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Capsule Enhances Pneumococcal Colonization by Limiting Mucus-Mediated Clearance▽†

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Expression of a polysaccharide capsule is required for the full pathogenicity of many mucosal pathogens such as *Streptococcus pneumoniae*. Although capsule allows for evasion of opsonization and subsequent phagocytosis during invasive infection, its role during mucosal colonization, the organism's commensal state, remains unknown. Using a mouse model, we demonstrate that unencapsulated mutants remain capable of nasal colonization but at a reduced density and duration compared to those of their encapsulated parent strains. This deficit in colonization was not due to increased susceptibility to opsonophagocytic clearance involving complement, antibody, or the influx of Ly-6G-positive cells, including neutrophils seen during carriage. Rather, unencapsulated mutants remain agglutinated within lumenal mucus and, thus, are less likely to transit to the epithelial surface where stable colonization occurs. Studies of in vitro binding to immobilized human airway mucus confirmed the inhibitory effect of encapsulation. Likewise, pneumococcal variants expressing larger amounts of negatively charged capsule per cell were less likely to adhere to surfaces coated with human mucus and more likely to evade initial clearance in vivo. Removal of negatively charged sialic acid residues by pretreatment of mucus with neuraminidase diminished the antiadhesive effect of encapsulation. This suggests that the inhibitory effect of encapsulation on mucus binding may be mediated by electrostatic repulsion and offers an explanation for the predominance of anionic polysaccharides among the diverse array of unique capsule types. In conclusion, our findings demonstrate that capsule confers an advantage to mucosal pathogens distinct from its role in inhibition of opsonophagocytosis—escape from entrapment in lumenal mucus.

Capsule, a surface coating generally comprised of polysaccharide, is a prominent feature of many pathogens, particularly those causing invasive infection. For example, the most common etiologic agents of bacterial meningitis, *Streptococcus pneumoniae*, *Haemophilus influenzae* type b, and *Neisseria meningitidis*, are encapsulated—a requirement for the sustained bacteremia needed to breach the blood-brain barrier. The importance of capsule to their pathogenicity results from its inhibition of opsonophagocytosis, a process involving the recognition of underlying structures by complement components and/or antibody and leading to engulfment by professional phagocytes (1, 3, 40, 41). Unencapsulated mutants rarely cause invasive infection and are highly attenuated in models of infection due to more efficient opsonophagocytotic clearance (3, 36).

Many encapsulated pathogens, including each of these species noted above, exist primarily in a commensal relationship with their human host, where they reside on the mucosal surface of the nasopharynx. This suggests that the main selective pressures for adaptation to the host occur during colonization, an organism's carrier state. Any advantage capsule confers to bacteria residing on mucosal surfaces, where complement and phagocytes may be less abundant, has not been established. It is thought that the ability of a microbe to colonize for extended periods (persistence) in this niche requires binding to host cells and tissues. However, in vitro studies consistently show an antiadhesive effect of capsule, suggesting a potential biological disadvantage to capsule expression that must be balanced by its contribution to survival during mucosal colonization (9, 24, 27, 28).

In this study, we focus on the role of the capsule during colonization by *S. pneumoniae* (the pneumococcus), a species capable of expressing a repertoire of at least 90 unique capsular polysaccharide "types" (PnPSs) (10). Each of these PnPSs differs in composition and linkage of its component sugars as well as other substitutes (12). A further source of heterogeneity among pneumococcal isolates of most types is due to phase variation between two forms (opaque [O] and transparent [T] colony forms) differing by up to 5.6-fold in amounts of PnPS/cell as well as other characteristics (13, 39). Similar to the capsules of many other species, the only common structural feature among this large array of polysaccharides is that none has a net positive charge. In fact, structures of more than half of the known PnPS types have been determined and all but four types are negatively charged due to the presence of acidic sugars, pyruvate, or phosphate, with the remainder being neutral (12). Despite the diversity of PnPS structures, shared physical characteristics are thought to contribute to a conserved function in protecting the underlying bacterial surface structures from the deposition of antibody and complement.

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Here we show that unencapsulated mutants successfully colonize nasal spaces but display altered colonization dynamics due to agglutination by mucus rather than enhanced susceptibility to opsonophagocytosis. These findings are consistent with capsule protecting underlying structures from host clearance mechanisms but suggest distinct functional roles during infection and colonization.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *S. pneumoniae* strains were grown as described elsewhere (39). Strains used in vivo were selected because of their ability to efficiently colonize the murine nasopharynx and included TIGR4 (type 4 clinical isolate, genome sequence strain), P303 (a mouse virulent, type 6A clinical isolate) and P1121 (a type 23F capsule-expressing *S. pneumoniae* isolate from experimental human colonization studies) (13, 19, 32). The *cps* operon was deleted from spontaneously streptomycin-resistant mutants (200 μg/ml) of TIGR4, P303, and D39 (type 2 clinical isolate) using the bicistronic positively and negatively selectable Janus cassette (29). The Janus cassette was replaced with *cps6A*, *cps7F*, *cps14*, or *cps23F* capsule operons as previous described (34). Encapsulation of streptomycin-resistant transformants was confirmed by positive quelling with type-specific antisera (Staten Seruminstitut, Copenhagen, Denmark). Colony opacity was visualized as previously described (39). All strains were passaged intranasally in mice prior to growth for preparation of frozen stocks.

Mouse model of nasopharyngeal colonization. Six-week-old, female C57BL/6J (wild type) or B6.129-S2-Igh-6^{tm1Cgn}/J (μMT) mice (Jackson Laboratories, Bar Harbor, ME) were housed in accordance with Institutional Animal Care and Use Committee protocols. μMT mice contain a targeted mutation in the heavy-chain locus of immunoglobulin M (IgM) and do not produce mature B cells or antibody (15). BALB/c mice were obtained from Taconic of Germantown, NY. Mice were colonized using a previously described model of nasopharyngeal colonization with *S. pneumoniae* (20). Briefly, groups of at least five mice per condition were inoculated intranasally without anesthesia with 10 μl containing 1×10^7 to 3×10^7 CFU of phosphate-buffered saline (PBS)-washed, mid-log-phase *S. pneumoniae* applied to each naris. At the time indicated, the animal was sacrificed, the trachea was cannulated, and 200 μl of PBS was instilled. Lavage fluid was collected from the nares for determination of viable counts of bacteria in serial dilutions plated on selective medium containing neomycin (5 μg/ml) to inhibit the growth of contaminants. The lower limit of detection for bacteria in lavage culture was 20 CFU/ml.

Neutrophil and complement depletion. Monoclonal antibody (MAb) RB6-8C5, a rat anti-mouse IgG2b directed against Ly-6G on the surface of murine myeloid (and limited subpopulations of lymphoid) lineage cells, was purified from ascites of nude mice given the RB6-8C5 hybridoma (4, 11). To deplete neutrophils, 150 μg of MAb/animal was administered by intraperitoneal (i.p.) injection 24 h prior to intranasal challenge with bacteria. This dose was previously shown to result in peripheral blood neutropenia (<50 granulocytes/μl) for a period of at least 48 h (17). Controls were given the equivalent i.p. dose of total rat IgG (Sigma Chemical Co., St. Louis, MO).

Hypocomplementemia was induced by i.p. injection of 25 μg/animal of cobra venom factor (Quidel, San Diego, CA) in PBS 18 h prior to bacterial challenge. This procedure was previously shown to reduce levels of immunodetectable C3 to <3% of normal and result in a period of hypocomplementemia of at least 48 h (30).

Histology and immunofluorescence. At the time indicated postinoculation, the animal was sacrificed and decapitated, and the head was fixed for 48 h in 4% paraformaldehyde in PBS. The head was then decalcified by serial incubations in 0.12 M EDTA (pH 7.0) over 1 month before freezing in Tissue-Tek O.C.T. embedding medium (Miles) in a Tissue-Tek Cryomold. Five-micrometer-thick sections were cut and stored at −80°C. Frozen imbedded tissue sections were stained with hematoxylin and eosin (H&E) following a 10-min fixation step in 10% neutral buffered formalin. Sections were then dehydrated in alcohol, cleared in xylene, and mounted in Cytoseal (Richard-Allan Scientific, Kalamazoo, MI).

For immunohistochemistry, sections were postfixed in 1:1 methanol-acetone at −20°C for 10 min followed by washing in distilled water (dH_2O). Endogenous peroxidase was blocked by incubation in 2.25% H_2O_2 in dH_2O for 15 min. Sections were blocked with avidin and biotin for 15 min each, followed by a further 10-min incubation in protein blocking reagent (PBR; Coulter/Immunotech, Miami, FL) to prevent nonspecific binding. Sections were then incubated in three steps in typing sera (Staten Serum Institute, Copenhagen, Denmark) at 4°C overnight, 1:500 in PBT (1× PBS, 0.1% bovine serum albumin, 0.2% Triton X-100), biotinylated goat anti-rabbit for 30 min, 1:200 in PBT, avidin-horseradish peroxidase-conjugated ABC reagent for 30 min (Vector Laboratories, Burlingame, CA), and the signal was developed using diaminobenzidine tetrahydrochloride (DAB) kit (Vector Laboratories). Sections were washed with PBS after each incubation step. After DAB staining, the slides were fixed in 10% NBF for 3 min followed by a 3-min wash in dH_2O. Sections were exposed to 3% acetic acid (pH 2.5) for 3 min, before being incubated with 1% alcian blue in 3% acetic acid (pH 2.5) for 30 min. After washing in water for 10 min, sections were counterstained with 0.1% nuclear fast red for 40 s and then dehydrated in alcohol, cleared in xylene, and mounted in Cytoseal. Bright-field imaging was performed on a Nikon E600 Eclipse microscope equipped with a liquid crystal (Micro-color RGB-MS-C, CRI Inc., Boston, MA) and a high-resolution charge-coupled device (CCD) digital camera (CoolSnap CF, Roper Scientific, Tucson, AZ) with Nomarski optics.

For immunofluorescent staining, tissue was postfixed with 1:1 acetone-methanol as described above, followed by blocking with PBR before addition of primary antibody, diluted 1:500 in PBT. Pneumococci were stained with typing sera as described above. Signal was detected with Cy3-conjugated species-specific secondary antibodies (Jackson ImmunoResearch, West Grove, PA) incubated in a 1:400 dilution of PBT for 2 h at room temperature. After washing with PBS followed by dH_2O, sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen, Carlsbad, CA) diluted 1:10,000 in dH_2O. All image analysis was carried out using IPLAB (Scanalytics, Fairfax, VA).

Human upper airway secretion binding assays. Mucus samples from human upper airway secretions obtained as previously described were used in a modified solid-phase mucin binding assay (8, 25). Secretions were diluted 1:1 in sterile PBS, sonicated until homogenous, and applied to 96-well Polysorp plate (Nunc) by centrifugation at $250 \times g$ for 3 min at room temperature (8). Following overnight incubation at 37°C, the unbound secretions were removed by washing with PBS and treated for an additional 3 h in sodium citrate buffer (50 mM, pH 6.0) with or without recombinant neuraminidase derived from *Clostridium perfringens* (25 U/ml; New England Biolabs, Beverly, MA). Bacterial cultures grown to mid-log phase (optical density at 620 nm [OD_{620}] of 0.4) were diluted in PBS to a density of 10^5 CFU/ml and applied by centrifugation at $250 \times g$ for 3 min at room temperature. Adherence was determined by incubation at 4°C for 60 min with gentle agitation. Supernatants and the first wash with PBS were removed for colony counts. Bacteria in the supernatant and first wash were considered to have not adhered to the well and were compared to the inoculum to calculate the proportion of bound or adherent bacteria. In the absence of fixed secretions (PBS-alone control), there was no significant adherence.

To generate a column to assess migration, mucins extracted from human upper airway secretions were covalently linked to acrylamide beads using UltraLink Biosupport according to the manufacturer's instructions (Pierce, Rockford, IL). Binding of mucins was monitored for saturation by assay of bound protein. Where specified, the mucin-treated beads were incubated with neuraminidase as described above. After equilibration with PBS, a 0.5-ml column of beads was loaded with 10^7 CFU of washed, mid-log-phase bacteria and fractions of the flowthrough were collected for quantitative culture.

Adherence to primary murine cells. Primary tracheal epithelial cells were isolated from C57BL/6 mice as described with two modifications (6). NuSerum (BD Biosciences) was used in differentiation medium in place of Ultroser G, and ITS+ tissue culture supplement (BD Biosciences) was used in the culture medium in place of insulin. Cells were cultured at an air-liquid interface for 3 weeks, and abundant ciliary activity was noted prior to adherence experiments. To assess the relative adherence of encapsulated and unencapsulated pneumococci, TIGR4 and TIGR4 *cps* were grown to OD_{620} of 0.5 in tryptic soy broth, pelleted, and washed twice with sterile PBS. Bacteria (1×10^8 CFU in 200 μl differentiation medium) were added to the apical chamber of the transwells and applied to the epithelial cells by centrifugation (150 x *g* for 5 min) prior to incubation at 37°C in 5% CO_2 for 2 h. Following incubation, monolayers were washed four times with sterile PBS. Cells were lifted by treatment with a 0.25% trypsin–1 mM EDTA solution (Gibco), and serial dilutions were plated for colony counting.

Statistical analysis. Colonization density was expressed as the log CFU/ml for calculation of means ± standard deviation. Statistical comparisons of colonization among groups were made by the nonparametric test indicated (GraphPad Prism 4).

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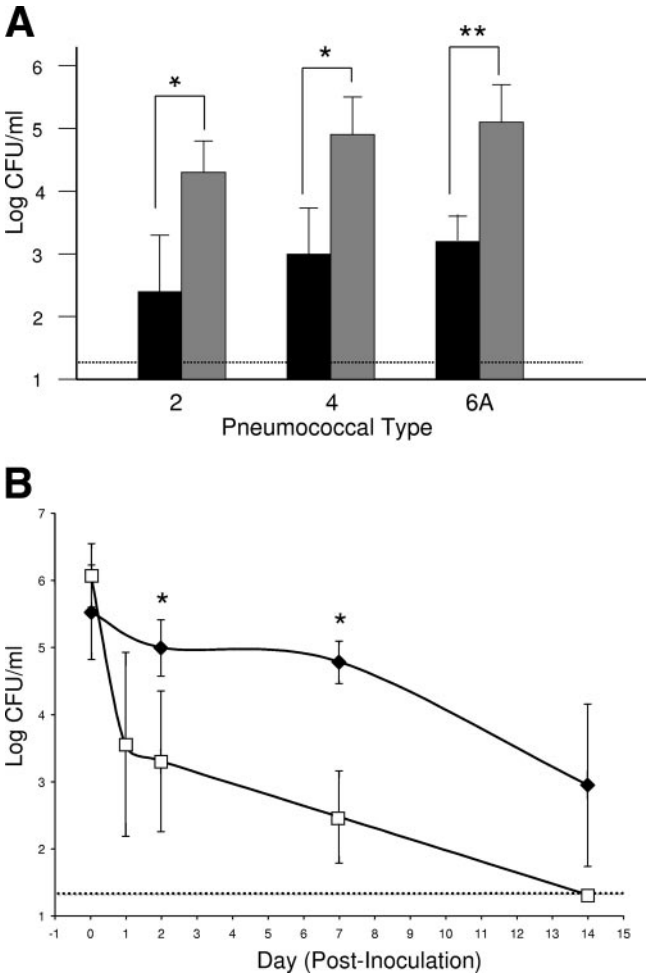

FIG. 1. Effect of pneumococcal capsule on the density and duration of nasal colonization. (A) Two days following intranasal challenge with 10^7 CFU of an isolate of the type indicated (gray bars) or its unencapsulated mutant (black bars) lacking the entire *cps* locus, C57BL/6 mice were sacrificed for quantitative culture of upper respiratory tract lavage fluid. Values represent the mean of 5 to 10 mice/group ± standard deviation. (B) Following intranasal challenge with 10^7 CFU of TIGR4 (closed diamonds) or TIGR4 *cps* (open squares), the density of colonization was assessed in quantitative culture of upper respiratory tract lavage fluid on the day postinoculation indicated. The dashed line indicates the limit of detection. Values represent the mean of 5 to 10 mice/strain at each time point ± standard deviation. *, $P < 0.05$; **, $P < 0.01$ compared to encapsulated parent strain (Mann-Whitney test).

RESULTS

Unencapsulated mutants colonize the nasal spaces. The contribution of capsule during colonization was assessed by comparing encapsulated isolates with their isogenic unencapsulated mutants in a murine model of colonization following intranasal inoculation. Mutants lacking the entire *cps* locus encoding PnPS were generated by use of Janus cassette technology that allows for construction of unmarked, in-frame deletions. TIGR4 *cps* consistently colonized C57BL/6 mice, but at a density 10- to 100-fold less than that of its parent strain, as assessed by quantitative culture of upper airway lavages at 2 days postinoculation (Fig. 1A). A similar contribution of encapsulation to colonization was also demonstrated by comparison of isolates of other types (2 and 6A) with and without *cps*.

To confirm that the decrease in fitness for colonization was due to the loss of the capsule, the deletion of *cps* in TIGR4 was corrected by insertion of the *cps* locus derived from TIGR4 or isolates of heterologous pneumococcal types. Phenotypic variants with opaque (O) or transparent (T) colony phenotypes were considered separately. Correction of capsule expression was sufficient to restore the density of colonization, as assessed at 2 days postinoculation, to wild-type levels for T but not O variants (Fig. 2). However, in these constructs of the same genetic background, the contribution of encapsulation depended on the capsule type with the greatest effect for negatively charged types, 4, 6A, and 23F and less effect for types with a neutral charge, 7F and 14.

To determine when encapsulation affects colonization, TIGR4 and TIGR4 *cps* were compared over the period during which pneumococci could be recovered from upper airway lavages. TIGR4 *cps* was able to persist for up to 7 days postinoculation (Fig. 1B). There was, however, a marked decline in colonization density between 30 min and 20 h postinoculation for TIGR4 *cps* that was not observed for TIGR4, which showed a more gradually decrease over 14 days postinoculation. After this initial decline for TIGR4 *cps*, the rate of decrease in the density of colonizing bacteria was similar regardless of the expression of capsule.

Together, these results showed that capsule is not necessary but may enhance colonization, particularly during initial events in the host, with the extent of its contribution dependent on its composition and amount.

Capsule does not impact on opsonophagocytic clearance during colonization. Histological examination of colonized nasal tissues confirmed that colonizing pneumococci induce a neutrophil influx into lateral nasal spaces by 1 day with a maximal response by 3 days postinoculation (see Fig. S1 in the supplemental material) (21, 35). Immunofluorescent staining of frozen tissue to detect bacteria demonstrated that these dense clusters of neutrophils have engulfed pneumococci but that not all pneumococci become associated with neutrophils (data not shown). To test whether neutrophil-mediated clearance accounted for the lower density of colonization by unencapsulated pneumococci, mice were treated with RB6-8C5, a rat MAB to murine Ly6.G, prior to intranasal challenge (11). This treatment effectively depleted neutrophils from peripheral blood and in tissue sections of colonized mice (17). If opsonophagocytic clearance were important during colonization, this treatment would be expected to enhance colonization by more readily opsonized unencapsulated pneumococci. However, there was no significant effect of RB6-8C5 treatment compared to that of controls on the density of TIGR4 *cps* at 2 days postinoculation (Fig. 3). Similarly, no effect on the colonization density of TIGR4 *cps* was observed following complement depletion by systemic administration of cobra venom factor prior to nasal challenge. In addition, genetically modified congenic mice (μMT) that fail to generate specific antibody did not demonstrate significantly enhanced colonization by TIGR4 *cps* compared to parental mice. These findings indicated that neutrophil-, complement-, or antibody-mediated clearance mechanisms do not contribute substantially to the

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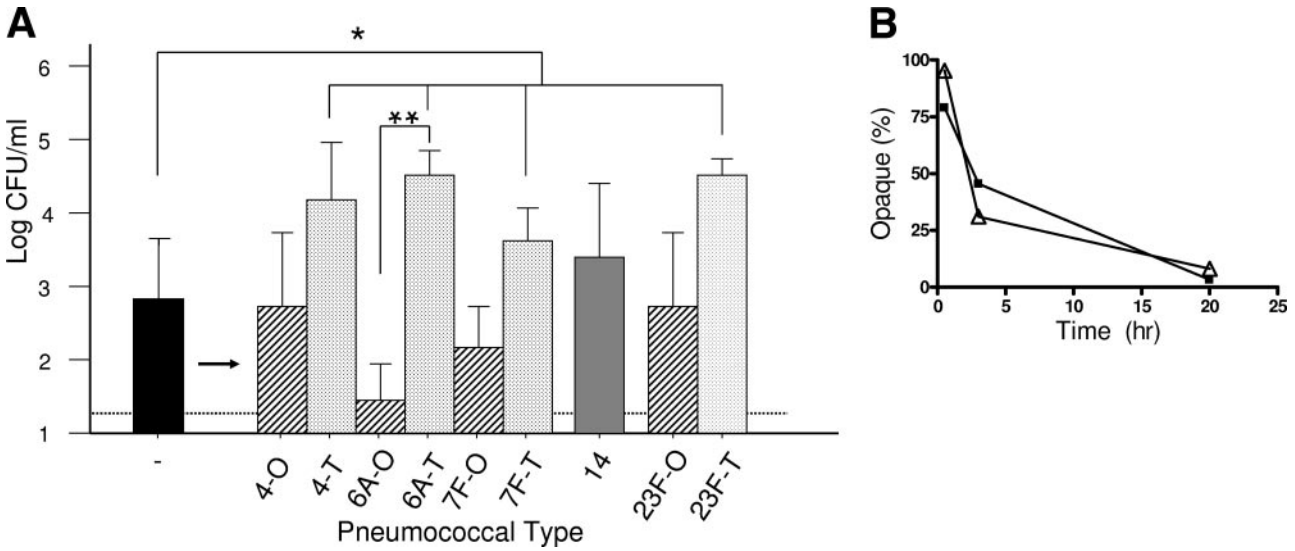

FIG. 2. Capsule type and amount determine colonization denisity. (A) Effect of restoration of encapsulation by different types. Unencapsulated TIGR4 *cps* (black bar) or transformants with the *cps* locus of the type indicated that corrected the loss of capsule expression were compared for their ability to colonize mice at 2 days postinoculation. Acquisition of the type indicated was confirmed by quelling. For encapsulated transformants of types 4, 6A, 7F, and 23F, opaque (O; hatched bars) and transparent (T; stippled bars) variants were tested separately. For the type 14 transformant (gray bar), only one phenotype was observed. The dashed line represents the limit of detection. Values represent the mean of 5 to 10 mice/group ± standard deviation. *, $P < 0.05$ compared to transparent encapsulated transformants (Kruskal-Wallis test with Dunn's post-test for multiple comparisons); **, $P < 0.03$ compared to homotypic transparent variant (Mann-Whitney test). (B) Following inoculation of equivalent numbers of O and T variants, colony phenotype was determined in nasal lavage cultures at the time postinoculation indicated. Representative experiments with isolates of types 6A (solid squares) or 23F (open triangles) are shown.

lower density of colonization by unencapsulated mutants and left in question the role of capsule during colonization.

Effect of capsule on the dynamics of colonization. To define the role of capsule in pneumococcal colonization, the events during the initial 2-day period postinoculation, during which the majority of the deficit in colonization by unencapsulated mutants occurs, were visualized in tissue sections. For TIGR4, initially (30 min) bacteria were confined to the lumen of nasal spaces, where they associate with amorphous, acellular material (Fig. 4A). This lumenal material is mucus based on its staining with alcian blue, which identifies acidic mucopolysaccharides (Fig. 4C). By 1 day postinoculation, these encapsulated pneumococci had transited to the mucosal surfaces, where they were found along the glycocalyx, a thin mucus-containing layer overlying epithelial cells (Fig. 4B and D). At later time points up to 14 days, pneumococci remained in the glycocalyx, indicating that this was the site of stable colonization (see Fig. S1 in the supplemental material).

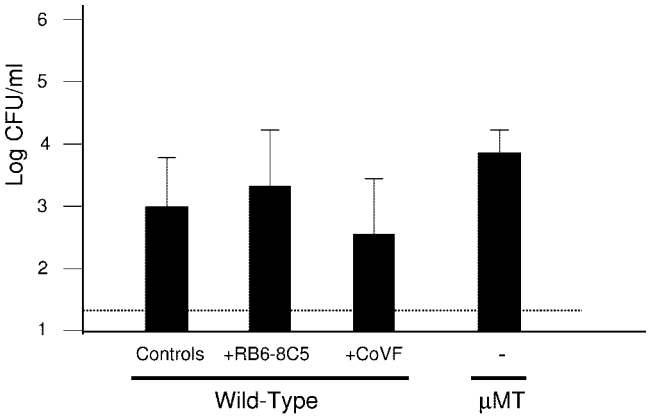

FIG. 3. Effect of neutrophils, complement, and functional antibody on colonization by an unencapsulated mutant. C57BL/6 mice were pretreated with RB6-8C5 to deplete neutrophils (or rat IgG control) or cobra venom factor (CoVF) to deplete complement (or vehicle control) prior to intranasal challenge with 10^7 CFU of TIGR4 *cps*, and the density of colonization was assessed in quantitative culture of upper respiratory tract lavage fluid on postinoculation day 2. Colonization of congenic μMT mice was determined in parallel experiments. Values represent the mean of 5 to 10 mice/strain at each time point ± standard deviation. $P > 0.05$ (Kruskal-Wallis test with Dunn's post-test for multiple comparisons).

TIGR4 *cps* was also seen initially in the lumenal mucus (30 min), but unlike the encapsulated parent was heavily agglutinated in mucus (Fig. 5A versus B). Similar results were noted when isolates P303 and P303 *cps* were compared at 30 min postinoculation (Fig. 5C versus D). When rare unencapsulated mutants of TIGR4 were seen later at 20 h postinoculation, these were still confined to lumenal mucus rather than along the epithelial surface, suggesting an inability to transit to the epithelial surface as occurred for the encapsulated parent (Fig. 5E versus F).

We considered whether these in vivo observations could be explained by increased adherence of encapsulated pneumococci to epithelial cells. However, when the abilities of TIGR4 and TIGR4 *cps* to adhere to ciliated monolayers of primary mouse tracheal epithelial cells in culture were compared, the presence of capsule was associated with a >500-fold decrease in the percentage of the inoculum bound (data not shown).

Effect of capsule on interaction with mucus. Consequently, the role of capsule in allowing for escape from mucus was

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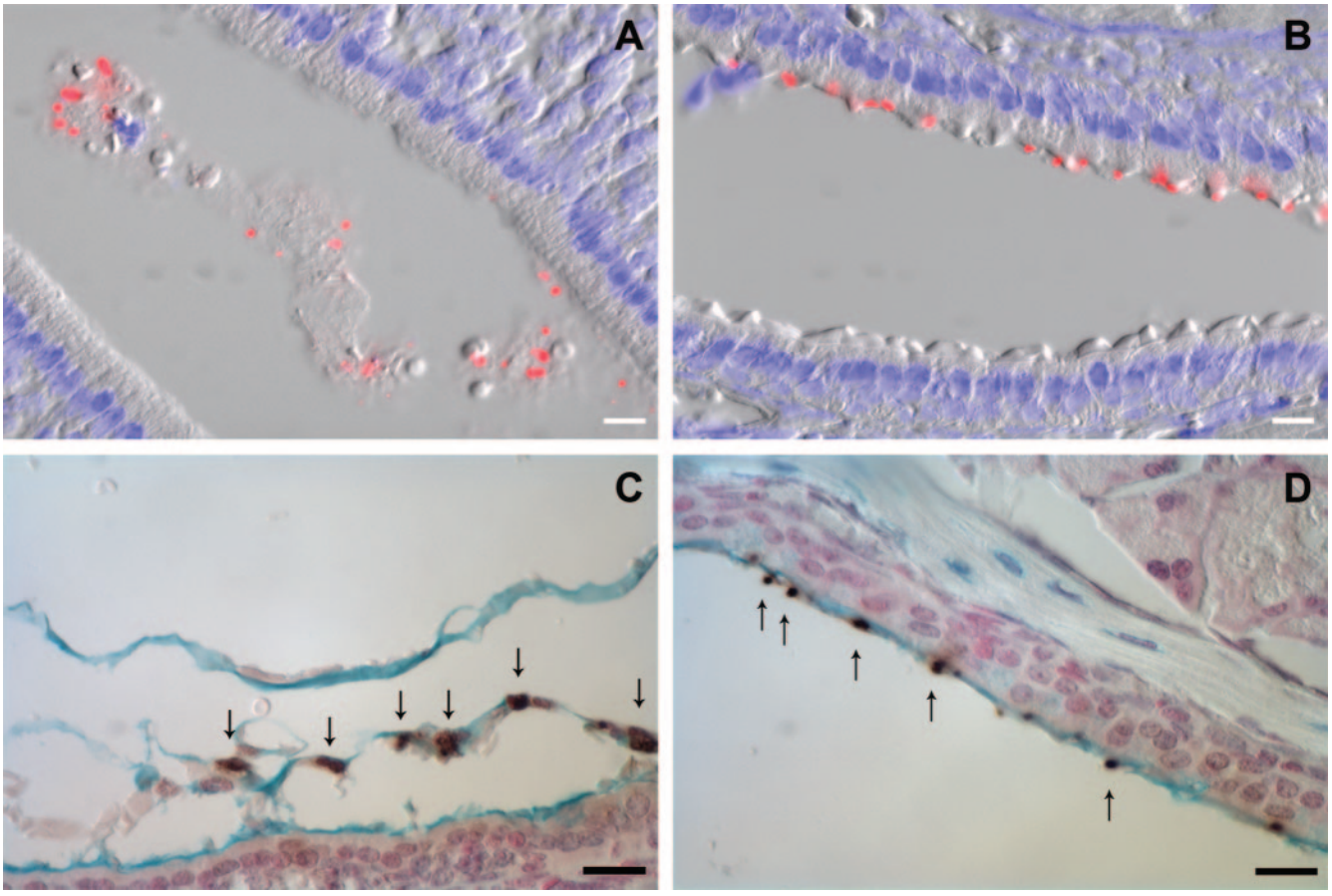

FIG. 4. Early events in colonization showing transition from mucus to the epithelial surface for encapsulated pneumococcal strain TIGR4. (A and B) Frozen nasal tissue from C57BL/6 mice colonized for 30 min (A) or 2 days (B) and stained with type-specific sera detected with Cy3 secondary antibody (red) and DAPI (blue) superimposed with Normarski bright-field optics to show the epithelial border. (C and D) Frozen nasal tissue from C57BL/6 mice colonized for 30 min (C) or 2 days (D) stained with type-specific sera and detected with horseradish peroxidase-conjugated secondary antibody and DAB substrate (brown), alcian blue (pH 2.5) (blue), and nuclear fast red (red). Arrows indicate bacteria. Scale bar, 10 μm.

examined. When TIGR4 and P303 were compared to their unencapsulated mutants for their relative ability to adhere to immobilized human airway mucus in vitro, the encapsulated strains showed significantly reduced mucus binding in a solid-phase assay (Fig. 6A). The ability of these encapsulated strains, but not their *cps* mutants, to evade binding to mucus was reduced by modifying the mucus by pretreatment with neuraminidase.

These results suggested that the larger amounts of PnPS/cell on O variants may increase their fitness during early events of colonization when escape from lumenal mucus is required. This was tested in vivo using inocula consisting of equal proportions of O and T variants of the same isolate. For isolates of two pneumococcal types, there was a strong selective advantage for O variants during initial colonization (<3 h) corresponding to the period when pneumococci are still predominately within the lumen of the nasal spaces (Fig. 2B). The effect of opacity was also examined in vitro using a model whereby the larger size and surface characteristics of opaque variants inhibit their passage through a column of acrylamide beads. When these beads were coated with human mucin, the relative efficiency of bacterial migration through the column was increased more for O than T variants (Fig. 6B). Once again, depletion of sialic acid from the mucin-coated beads reduced the advantage of increased encapsulation. Together these findings suggest that capsule acts to inhibit the association with lumenal mucus that promotes clearance in the early phases of colonization.

DISCUSSION

This study examined the role of capsule in the biology of the mucosal pathogen *Streptococcus pneumoniae*. We focused on the commensal state of the organism since colonization is the first step in all pneumococcal disease and by far the most common outcome of the bacterium-host interaction (2). We utilized a murine model of nasal colonization that recapitulates many of the key features of experimental and natural colonization of humans (19). Findings in this study reveal that only encapsulated organisms are able to transit efficiently from their initial site in a host, the lumenal mucus, to the epithelial surface. The capacity of encapsulated, but not unencapsulated, pneumococci to escape from lumenal mucus would allow access to host cell receptors on the epithelial surface of the nasal

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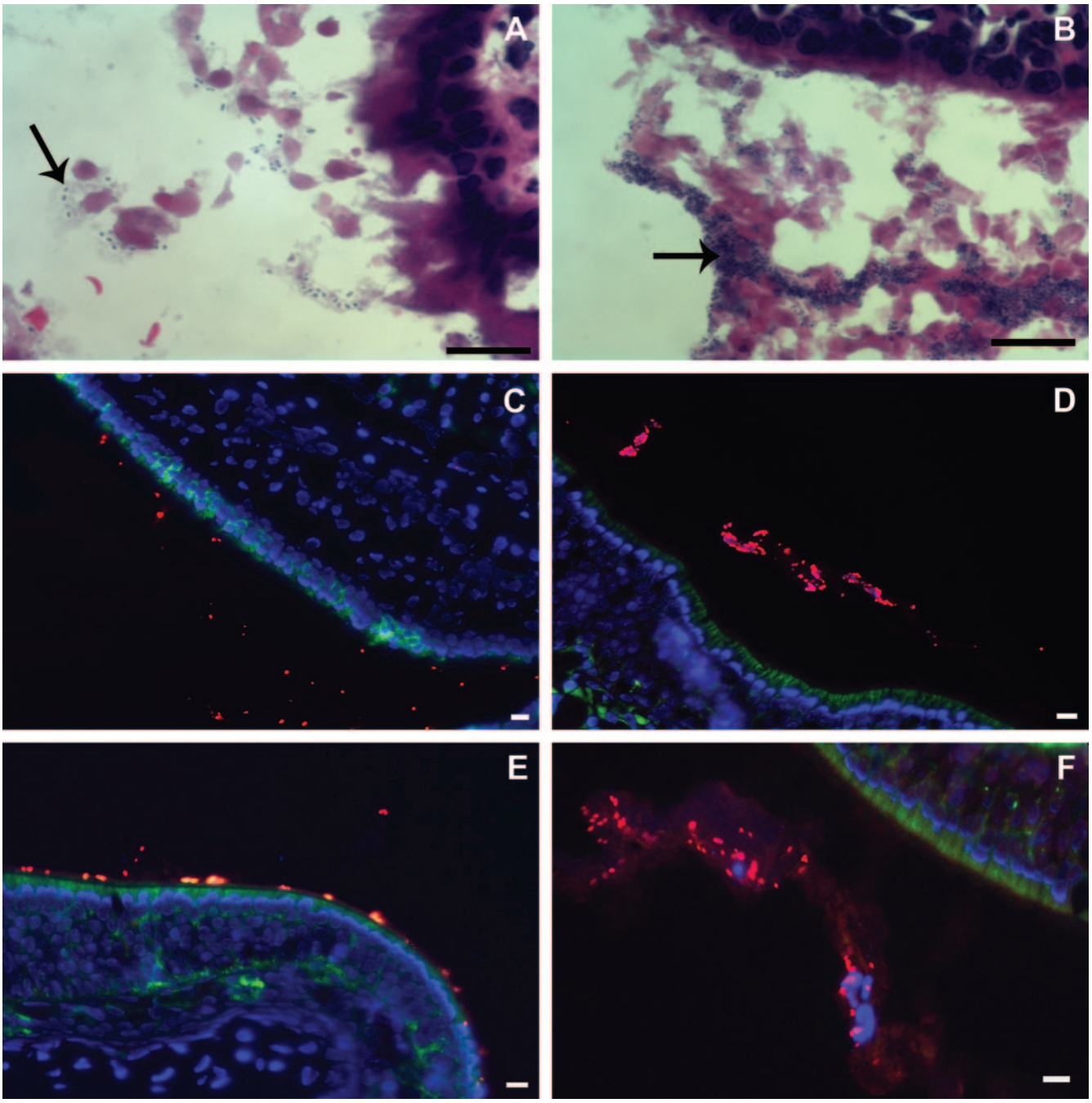

FIG. 5. Unencapsulated pneumococci remain trapped within lumenal mucus. (A and B) H&E-stained frozen nasal tissue from C57BL/6 mice 30 min postinoculation with (A) TIGR4 or (B) TIGR4 *cps*. Only the unencapsulated mutant is heavily agglutinated in mucoid material in the lumen. Pneumococci are marked by arrows. (C to F) Frozen nasal tissue from C57BL/6 mice comparing P303 (C) and P303 *cps* (D) or TIGR4 (E) and TIGR4 *cps* (F) at 30 min (C and D) or 20 h postinoculation (E and F) using staining with type-specific sera detected with Cy3 secondary antibody (red) and DAPI (blue). Tissue autofluorescence (green) reveals the epithelial border. Scale bar, 10 μm.

mucosa. Since only the bacteria closely approximated to these epithelial surfaces demonstrate stable colonization, escape from the mucus appears to be an important step in persistence and may explain the contribution of encapsulation to this mucosal pathogen.

In considering the mechanism for these observations, it seems unlikely that capsule facilitates the transition from the mucus to the epithelial surface by increasing the adhesive properties of the organism. Capsules are highly diverse in structure, and in vitro studies, including the testing of primary murine epithelial cells in this report, confirm that capsule substantially inhibits, rather than promotes, bacterial adhesion to host cells. We cannot fully exclude the possibility that capsule inhibits killing by antimicrobial substances. However, the

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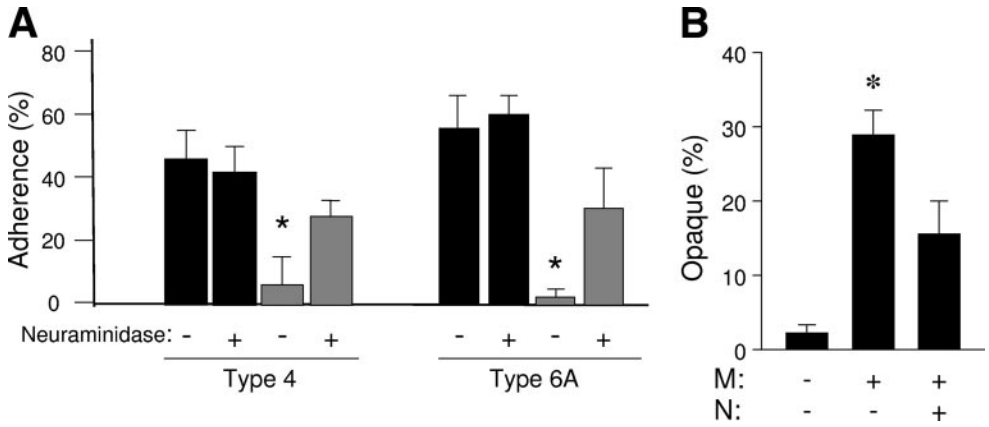

FIG. 6. Capsule inhibits mucus association. (A) The levels of binding of unencapsulated (black bars) compared to encapsulated (gray bars) strain TIGR4 (type 4) or P303 (type 6A) to immobilized human airway mucus were compared. Where indicated, mucus was pretreated with neuraminidase. Adherent bacteria were quantified by determining the proportion of the inoculum removed by a 60-min incubation at 4°C (nonadherent bacteria). Means of three independent experiments in duplicate ± standard deviation are shown. *, $P < 0.03$ compared to other experimental conditions for the same strain (Kruskal-Wallis test with Dunn's post-test for multiple comparisons). (B) The relative abilities of immobilized human airway mucins to inhibit the migration of O compared to T variants of a type 23F isolate were compared. A column of acrylamide beads was loaded with an equal mixture of O and T variants, and the colony phenotype was determined in cultures of the flowthrough. Where indicated, beads coated with mucin (M) were pretreated with neuraminidase (N). Values represent the mean of three independent determinations ± standard deviation. *, $P < 0.05$ compared to other experimental groups (Kruskal-Wallis test with Dunn's post-test for multiple comparisons).

marked effect of capsule in the rate of decrease in the density of colonization during the first hours following challenge, but not in subsequent days, is not supportive of such a hypothesis. We conclude, therefore, that the events seen by histological examination during early colonization are most consistent with the capsule enhancing escape of the organism from mucus and evasion of clearance by mucociliary flow. A further implication is that the optimal strategy for successful colonization requires a balance between sufficient PnPS to escape mucus and excessive PnPS inhibiting adherence to the epithelium. Pneumococci appear to deal with the advantages and disadvantages of capsule by spontaneously varying its expression between two phases. This study provides an explanation for O variants, previously shown to be relatively deficient at adherence and sustained colonization but more likely to survive in the bloodstream in animal models and humans because of increased amounts of PnPS/cell (5, 13, 37). Increased encapsulation by the more virulent O variants facilitates escape from lumenal mucus, ensuring a portion of the population will overcome this initial clearance mechanism. Our findings also offer an explanation for a prior report showing that a minimal amount of PnPS is needed for efficient colonization (18).

Why does capsule allow for escape from mucus? Mucus, comprised largely of mucopolysaccharides that are highly negatively charged due to an abundance of sialic acid and other anionic residues, traps and removes particles such as bacteria (26, 31). Microbes with surface characteristics that promote interaction with the mucus and subsequent removal by mucociliary flow may benefit from an altered surface charge that increases electrostatic repulsion. This hypothesis is supported by data showing that (i) restoration of capsule expression with negatively charged polysaccharides had the greatest impact on the density of colonizing bacteria and (ii) treatment of mucus with neuraminidase to remove negatively charged sialic acid residues increased the adherence of encapsulated bacteria. In the case of the pneumococcus, the negatively charged (or in a few instances uncharged) capsule may act to obscure positively charged surface features such as the quaternary amines on choline residues on its structurally conserved teichoic acids (7). In this regard, mucin glycoproteins have been shown previously to agglutinate nonencapsulated oral streptococci in a manner dependent on their sialic acid content (16). Other surface characteristics of the pneumococcus may also serve to release it from entrapment in mucus. These include its three exoglycosidases, which sequentially remove sugars, including sialic acid found on mucus and other human glycoconjugates that bind to the organism, and its IgA1-specific protease, which cleaves off the Fc_{α}-containing fragment linked to the mucus through a secretory component (14, 23, 38). Different experimental systems have shown conflicting evidence about the role of pneumococcal neuraminidase (NanA) in initial colonization (14, 22, 33).

Although capsules also act to inhibit opsonization of underlying bacterial surface components, our findings suggest that this may not be the primary function for which these structures have evolved on mucosal organisms. Despite an influx of neutrophils during colonization, opsonophagocytosis-mediated clearance of unencapsulated mutants did not account for their lower density during colonization. Pathogenicity, therefore, may not have been selected for among these organisms but may be a consequence of encapsulation that evolved primarily to serve a different purpose: escape from mucociliary clearance during their commensal state.

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