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Nonencapsulated *Streptococcus pneumoniae*: Emergence and Pathogenesis

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ABSTRACT   While significant protection from pneumococcal disease has been achieved by the use of polysaccharide and polysaccharide-protein conjugate vaccines, capsule-independent protection has been limited by serotype replacement along with disease caused by nonencapsulated *Streptococcus pneumoniae* (NESp). NESp strains compose approximately 3% to 19% of asymptomatic carriage isolates and harbor multiple antibiotic resistance genes. Surface proteins unique to NESp enhance colonization and virulence despite the lack of a capsule even though the capsule has been thought to be required for pneumococcal pathogenesis. Genes for pneumococcal surface proteins replace the capsular polysaccharide (cps) locus in some NESp isolates, and these proteins aid in pneumococcal colonization and otitis media (OM). NESp strains have been isolated from patients with invasive and noninvasive pneumococcal disease, but noninvasive diseases, specifically, conjunctivitis (85%) and OM (8%), are of higher prevalence. Conjunctival strains are commonly of the so-called classical NESp lineages defined by multilocus sequence types (STs) ST344 and ST448, while sporadic NESp lineages such as ST1106 are more commonly isolated from patients with other diseases. Interestingly, sporadic lineages have significantly higher rates of recombination than classical lineages. Higher rates of recombination can lead to increased acquisition of antibiotic resistance and virulence factors, increasing the risk of disease and hindering treatment. NESp strains are a significant proportion of the pneumococcal population, can cause disease, and may be increasing in prevalence in the population due to effects on the pneumococcal niche caused by pneumococcal vaccines. Current vaccines are ineffective against NESp, and further research is necessary to develop vaccines effective against both encapsulated and nonencapsulated pneumococci.

*S*treptococcus pneumoniae (pneumococcus) is a significant human pathogen. Humans are the main reservoir for the pneumococcus, and asymptomatic carriage in the nasopharynx typically occurs at least once by the age of 2 years in the United States (1). However, when the pneumococcus gains access to normally sterile body sites, immune dysregulation and disease can occur. The pneumococcus is a common etiological agent of several diseases such as bacterial meningitis, pneumonia, otitis media (OM), sinusitis, and conjunctivitis (2–4). According to the World Health Organization, the pneumococcus is responsible for 1 million deaths yearly (5). In 2000, over 14 million children worldwide under the age of 5 years were diagnosed with an invasive pneumococcal disease, with the highest incidence seen in Africa (5). Bacterial OM is most commonly caused by the pneumococcus, *Haemophilus influenzae*, and *Moraxella catarrhalis* (3). In the United States, OM is the number 1 reason for pediatric clinical visits and antibiotic prescriptions. Currently, an estimated cost of 1.5 to 3 billion dollars is associated with OM despite high rates of childhood vaccination (6–8), while the cost was as high as 5 billion dollars before routine vaccinations (9). Due to high rates of OM despite pneumococcal vaccination, further investigation and increased insight into how these versatile pathogens continue to cause disease are necessary to produce widespread and effective prevention methods.

CLASSIFICATION

One of the most important pneumococcal virulence factors is the polysaccharide capsule, of which there are at least 97 antigenically distinct serotypes (10). The pneumococcal capsule is necessary for virulence of encapsulated pneumococci as it not only protects against opsonophagocytosis but also allows nasopharyngeal (NP)

colonization (11, 12). NP colonization is required for progression to pneumococcal disease, and the capsule has been implicated in reducing mucosal clearance from the NP, allowing effective colonization (13). Specific pneumococcal serotypes are associated with invasive pneumococcal disease (IPD), as some serotypes cause more IPD than others even after accounting for the more frequent colonization of some serotypes (14–16). The pneumococcal conjugate vaccines (PCVs) have significantly reduced the rates of IPD while reducing rates of noninvasive disease such as OM, along with NP colonization, only slightly (17–22). Smaller reductions in rates of noninvasive disease were observed because of serotype replacement, which was characterized by increased levels of some pneumococcal nonvaccine serotypes (NVT) (23).

The capsular polysaccharide synthesis (*cps*) locus, which is required for capsule production, is situated between the highly conserved *dexB* and *aliA* pneumococcal genes. The *dexB* and *aliA* genes are not known to be used for capsule production (24). Homologous recombination at the *cps* locus can occur because of these highly conserved flanking regions within the chromosome (25, 26). There have been reports of increased isolation of nonencapsulated *Streptococcus pneumoniae* (NESp) from patients with

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TABLE 1 Prevalence of NESp within carriage isolates along with rates of resistance

Country	Total no. of isolates[a]	No. of NESp isolates	% NESp isolates	% resistant NESp isolates[b]	Typing method(s)	Reference
Portugal	254	18	7.1	100	Quelling	49
Portugal	202	25	12.4	100	Quelling	50
Spain	194	12	6.2	58	Quelling/dot blot	51
Brazil	253	48	19	100	Quelling/PCR	52
Brazil	166	22	13.3	47.4 (MDR)	Quelling/latex beads/PCR	53
Italy	184	10	5.4	80	Quelling/latex beads	54
France	170	16	9.4	94	Quelling	55
Israel	1,763	90	5.1	30 (MDR)	Quelling	56
Poland	139	5	3.9	100 (MDR)	Quelling/PCR	57
Thailand	3,085	512	16.6	83.6	Genome sequencing	58

[a] Data represent numbers of isolates that were serotyped in the study.
[b] Data represent percentages of resistance to at least one antibiotic tested. Multiple drug resistance (MDR) data represent resistance to at least three classes of antibiotics and are included where individual rates of resistance were not available.

NP colonization and noninvasive disease and occasionally from patients with IPD (27–36). NESp can be classified into either group I or group II isolates on the basis of the composition of the *cps* locus (28). Group I NESp isolates have *cps* genes, but they do not produce capsule due to mutations or deletions of specific *cps* genes (28). Group II NESp isolates have novel genes in place of the *cps* genes. Two *aliB*-like homologues, called *aliC* and *aliD* (also called *aliB* ORF1 and ORF2, respectively), as well as the gene coding for pneumococcal surface protein K (*pspK*; also called *nspA*), replace the *cps* locus of group II isolates (28, 31, 37). These different variants of group II NESp have been subdivided into so-called null capsule clades (NCCs) based on which genes are located within the *cps* locus (31). NCC1 strains are *pspK*-positive (*pspK$^+$*), *aliC* negative, and *aliD* negative strains. NCC2 strains are *pspK* negative, *aliC$^+$*, and *aliD$^+$* strains and are classified as either NCC2a or NCC2b on the basis of the intergenic length polymorphism between a remnant *capN* gene and the flanking *aliA* gene (31). To date, strains containing only the *aliD* gene (NCC3 [*pspK* negative, *aliC* negative, and *aliD$^+$* strains]) have been identified as closely related streptococcal species and not true pneumococci. Interestingly, *aliD*, but not *aliC*, is also found within the *cps* locus of serogroups 25 and 38 and a truncated version is found within 66 other serotypes, while *pspK* has not been shown to occur with any other *cps* genes (24).

Speculation on the increase in NESp isolation has been based on two thoughts: (i) the introduction of PCVs as mentioned above and (ii) an increase in awareness and more stringent typing of collected samples. Pneumococcal colony morphology on agar plates varies based on capsule status. NESp colonies have a classical "rough" morphology that contrasts with the "smooth," and sometimes "mucoid," morphology of encapsulated pneumococci. Also, NESp colonies may be smaller than those of encapsulated pneumococci, making identification and collection more challenging. Together, these characteristics may cause NESp to be overlooked in laboratories and underreported (38, 39). NESp strains produce a null result when typed with capsular serotype-specific antisera and are termed nontypeable (38–40). In general, nontypeable *S. pneumoniae* strains include both NESp strains, which do not produce capsule, and strains that express novel serotypes or amounts of capsule below the limit of detection. This distinction is important and necessary for understanding the true prevalence of NESp in contrast to the emergence of novel pathogenic serotypes. The U.S. introduction of the PCV in the year 2000 and in other countries shortly after preceded many of the reports

about NESp in the population. To draw a connection to the general prevalences of NESp before and after the introduction of the PCV is challenging due to the refinement of and greater sensitivity in typing methods that developed over the years. Despite this, some studies have been able to more directly address pre- and postvaccine NESp prevalences (27, 29, 32, 35, 36). In general, these studies have reported differing results, with some showing no change in NESp prevalence and others showing greater or lesser prevalences.

It is also important to differentiate NESp from other closely related nonencapsulated streptococcal species, such as *S. pseudopneumoniae* (41) and *S. tigurinus* (42), which are often confused with *S. pneumoniae*. Molecular approaches can be used to confirm both species and capsule status with greater precision and accuracy to demonstrate true prevalence (43, 44). These approaches include multilocus sequence typing (MLST), which has allowed reliable species identification of streptococcal isolates (45). Analysis of the *cps* locus can be used for verification of nonencapsulated status. For example, the first and essential gene of the *cps* locus, *cpsA* (also called *wzg*), is detected by several PCR assays and can differentiate NESp strains as group I or group II (46).

PREVALENCE AND ANTIBIOTIC RESISTANCE

Prevalence. The human population most susceptible to pneumococcal infections is that of children due to their immature immune system (47). Children within day care centers are in close contact, which promotes pneumococcal transmission (48). Therefore, many studies that document NESp colonization involve children (Table 1). One study in Portugal found that 7% of carriage isolates collected in one day care center were NESp. Interestingly, the only examples of concurrent colonization by multiple strains in that study were of colonization with a NESp and either an encapsulated 23F or 16F strain (49). In another study of Portuguese children attending a day care center, it was found that 12% of pneumococcal isolates were NESp (50). Within a Spanish population of children, 6.2% of carriage isolates were NESp (51), while a study of pneumococcal carriage isolates from Brazilian children found that almost 19% of isolates were NESp (52). Over 13% of the strains isolated from their sampling of children colonized with pneumococcus in another Brazilian study were NESp strains (53). A population of Italian children contained 5.4% NESp carriage isolates (54), and in southeastern France, children had a NESp carriage rate of 9.4% (55). In Israeli pneumococcal carriage isolates, NESp consisted of over 5% of the total number of collected samples (56),

while a study in an unvaccinated population of children in an orphanage in Poland found that 3.9% of the pneumococcal strains isolated were NESp (57). In a cohort of 770 infants and mothers in Thailand that had not received any pneumococcal vaccines, up to 16% of carriage isolates were found through genome sequencing to be NESp (58).

Resistance. Resistance profiles for the collected NESp isolates from the studies described above were also reported in most cases and showed that high rates of resistance are common. For example, in the study from Portugal described above, all 18 NESp isolates were resistant to at least one antibiotic and 16 were resistant to multiple drugs (49). Among these isolates, all of the NESp strains of the NNN clonal cluster and 14 of 18 strains of the BE clonal cluster, as defined by pulsed-field gel electrophoresis (PFGE), were resistant to erythromycin, clindamycin, tetracycline, and sulfamethoxazole-trimethoprim, and some were resistant to penicillin. All isolates of the NNN and BE clusters were multilocus sequence type (ST) ST344 or double-locus variants (DLV) of ST344. Interestingly, in a previous study by this group, the NNN cluster was found in only one day care center, while this study isolated NNN clones from four different day care centers (59). Sánchez-Tatay (51) found that 50% of NESp isolates were resistant to penicillin and erythromycin (6 of 12 isolates) and that all of the resistant isolates were ST344. One other NESp isolate was resistant to only penicillin and was ST942 (51). One of the studies of Brazilian children found that all of the NESp isolates were resistant to penicillin and were ST2315 or a single-locus variant (SLV) of ST2315 (ST4746) (52). Another study of Brazilian children did not provide data for all resistance profiles determined, but the NESp isolates had higher rates of multiple drug resistance (MDR [resistance to at least three antibiotic classes]) than other isolates, with 19 of 22 isolates being ST2315 (53). NESp isolates from Italian children were all ST344, and 80% were resistant to at least two antibiotic classes (54). Sixteen NESp isolates were collected from children in southeastern France, and 15 were ST344 or ST344 SLVs, with the remaining NESp isolate being ST63. Of these 16 NESp isolates, 15 were resistant to erythromycin and intermediately susceptible to penicillin, but it was not stated if all 15 resistant NESp isolates were ST344 (55). NESp isolates collected from Israel were divided into 6 clusters (clusters A to F) based on PFGE results, with clusters A and B containing 44% of all 148 isolates. MLST of clusters A and B was done and showed an ST448 SLV for cluster A and a new ST for cluster B that varied from ST344 by three alleles. Resistance profiles for these clusters indicate that at least 70% of cluster A isolates were resistant to a least one antibiotic, while 96% of cluster B isolates were MDR. Rates of resistance were also high in the other clusters (clusters C to F), with the exception of cluster F, which was susceptible to all antibiotics tested. NESp isolates from Poland were all ST344, and all were MDR (57). The genomes of the NESp isolates from Thailand were analyzed for known resistance genes for β-lactam antibiotics or for allelic forms conferring resistance to co-trimoxazole (58). Over 83% of the NESp isolates were predicted on the basis of their sequences to be resistant to either of these antibiotic classes but included no isolates from the ST344 cluster. Of the highly recombinogenic NESp strains contained within a polyphyletic sequence cluster (SC) called BC 3-NT, 86% of isolates were ST4133; of these isolates, 30% had no genes or single nucleotide polymorphisms (SNPs) that would confer resistance to the tested antibiotics. This is in contrast to the ST448 cluster, all isolates of which

contained genes or SNPs for resistance to both antibiotic classes. Interestingly, the genes for β-lactam antibiotics and co-trimoxazole resistance occurred together more often than not despite not being closely linked in the genome. Of the NESp isolates, 16.6% were sensitive to both antibiotics compared to the 1.17% that were sensitive to only one antibiotic, showing that multiple drug resistances were more common than resistance to a single antibiotic.

These studies indicated that a significant proportion of colonizing pneumococcal isolates are NESp, and this rate could be higher in vaccinated individuals, as increases in NESp levels have been observed following the introduction of the PCV (36). Also, the results of these various studies show that the global distribution of NESp varies according to geographic location. This is not surprising since the global distribution of pneumococcal serotypes also varies based on geographic location (60, 61). While the currently available data demonstrate that the highest rates of NESp carriage are in Asia and South America, this may simply reflect an increased awareness of NESp isolates in these regions. Additionally, high rates of antibiotic resistance are seen for NESp, specifically, within the ST344 cluster (49, 54, 55, 57). Together, these results indicate that multiple antibiotic resistances may be a common feature of NESp.

COLONIZATION

Colonization is requisite for pneumococcal disease (62, 63). Effective colonization within the NP allows the necessary dissemination into sterile tissue sites leading to disease (62, 63). The loss of capsule from strains that normally express capsule abolishes colonization and virulence (12, 13). Current vaccines induce a strong antibody response to specific capsule serotypes, allowing efficient clearance from the host and reduced colonization of the targeted capsule types, but these vaccines are ineffective against NESp strains (64).

The phase-variable "transparent variants" of encapsulated strains have increased epithelial cell adherence due to reduced capsule expression (65, 66). This allows pneumococcal surface proteins greater access to host cell receptors for adherence and potential immunomodulatory effects (67, 68). Also, protein expression profiles alter depending upon the pneumococcal phase phenotype (69). Increased capsule expression is observed when encapsulated pneumococcal strains cause systemic infection (66, 70). It has been shown that the pneumococcus increases mucosal secretions, leading to dysregulation of mucosal clearance by host ciliary action (71, 72). While capsule is needed for encapsulated pneumococcal colonization, strains that naturally lack capsule, i.e., NESp strains, compensate for the absence of capsule and can still effectively colonize. A potential mechanism of colonization was found within group II NESp strains. Group II NESp isolates with the *pspK* gene showed significantly greater NP colonization of the mouse than an isogenic *pspK* mutant (31). Also, extrachromosomal expression of PspK in a PspK⁻ NESp strain increased NP colonization in a mouse (73). Furthermore, it was found that PspK⁺ NESp strains were able to colonize the NP as effectively as an encapsulated strain and that both colonize at greater densities than PspK⁻ NESp strains (31). The ability of PspK to increase adhesion to epithelial cells may explain how the loss of capsule has been compensated for in some NESp strains (73).

Despite the lack of capsule and PspK, group II NESp strains that contain *aliC* and *aliD* within the *cps* locus are also able to

colonize the nasopharynx (28, 31, 58, 74). Of 512 sequenced NESp isolates from Thailand, 197 contained *aliC* and *aliD*, while 258 contained *pspK* (58). Recent work led to the conclusion that AliC increased pneumococcal competence and AliD enhanced early NP colonization of the mouse (74). The oligopeptide transporter Ami-AliA/AliB was also found to be important for NP colonization (75). Thus, results of current studies indicate that both PspK and AliD have a role in colonization.

An important consideration during colonization and disease is evasion of the innate immune response. Despite lacking some important colonization and immune evasion proteins (see Virulence Factors below), NESp strains do colonize and are isolated from the nasopharynx. Pneumococcal clearance is mediated through several host factors, including neutrophil recruitment, macrophage opsonophagocytosis, and CD4$^+$ T cells (76, 77). Interestingly, results from studies of available genome sequences (78, 79) indicate that NESp strains contain DltA (80, 81) and EndA (82), which have been shown to inhibit neutrophil extracellular traps, an important method of bacterial clearance (83).

BIOFILM FORMATION

Pneumococcal biofilm is important for colonization and persistence within the NP (84, 85). Bacterial populations are almost exclusively contained within these surface-associated communities when they are part of the nasopharyngeal community (86–88). The ability to produce biofilm and interact with different bacterial species or different pneumococcal strains is vital for colonization (89). It has been shown that NESp strains produce larger amounts of biofilm than their encapsulated counterparts (90–92). This observation might be attributed to increased exposure to surface proteins that allow greater attachment and, subsequently, enhanced biofilm formation. Therefore, the very nature of NESp may allow increased biofilm formation, which not only enables greater colonization but also reduces susceptibility to antibiotics and the host response (93). NP colonization and biofilm formation allow the pneumococcus to disseminate to other body sites and cause disease (62, 63). In these sites, biofilm formation has been implicated in increased probability of disease, especially during OM (94–97).

VIRULENCE FACTORS

Pneumococcal virulence factors tend to fall within one of three groups: capsule, toxin, and surface proteins (11, 98, 99). The capsule is not necessary for NESp strains as, by definition, they lack this virulence factor. All pneumococci contain the cytolytic toxin pneumolysin, and, in addition, some NESp strains have been recently reported to contain a second pneumolysin gene that is closely related to other streptococcal cytolysins (100, 101). As no other toxins have been described for any pneumococcus, NESp virulence is mainly mediated through surface proteins and other surface structures. Surface proteins are attached to the bacterial cell through various mechanisms, which include attachment to choline on the pneumococcal surface by choline binding repeats, lipid attachment via recognition of lipobox motif (LxxC) by Lgt enzyme (102), and peptidoglycan anchoring through sortase-mediated attachment via the LPxTG motif and other pilus-specific sortases (103). The structure of pneumococcal surface proteins allows anchoring to the pneumococcal surface with an extracellular portion that interacts with the environment, typically to promote survival (98, 104). NESp strains, like encapsulated pneumo-

cocci, reside in the NP, and surface proteins can aid in colonization through attachment, immune modulation, or enhanced biofilm formation (67, 105, 106).

Interestingly, the first genome sequences of five genetically diverse NESp isolates lacked genes for common pneumococcal surface proteins such as PspA, PspC (also called CbpA), and PcpA (78). The absence of genes for PspA and PspC was also observed with comparative genomic hybridization of 34 other NESp isolates from diverse genetic backgrounds (107). The surface proteins PspA and PspC have been studied as vaccine candidates (108–111), but these proteins would not be effective immunogens for NESp since they lack these proteins. Other potential protein-based vaccine targets are the pneumococcal histidine triad proteins (Pht). PhtD has been found in all encapsulated strains regardless of serotype and shows a high degree of sequence identity between strains. Despite this, PhtD is absent in the sequenced PspK$^-$ NESp strains but is present in the sequenced PspK$^+$ strains (78, 112). Genome sequences of hundreds of NESp isolates were published during 2013 and 2014 (58, 78, 79, 113) and will provide more information about the distribution of NESp virulence factors. However, the genes for some virulence factors, such as PspA and PspC, are difficult to assemble with short-read sequence data, as noted previously (114), so molecular confirmation of their presence or absence may be needed.

Current results indicate that NESp strains lack surface proteins that are common in encapsulated pneumococci. Thus, it is reasonable to propose that the bacterial surface of NESp differs from that of encapsulated strains. In fact, several novel surface proteins were recently discovered in a genomic study of 21 NESp isolates from patients with conjunctivitis (79). Some of these proteins were reported to be divergent forms of choline-binding and LPxTG-attached proteins (Fig. 1 [ECC_3504]), and others were reported to have glycan and gp-340 binding domains that may contribute to bacterial aggregation (79). As shown in Fig. 1, the PspC (CbpA) of strain EC_3504, which was named CpbAC (79), is divergent from the PspC protein of strain TIGR4. It is interesting that the first 279 amino acids of CbpAC have 77% identity to PspK of MNZ11 but show only 55% identity to the R1 region of the PspC protein of TIGR4. We have shown that PspK enhances epithelial cell attachment and binds secretory IgA (sIgA) (73), while it was predicted that CbpAC binds dimeric IgA based on homology to the beta antigen of *S. agalactiae* (79). PspK and CbpAC may perform redundant roles, which could explain why CpbAC-containing NESp strains have *aliC* and *aliD* in the *cps* locus and not *pspK*. This may also indicate that the *aliC* and *aliD* genes, which conjunctival isolates often contain, perform a function during conjunctivitis (see Disease below). Another possible explanation for the fact that PspK$^+$ NESp strains have not been observed to cause conjunctivitis is their potentially recent emergence, with the first known PspK$^+$ isolate collected in 2008 (31). While the answer has yet to be experimentally verified, it is important to determine what NESp virulence factors or combinations of virulence factors can lead to various pneumococcal diseases. Therefore, in-depth studies of the way NESp interacts with the host are necessary because these strains have virulence gene repertoires different from those of encapsulated pneumococci.

DISEASE

Conjunctivitis. The best-documented disease caused by NESp is conjunctivitis, which is associated with sequence type 448 (ST448)
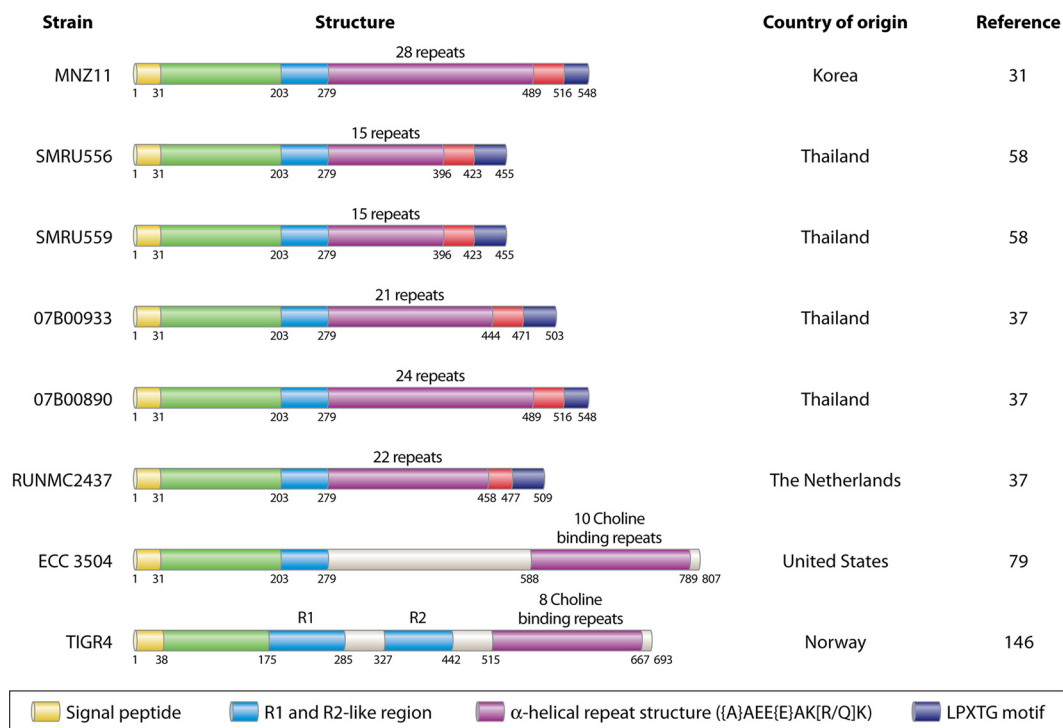
FIG 1 Schematic of PspK from several geographic locations, highlighting homology to PspC and newly described CbpAC1. Representations of various PspK proteins at the amino acid level, CbpAC1 from ECC_3504, and PspC from TIGR4 are shown. Countries of strain isolation and strain references are provided adjacent to the representations. All representative structures have a highly conserved amino terminus for the first 279 amino acids. Variations in amino acids 203 to 279 have been observed in other strains, but the number of repeats after amino acid 279 is what most often varies between PspK sequences. The protein from strain ECC_3504 was isolated from a conjunctival sample and was encoded at the *pspC* (also known as *cbpA*) locus instead of at the *cps* locus as PspK is. Despite identical sequences for the first 279 amino acids, the location on the genome and the presence of choline binding repeats led to this structure being referred to as a divergent PspC called CbpAC1. CbpAC1 also has an additional ~300 amino acids after the conserved region that are not seen in any known PspK sequences. The final protein is PspC from TIGR4 (146) for homology comparison with PspK and variant PspC. Sequence data were obtained from an NCBI database query. Each color in the color-coded key at the bottom of the figure corresponds to regions of the same color on the protein structures except where indicated.

and ST344 (56, 115, 116). There were reports of conjunctivitis caused by NESp as early as 1980, indicating the presence of NESp in the population for at least several decades (117). Of 271 conjunctival isolates from across the United States, over 90% were NESp and over 85% were represented by 4 STs, ST448, ST344, ST1186, and ST2315, all of which are nonencapsulated (79). An earlier Spanish study found that only 23.2% of conjunctival isolates were nonencapsulated, all of which were represented by 4 STs, but only ST344 was common to that study and the study described above (118). Another Spanish study reported that 34.8% of conjunctival isolates were NESp, with the majority related to ST344, ST448, or ST941. Rates of antibiotic resistance were in agreement with the resistance rates of carriage isolates as outlined above. ST344 were commonly MDR, with ST941 being resistant to at least two antibiotics and ST448 being susceptible to the tested drugs (119). In general, ST344 and ST448 NESp strains commonly cause conjunctivitis outbreaks, while sporadic cases have shown greater variation in both capsule status and ST. Among those involved in outbreaks of pneumococcal conjunctivitis, the majority are of the ST448 clonal cluster, in contrast to reports of the high rates of carriage by the ST344 cluster (119–122). While it is unknown why NESp strains, and specifically the ST448 cluster, cause conjunctivitis at high rates, Valentino et al. reported that all epidemic conjunctival isolates contain the *aliC* and *aliD* genes within the capsule locus, indicating a potential role in the virulence of NESp (79). Recent work has elucidated several differences between the gene contents of encapsulated and nonencapsulated *S. pneumoniae* strains. Some of these differences increase colonization and virulence, but the majority of these differences are uncharacterized, necessitating increased research on these emerging pathogens (73). Further information about conjunctivitis can be found in a review article by Tarabishy and Jeng (123).

Otitis media. NESp strains have been isolated from children with otitis media (OM) (27, 29, 124). In fact, one study found that as many as 8% of OM isolates were NESp (27), and, more recently, NESp strains were found in 6.4% of OM isolates (125). This is interesting because complement hinders pneumococcal infections in the middle ear (126) and NESp strains lack the protection from complement afforded by the capsule and PspC (78, 107). Other mechanisms to avoid complement-mediated clearance must be used, and several other pneumococcal factors that reduce the effect of complement were found in several NESp genomes. These complement evasion proteins that NESp strains contain include a phosphoglycerate kinase (PGK) (127) that inhibits membrane attack complex formation; endopeptidase O (128), which binds C1q; and Tuf (129), which binds various proteins in the complement-regulating factor H family (130), including factor H. It is currently unknown if these proteins serve the same function in NESp as they do in encapsulated strains.

While there have been almost no studies that have examined the mechanisms that allow NESp to persist and cause disease, the chinchilla has emerged as an effective model of OM caused by NESp (124, 131–133). In this model, the replacement of the *cps* locus with *pspK* allowed efficient colonization in the NP along with increased virulence in the middle ear (31, 132, 134). Our study examined the ability of NESp to cause OM in the chinchilla along with the function of PspK during infection. All strains that contained *pspK* produced OM (132). In contrast, only half of the NESp isolates tested that did not naturally contain *pspK* were able to cause OM. Additionally, the PspK⁻ NESp isolates that caused disease had higher relative epithelial cell adhesion to nasopharyngeal epithelial cell line Detroit 562 than avirulent PspK⁻ NESp (132). While there was no correlation between epithelial cell adherence and levels of recovered bacteria in PspK-containing strains, it was postulated that PspK allows sufficient attachment for other factors to then increase virulence (132). A recent study determined the prevalence of *pspK* in NESp isolates from 12 Asian countries (135). That study found that over 42% of the NESp isolates from Thailand contained *pspK* and that over 15% of the NESp isolates from Asia had the *pspK* gene (135). Interestingly, the most common ST found was ST1106, which has been previously associated with serotype 14 isolates, and all of the ST1106 isolates were multiple drug resistant (135).

Invasive pneumococcal disease. NESp strains are able to cause IPD, but the frequency is much lower than that caused by encapsulated strains (28, 30, 37, 136). This can potentially be explained by the innate immune response, specifically, the complement system. Activation of the host complement system by the pneumococcus is the main mechanism of clearance during infection (137, 138). The classical complement pathway is responsible for initiating complement deposition and ultimately leading to clearance through opsonophagocytosis (139). The presence of capsule hinders the recognition of common pneumococcal antigenic sites on the bacterial surface, while the absence of capsule allows rapid recognition and efficient complement activation (140). This lack of capsule reduces the ability of NESp to produce relevant infection in sterile sites that are inundated with complement. Despite this, a review of South East Asian cases of IPD found that anywhere from 0% to 15% were caused by NESp, with some variation by country (141).

The largest study to date on IPD isolates from the United States found that 88 (0.61%) of 14,328 isolates tested were NESp, with the majority being group I isolates (30). Of the 79 group I isolates, 22 were serotype 8 strains that expressed a dysfunctional *cpsA* gene that stopped capsule production (30). It was suggested that opsonization of capsule-bearing pneumococci would deplete complement levels in the microenvironment to allow transient survival of the *cpsA* escape mutants. Of the nine group II isolates found in this survey, six contained *aliB*-like homologues *aliC* and *aliD*, and the remaining three isolates were either unclassified or lacked all genes in the *cps* locus (30). Another study of nearly 5,000 IPD isolates from Chile found that 3.43% of pneumonia cases were caused by NESp, while only 1.5% of bacteremia cases were caused by NESp (142). The first IPD study by Park et al. (30) described above did not differentiate between types of IPD. Since, as reported by Lagos et al. (142), NESp strains are more likely to cause pneumonia than bacteremia, the study by Park et al. (30) may have included higher numbers of isolates from patients with bacteremia than from patients with pneumonia. This could result

in the observed lower prevalence of NESp IPD cases than that reported from the earlier Chilean study. Alternatively, the differences between these studies in reported rates of IPD caused by NESp could have been due to geographic differences in the host or the pathogen (30).

These studies indicate that group II isolates may be specialized for NP colonization. Thus, these upper respiratory commensals may be important pathogens of noninvasive diseases such as otitis media, sinusitis, and nonbacteremic pneumonia. A general model of NESp pathogenesis is presented in Fig. 2.

GENETIC EXCHANGE

The pneumococcus has several mechanisms to mediate horizontal gene transfer and recombination (here referred to simply as "recombination"), including transformation, integrative conjugative elements (ICEs), and transducing phage. Several studies have shown, *in vitro*, that capsule-negative mutants acquire genes through recombination at a higher frequency than their encapsulated progenitors (143–145). These results may indicate that capsule is an anatomical barrier that impedes the receipt of DNA or possibly that bacterial physiology changes with loss of capsule in a way that promotes the receipt of DNA. However, inferences of the frequency of recombination in natural populations of pneumococci are affected by many additional factors, including population sizes, selection for or against the incoming DNA, and the power of recombination tests to detect the recombinant DNA. Several recent genomic studies have provided a nuanced view of the frequency of recombination in natural populations of NESp compared to encapsulated pneumococci, which we discuss below.

A study of the genomes of NP colonization isolates from Massachusetts classified ST448 and ST344 NESp into an early-branching, monophyletic sequence cluster (SC) called SC12 (114). Various other NESp strains were classified into a polyphyletic sequence cluster called SC16 and were not further examined. The per-site recombination-to-mutation ratio (r/m; the ratio of the numbers of single nucleotide polymorphisms accumulated by recombination versus point mutation) of SC12 was 12.3, which ranked it only 7th among 15 SC's in the order of highest per-site r/m (114). These results indicate that the ST448 and ST344 NESp strains are not particularly recombinant compared to most encapsulated pneumococcal strains isolated in Massachusetts. This conclusion is supported by a study of the genomes of globally sampled ST448 and ST344 NESp strains, where the range of per-event r/m (ratio of the number of recombination events to the number of point mutation events) was 0.15 to 0.18 and was comparable to the range of 0.13 to 0.23 determined for many encapsulated lineages (58, 113). On the other hand, there is evidence that the sporadic, polyphyletic NESp strains are much more recombinant than other pneumococcal strains. The previously mentioned study of colonization isolates from Thailand classified many NESp strains into a polyphyletic sequence cluster called BC 3-NT (58). The per-event r/m of BC 3-NT was 0.32, significantly higher than that for all other sequence clusters. More impressively, the 128 NESp isolates of BC 3-NT, which do not contain NESp of the so-called classical lineages of ST448 and ST334, were significantly more recombinant than the 74 capsular serotype 14 isolates of BC 3-NT (per-event r/m of 0.34 versus 0.20, respectively) (58).

The classical NESp lineages of ST448 and ST344 have been noted to have relatively large genomes with novel gene content in comparison to other pneumococci (77, 112), including an
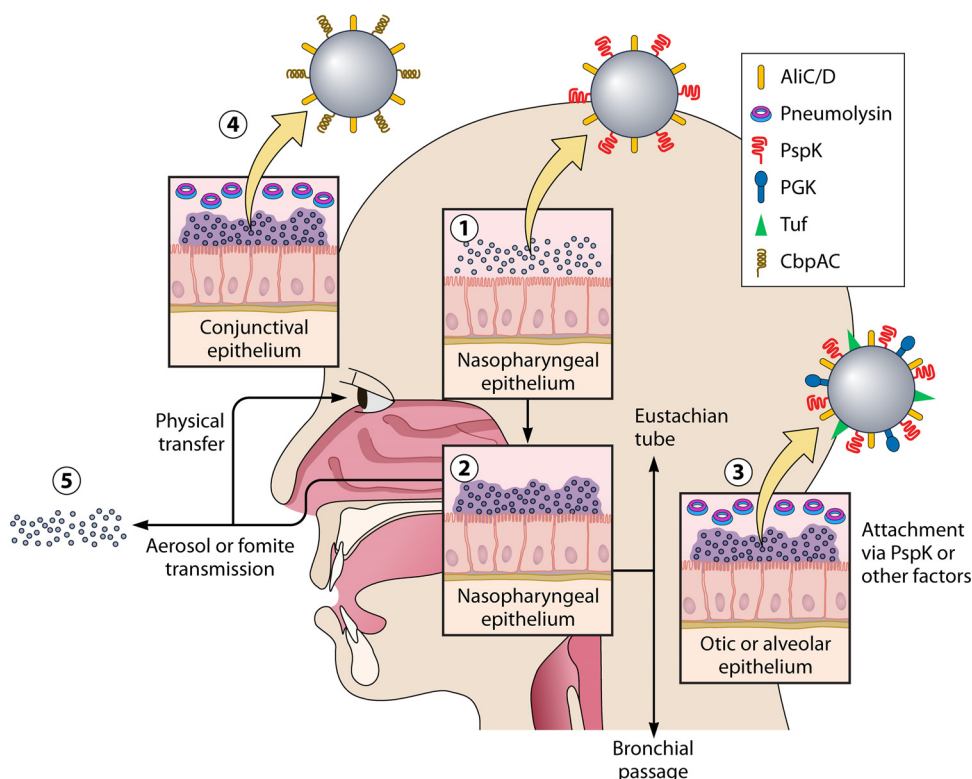
FIG 2 Proposed model for NESp colonization and disease. Initial attachment and colonization is mediated through surface proteins (1), including unknown methods of immune evasion. NESp biofilm formation and persistence are aided by enhanced adhesive properties (2). Planktonic bacteria dissociate from the biofilm matrix and ascend into the middle ear through the Eustachian tube or descend to the lung through the pharynx (3). Disruption of surface-associated communities is followed by physical transmission to eye (4) or transmission to other hosts through aerosol droplets or fomite intermediates (5).

abundance of ICEs and novel regions of difference/genomic islets in addition to the previously mentioned virulence factors (77, 112). Apparently, the presence of multiple ICEs in the ST448 and ST344 NESp strains has not resulted in increased recombination frequency within these lineages or in export of their novel genes to other pneumococci. These classical NESp lineages contain 116 unique clusters of orthologous groups (COGs), including 33 COGs within two ICEs, ICE$_1$ *S. pneumoniae* ST344 (ICE$_1$*Sp*ST344) and ICE$_2$*Sp*ST344, and two COGs within pneumococcal pathogenicity island 1 (113). ICE$_1$*Sp*ST344 was found within both the classical and sporadic NESp lineages and was shown to aid in epithelial cell adhesion but had reduced growth rates compared to NESp lineages lacking this ICE (113). The remaining COGs unique to NESp include mobilization and conjugation proteins, a zinc ABC transporter, a zinc metalloprotease, a lipoprotein, and numerous hypothetical proteins of unknown function. As discussed earlier, NESp strains are also distinguished by novel gene content that includes variants of surface proteins found in the epidemic conjunctivitis cluster (79) and an altered *cps* locus (31, 37, 73).

Despite potential colonization advantages conferred by some of these novel elements, the large genomes of classical NESp may hinder growth and result in reduced fitness (113). NESp strains harbor multiple unique mechanisms to aid in colonization, and a balance between growth, colonization, and unique gene content must be maintained. In contrast to classical lineages of NESp, sporadic lineages of NESp have higher rates of recombination than other pneumococci (58, 113). Despite these differences, pen-

icillin nonsusceptibility levels of 0.06 μg/ml to 2.0 μg/ml are common in both classic and sporadic lineages. Moreover, resistance to tetracycline and erythromycin is prevalent regardless of lineage. ST448 bacteria were generally susceptible to trimethoprim-sulfamethoxazole, but this was not true with ST344 (58, 113). Thus, NESp could conceivably represent a growing reservoir of antibiotic resistance genes that could be exported to encapsulated strains, reducing treatment options and survival outcomes.

In summary, these results indicate that the early-branching, monophyletic, classical lineages that include ST448 and ST344 NESp are not unusually recombinant, but they carry novel genes and genetic elements that may aid survival. The polyphyletic, sporadic lineages that include other NESp sequence types such as ST1106 are highly recombinant and also carry novel genes such as *pspK* that may aid survival (58, 113). Therefore, both sorts of NESp may be poised to increase in prevalence in the wake of PCV.

CONCLUSIONS

A century of research has led to the view that pneumococcal capsule is required for colonization and virulence (12, 13); however, a growing body of evidence is showing that this is not always the case. The loss of the polysaccharide capsule does significantly decrease the chance of IPD but does not eliminate the possibility (30, 142). More commonly, noninvasive diseases such as OM and conjunctivitis are caused by NESp (27, 79). The total number of clinical cases caused by NESp is probably underestimated because serotyping is not routinely performed and serotype-nontypeable strains may not be further classified. Also, the numbers of pneu-

mococcal carriage isolates that lack capsule have been underestimated, with prevalences ranging from 3% to 19% of the pneumococcal population (52, 57). NESp strains have been isolated from the human population for several decades and have most likely been present for much longer (117). Indeed, some of these NESp strains appear to represent early-branching lineages of pneumococcus whereas other NESp strains are highly recombinant, but more work is needed to understand their evolutionary history (58, 113, 114). Increasing pressure against a specific subset of pneumococcal isolates, namely, capsule types within the PCV, affects the environmental niche that NESp strains are able to exploit. This allows increasing numbers of NESp strains to occur within the human population, increasing the risk of pneumococcal disease caused by NESp. In addition, NESp strains have novel gene content that is just beginning to be cataloged and that has been rarely studied from the functional and pathogenic perspectives. Understanding how these unique organisms colonize and have survived within the human population despite the lack of an antiphagocytic capsule will lead to greater insights into bacterial methods of persistence and evasion of the host defense.

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