerea* strains; 5, commensal strains (specifically, the eight strains shown right to left for each species correspond to the first eight strains listed for each species in Table 1). The final position in row 5 represents *Branhamella catarrhalis*, which serves as a negative control. Each panel was probed with a different DNA probe. (A) Meningococcal H.8 (pFLOB802); (B) gonococcal H.8(s6H.8); (C) P.II(pFLOB709); (D) pilin(pNG1102).

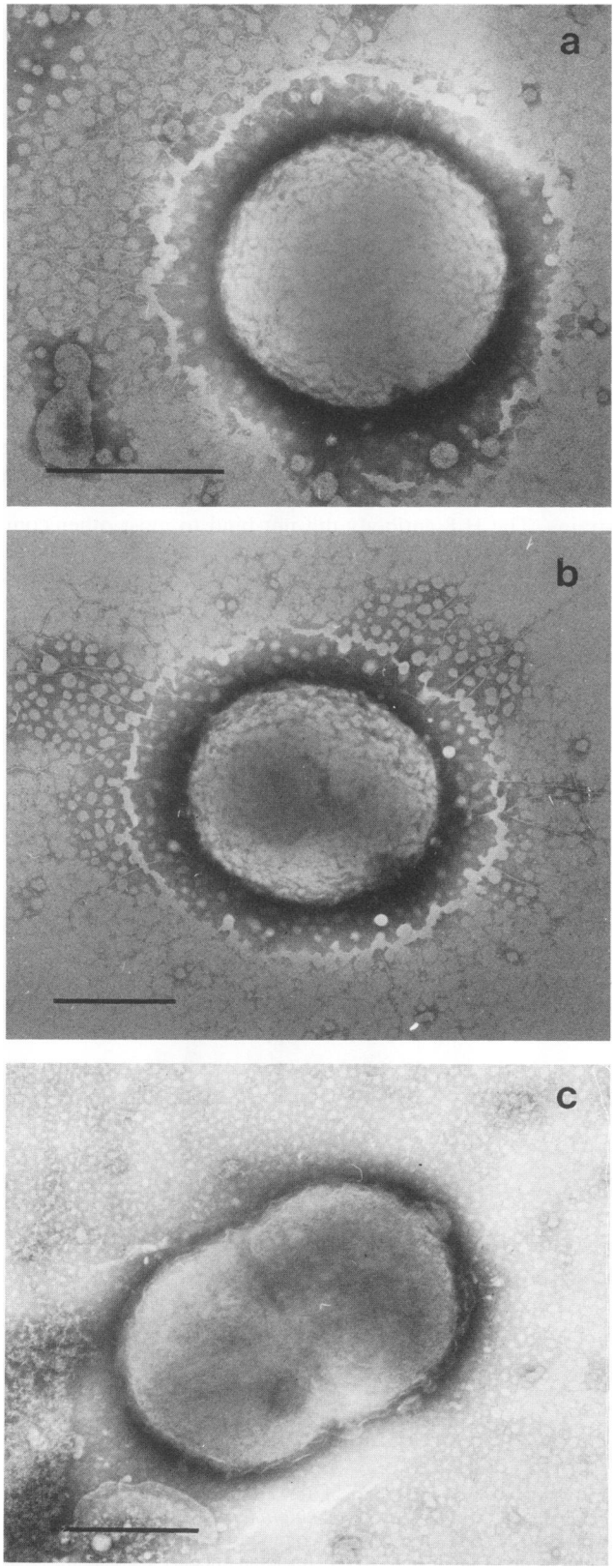

FIG. 2. Electron micrographs of negatively stained *Neisseria spp.* (a) presence of pili on *N. flava* NRL9993, which does not have homology to the cloned gonococcal pilin gene; (b) positive control with pili visualized on *N. gonorrhoeae* FA19; (c) negative control with pili absent from *N. lactamica* NRL 30011. Cells were prepared for electron microscopy by suspending the top of a 12- to 16-h colony, grown on GCB agar, in a drop of phosphate-buffered saline. They were then negatively stained with 1% potassium phosphotungstate (pH 7.0) on carbon-coated Formvar grids as described by McGee et al. (24). The specimens were examined with a JEOL 100 CX transmission electron microscope at 80 kV.

28). These species represent an interesting subgroup within the *Neisseria* genus.

We examined the outer membranes of five *N. lactamica* and *N. cinerea* strains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to look for expression of proteins similar to gonococcal P.II and meningococcal class 5 proteins. We found no evidence for a heat-modifiable P.II-like protein in any of these strains (data not shown), even though four of these five strains showed hybridization to the P.II DNA probe. Thus, whereas all organisms known to have P.II or equivalent proteins hybridized to the P.II DNA probe, not all organisms that hybridized to the DNA probe showed expression of such a protein.

We also examined 11 strains of both pathogenic and commensal *Neisseria* species by electron microscopy for the presence of pili. We did not find a strong correlation between the presence of pili and hybridization to the pilin DNA probe. Pili were not observed on some strains that hybridized to the probe, probably because of phase variation resulting in lack of pilus expression under the growth conditions we imposed (26). Also, interestingly, pili were seen on two strains, *N. flava* NRL 9993 (Fig. 2) and *N. lactamica* F-6865, that did not hybridize to the cloned gonococcal pilin gene we used as a probe. This was in contrast to the H.8 and P.II expression data, for which all organisms that expressed the proteins showed hybridization to the cloned genes. Some commensals may produce pili that are fundamentally different from the pili found on pathogens, which is of particular interest, since the pilin gene was the most pathogen specific of the genes we tested.

In conclusion, we have examined the distribution of several specific DNA sequences among *Neisseria* species. These sequences were conserved in pathogenic *Neisseria* species and generally absent from commensal species other than *N. lactamica* and *N. cinerea*. This study has provided additional evidence indicating that *N. lactamica* and *N. cinerea* share specific traits with the pathogenic *Neisseria* species. In addition, examining the distribution of the gonococcal pilin gene revealed the probable existence of two types of neisserial pilin genes (those with extensive homology to the gonococcal pilin probe and those with reduced or no homology to it).

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