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Mechanisms of Bacterial Colonization of the Respiratory Tract

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Abstract

Respiratory tract infections are an important cause of morbidity and mortality worldwide. Chief among these are infections involving the lower airways. The opportunistic bacterial pathogens responsible for most cases of pneumonia can cause a range of local and invasive infections. However, bacterial colonization (or carriage) in the upper airway is the prerequisite of all these infections. Successful colonizers must attach to the epithelial lining, grow on the nutrient-limited mucosal surface, evade the host immune response, and transmit to a susceptible host. Here, we review the molecular mechanisms underlying these conserved stages of carriage. We also examine how the demands of colonization influence progression to disease. A range of bacteria can colonize the upper airway; nevertheless, we focus on strategies shared by many respiratory tract opportunistic pathogens. Understanding colonization opens a window to the evolutionary pressures these pathogens face within their animal hosts and that have selected for attributes that contribute to virulence and pathogenesis.

Keywords

nasopharynx; commensal; opportunistic pathogen; *Streptococcus pneumoniae*; inflammation

INTRODUCTION

Acute respiratory tract infections are a major source of disease worldwide, with pneumonia alone responsible for more than 1.3 million child deaths annually (84). Among the most frequent bacterial causes of pneumonia are *Streptococcus pneumoniae* (the pneumococcus), *Haemophilus influenzae*, and *Staphylococcus aureus*. Common to all these pathogens, as well as other opportunistic invaders of the respiratory tract, is the requirement for asymptomatic bacterial colonization, or carriage, to precede local and systemic disease (9, 31, 120).

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Upper respiratory tract colonization not only causes disease but also drives the evolution of these opportunistic pathogens. Transmission to new susceptible hosts occurs from the reservoir of bacteria colonizing the oro- or nasopharynx, rather than from invasive infections. As a result, the demands of colonization form the selective pressures that promote pathogen adaptation and virulence (129). Colonizing bacteria must avoid a range of host challenges, from penetrating the mucous barrier to evading professional phagocytes and obtaining a source of nutrients on the harsh mucosal surface (Table 1). These essential activities are summarized in the stages of colonization: bacterial acquisition and attachment to the epithelial lining, obtaining nutrients, replication, persistence in the face of the host immune response, and transmission to a new host (Figure 1). Here, we review the mechanisms underlying each of these steps. We focus on colonization by opportunistic pathogens that have the potential to cause disease, rather than bacteria that exclusively share a commensal relationship with their host. Most notable, and most studied, among these is the pneumococcus. We also focus on evidence from in vivo experimental models—primarily rodents, with some work in humans and nonhuman primates, as these manipulable models provide mechanistic insight. This review highlights broad themes and commonalities shared by many of these opportunistic pathogens that colonize the nasopharynx as the first step in pathogenesis.

ACQUISITION AND ATTACHMENT

Stable colonization of the nasopharynx requires adherence to the mucosal surface, lest colonizing bacteria be rapidly swept away by mucociliary clearance (29). So important is remaining on the epithelial surface, at least until the time of transmission, that opportunistic pathogens of the airway have developed multiple strategies for attachment, which span three broad categories: transient association with mucus, weak association with host carbohydrates, and strong association with host surface proteins.

The importance of mucus in protecting against bacterial acquisition was demonstrated by experimental colonization of ferrets with *Staphylococcus aureus*. Early after inoculation, nearly all staphylococci were in the mucous gel layer, rather than being bound to epithelial cells (101). Whereas some bacteria may bind to glycoconjugates in the mucous layer, others exit it to reach the epithelial surface by repelling anionic mucus with a negatively charged polysaccharide capsule (81). Using capsule to pass through mucus, however, complicates attachment to the epithelium, as the thick polysaccharide capsule also repels host cells. Airway colonizers have evolved multiple mechanisms of phenotypic variation to adjust quickly to different microniches during carriage. Pneumococci, for example, undergo phase variation, or rapid on-off switching, apparent as differences between opaque and transparent colony morphologies. Opacity is a reflection of bacteria-bacteria interaction, and differences stem from cell surface changes that affect these associations. Opacity phase variation has recently been linked to epigenetic changes in DNA methylation (62). Opaque variants display more capsule but less teichoic acid and less of other adhesins such as PspC (CbpA) (48). Only transparent variants could effectively colonize infant rats (48). Opacity variation, while leading to the emergence of more virulent, highly encapsulated organisms during sepsis, could in the nasopharynx serve to promote exit from mucus (highly encapsulated opaque variants) and then binding to the epithelium (transparent variants with less capsule to

block adhesin interactions). Lipopolysaccharide (LPS) modifications causing colony opacity variation also contributed to colonization of infant rats by *Haemophilus influenzae* (128). Not all bacteria need to downregulate capsule to bind the epithelial surface stably, however. For example, the hyaluronic acid capsule of *Streptococcus pyogenes* is bound to CD44 on host cells (21).

The second stage of attachment is thought to involve weak attachment by bacterial lectin-like proteins to host surface carbohydrates (121). In studies of the chinchilla airway, pneumococcal colonization led to desialylation that was dependent on expression of the bacterial neuraminidase NanA. A *nanA* mutant was unable to colonize chinchillas, indicating that sialic acid cleavage was important either to exit the heavily sialylated mucus or to reveal underlying host glycoconjugate receptors (112, 113). The effect of NanA may depend on the animal model, however, as other work found no effect during colonization of infant rats (49). In vitro, the pneumococcal exoglycosidase BgaA binds specific host surface carbohydrate residues revealed by the removal of sialic acid (108).

More stable adherence comes through interaction with host cell surface proteins (121). Pili are important for adhesion and colonization for pneumococci (6), *H. influenzae* (42), and *Moraxella catarrhalis* (56); but they are not the only adhesive factor. In pneumococci, RrgA is a pilus-associated adhesin required for adhesion in both the presence and absence of pili (80). Pili may aid in extending adhesins, including beyond the capsule, to allow for binding to the host (80). Another pneumococcal adhesin is PspC (CbpA), which binds secretory component on the polymeric immunoglobulin receptor (85). Fibronectin-binding proteins PavA and PavB were also important during pneumococcal colonization (41, 43). The filamentous hemagglutinin (FHA) of *Bordetella* species has been implicated in adherence in vivo. In *Bordetella bronchiseptica*, FHA was necessary for colonization of mice, and the gene encoding FHA is one of the most expressed in Bvg^+ *Bordetella*, the phase of growth when virulence genes are expressed under the control of the BvgAS two-component system (20). However, ectopic expression of FHA during the Bvg^- phase was not sufficient to promote adherence in vivo (20), underscoring the importance of tight control over adhesin expression for effective colonization. Similarly, pilus expression in pneumococci appears to be regulated, as it peaks during the early phases of murine colonization (86).

Adhesins in *S. aureus* follow a similar pattern of modulated expression. Wall teichoic acids on the *S. aureus* cell surface were required for colonization of cotton rats (126), binding to the nasal cavity surface receptor SREC-I (7). Clumping factor B (ClfB) was necessary for experimental human colonization and bound cytokeratin 10 in vitro (130). More recent work using knockout mice and bacterial mutants demonstrated that the receptor for ClfB was not cytokeratin 10 but rather loricrin, a protein found on the cornified envelope of the squamous epithelium in the anterior nares, the site of *S. aureus* colonization (76). When exogenously expressed in *Lactococcus lactis*, ClfB was sufficient for adherence and colonization of wild-type, but not loricrin-deficient, mice (76). These results emphasize the importance of in vivo studies; in vitro studies did not identify the relevant ligand-receptor interaction necessary for colonization. Using cotton rats, Burian et al. found that wall teichoic acid adhesins were expressed in vivo early during colonization and replaced over time as ClfB and the adhesin IsdA were expressed (14).

GROWTH AND NUTRIENT SOURCES

After establishing a stable presence on the mucosal surface, colonizing organisms must find a source of nutrients to promote replication. Growth during colonization amplifies bacterial density, potentially increasing the likelihood of transmission to the next host. However, proliferation is particularly challenging in the nasopharynx. Free carbohydrates are not abundant in the normal, uninflamed upper respiratory tract (90), and the host actively depletes glucose from the airway surface fluid as a form of nutritional immunity (89).

van Opijnen & Camilli demonstrated the difficulties of replication in the nasopharynx using transposon mutagenesis of pneumococci and the method Tn-seq to catalog the genes necessary in different host and in vitro environments (115). The nasopharynx was the harsher microniche: When genes that were required in both the upper and lower respiratory tracts were absent in the nasopharynx, larger average fitness defects were observed, suggesting that these genes were more important for establishing colonization than pneumonia (115). Using dilution of a temperature-sensitive, antibiotic-resistance-expressing plasmid, they calculated pneumococcal doubling times for replication in the mouse nasopharynx (161 min) and lungs (108 min), which suggested that nutrients are less readily available at the site of initial colonization (115). This doubling time was consistent with results obtained using a flow cytometric method to quantify the dilution of CFSE, a fluorescent dye commonly used to track eukaryotic cell division, from pneumococci colonizing the mouse upper respiratory tract. Influenza coinfection accelerated CFSE dilution, and therefore replication, by providing a carbon source via mucin secretion (107).

Pneumococci depend on carbohydrates as a source of carbon for growth and are particularly adept at accessing them on the airway surface. Among commensal and pathogenic bacteria that colonize the upper respiratory tract, pneumococci express the most diverse array of sugar transporters and utilization genes (13). More than 30% of transporters in the pneumococcal genome were predicted to import carbohydrates as substrates, a higher proportion than any other bacterium sequenced (111). By contrast, *H. influenzae* and *Neisseria meningitidis* had few carbohydrate transporters (111). Pneumococcal colonization independently requires catabolism of sialic acid (65, 114), hyaluronic acid (66), and sucrose or a related disaccharide (39). It also requires an ATPase to energize multiple ABC transporters used for sialic acid, raffinose, and maltotetraose (64). This wide range of transporters and bioavailable sugars does not reflect redundant pathways, however, as suggested by the decreased colonization seen with any individual mutation (13). Rather, each transporter may be important in specific microenvironments encountered during colonization or in certain hosts, such as the particular importance of sialic acid during colonization following influenza (107).

Other opportunistic pathogens of the airway use additional sugars during colonization, though in vivo evidence is more lacking. *N. meningitidis* required lactate permease to utilize this carbon source in vitro and to grow in nasopharyngeal explants (28). Sialic acid catabolism was not required for *H. influenzae* to colonize the infant rat, though sialylation of the bacterial surface contributed to immune evasion (118). During experimental *S. pyogenes* pharyngitis in macaques, microarray analysis revealed high levels of maltodextrin and

mannose catabolic gene expression in bacteria early during colonization (119). A maltodextrin-binding protein was required for *S. pyogenes* to colonize the mouse oropharynx (103). A transcriptional repressor in the same operon was also necessary for colonization, implying that tight regulation of carbohydrate utilization genes is important during carriage (102). Similarly, the nutritional repressor CodY was required during pneumococcal colonization, and its transcript was expressed in vivo in the nasopharynx (35). These modes of regulation imply there may be a temporal switch during colonization from rapid replication to a later, more silent phase of quiescent persistence.

Nearly all bacteria, including all opportunistic pathogens of the airway, require an iron source to survive (127). Mammalian hosts have developed mechanisms to combat this essential microbial activity by sequestering ferric iron or targeting bacterial uptake mechanisms (127). These mechanisms are active in the nasopharynx, as evidenced by the high levels of *isdA* expression in *S. aureus* colonizing the human airway (15). Moreover, that expression of this tightly-iron-regulated gene implies *S. aureus* is growing under iron-limited conditions in vivo (15). One bacterial strategy to acquire iron is secretion of siderophores, low molecular weight proteins that bind iron with high affinity (127). In contrast to inhabitants of the gut, respiratory colonizers, including pneumococci, *H. influenzae*, and *N. meningitidis*, tend not to secrete siderophores and instead obtain iron by directly retrieving iron-containing molecules such as lactoferrin, transferrin, hemin, and hemoglobin (79, 110, 127). This strategy may have evolved to avoid host antisiderophore defenses, including secretion of lipocalin-2 (siderocalin), which binds enterobactin-type siderophores to prevent their uptake by bacteria (79, 127). Lipocalin-2 was induced in the murine nasopharynx during colonization with pneumococci and *H. influenzae*, as well as in the human nasal mucosa, raising the possibility that these bacteria were actively inducing a host response that could eliminate competitors without being susceptible themselves (79). *S. aureus* and bordetellae produce siderophores that are structurally distinct from enterobactin and, as a result, can evade lipocalin-2 (79). The precise in vivo importance of many of these iron-acquisition mechanisms has not been tested in nasopharyngeal colonization, though *S. aureus* colonization of the infant rat airway was enhanced by the presence of hemoglobin (96).

Nutritional immunity extends to other transition metals, particularly zinc and manganese, additional critical cofactors for many bacterial functions (46). Pneumococci obtain both zinc and manganese via the PsaA protein, but excess zinc can be toxic to colonizing bacteria and can compete with manganese for uptake by pneumococci (71). The ratio of Zn^{2+} to Mn^{2+} becomes elevated in the nasopharynx during pneumococcal colonization, suggesting the host may be actively increasing zinc or limiting manganese to combat incoming bacteria (71).

IMMUNE EVASION AND PERSISTENCE

Scarcity of resources is not the only reason the mucosal surface is harsh. From the time of acquisition, colonizing bacteria interact with and must evade the host mucosal immune system long enough to spread to susceptible hosts. Bacteria have evolved a range of strategies to evade the different branches of host immunity. Innate responses can be actively thwarted by specific effectors that target host cells. Bacteria can evade detection by

shielding themselves with host-mimicking or antigenically varying molecules. Rapid transmission can also be thought of as an evasion strategy, as exit to a new host can occur before the onset of adaptive immunity.

Bacterial contact with the immune system begins on entry into the nasopharynx, where colonizers are met by the layer of mucus that coats the upper respiratory tract. Mucus itself provides a physical barrier bacteria must traverse to avoid entrapment and removal by mucociliary clearance. Additionally, mucins serve as a scaffold for antimicrobial proteins (29). So important is the mucous barrier to infection that the primary virulence factor common to many opportunistic pathogens of the upper respiratory tract, capsular polysaccharide, may have evolved to combat it. While capsule is well known for its role in limiting opsonization by complement and antibody, it also contributes to evading mucus-mediated clearance. Unencapsulated pneumococci were inhibited in early colonization of mice compared with isogenic bacteria expressing a capsule (81). All but a few of the >90 pneumococcal capsule types are negatively charged, allowing for repulsion from the anionic mucous barrier. The lack of colonization without capsule was not due to increased susceptibility to neutrophils, complement, or antibody, and unencapsulated mutants were observed stuck in mucus rather than reaching the nasopharyngeal epithelium (81).

Even without capsule, successful respiratory tract colonizers have alternative methods to resist antibody-mediated clearance. Pneumococci, pathogenic *Neisseria* species, and *H. influenzae* all encode a protease specific to human immunoglobulin A1 (IgA1), the dominant IgA present in the human respiratory tract. In vitro, these proteases cleave IgA1, retaining Fab fragment binding to the bacterial surface but preventing antibody effector functions by removing the Fc fragment (93). Mice passively immunized with human protease-sensitive IgA1, but not protease-insensitive IgA2, were protected from intranasal infection with IgA1-protease-producing, but not IgA1-protease-deficient, pneumococci (40). Other bacteria do not effectively evade humoral immunity. Clearance of *Bordetella pertussis*, and the related species *Bordetella parapertussis* and *B. bronchiseptica*, was delayed in mice lacking mature B cells (50).

Colonizing bacteria modify their cell surface to resist antibodies and other mechanisms of targeting microbes for clearance. Phosphorylcholine (ChoP), a constituent of the surface of multiple respiratory tract bacteria, is poorly immunogenic, as it mimics host structures including phosphatidylcholine found in membrane lipids. For example, ChoP modifies the LPS of nontypeable *H. influenzae* (NTHi), preventing binding of bactericidal antibody by altering accessibility to underlying bacterial antigens (17). During colonization, adaptive immunity was required to select for ChoP-expressing NTHi, a process that occurs through phase variation, the rapid switching of translation of a choline kinase gene on or off through slipped-strand mispairing in a repetitive DNA motif (17). LPS biosynthetic gene phase variation and selection for the ChoP-on phase has also been observed during experimental human colonization (95). In a different form of genetic regulation, the LPS of *B. bronchiseptica* is modified by *pagP*, a palmitoyl transferase that is under the control of the BvgAS two-component system that regulates virulence gene activity (92). These LPS modifications increase evasion of antibody-dependent complement-mediated lysis (92).

After transiting the mucous layer, colonizing bacteria reach the epithelial surface, where they are rapidly sensed by multiple innate pattern recognition receptors that contribute to clearance. Lipoteichoic acids of pneumococci (116) and *S. aureus* (30) are sensed by Toll-like receptor (TLR) 2, whereas lipoproteins and LPS from *H. influenzae* can be recognized by TLRs 2 and 4, respectively (133). TLR4 has also been reported to recognize the pneumococcal pore-forming toxin pneumolysin (60). In addition to being sensed by surface receptors, these predominantly extra-cellular pathogens are recognized by host cytosolic detectors that contribute to clearance, such as nucleotide-binding oligomerization domain (Nod) receptors that detect peptidoglycan from bacterial cell walls. Signals from Nod1, which recognizes gram-negative peptidoglycan, are important in controlling *H. influenzae* carriage (133), whereas Nod2, which senses peptidoglycan fragments from both gram-positive and gram-negative bacteria, contributes to pneumococcal clearance (24). Further in vivo evidence of intracellular sensing of extracellular pathogens stems from findings that type I interferons are produced during pneumococcal colonization and required for clearance of the organism (88). Other work has shown, however, that synergistic, far larger increases in type I interferon during influenza-pneumococcal coinfection impairs pneumococcal clearance (78).

The initial response to colonization with a range of opportunistic pathogens is generally a mild suppurative rhinitis that can often be subclinical and inapparent but reflects infiltration of neutrophils into the nasopharynx (1, 30, 116, 133). For many respiratory tract colonizers, this initial influx is insufficient to clear colonization, either because the phagocytes are ineffective at clearing these encapsulated microbes prior to development of specific antibody or because the bacteria actively evade neutrophil-mediated killing (68). The adenylate cyclase toxin of *B. bronchiseptica*, for example, inhibits neutrophil effector functions by inducing cAMP overproduction from ATP, allowing the bacteria to avoid mucosal clearance (34, 72).

Neutrophils are recruited in response to pneumococcal acquisition but are insufficient for clearance from the upper respiratory tract in the absence of cellular and adaptive immunity (116). CD4 T cells are necessary and sufficient to provide acquired immunity to secondary pneumococ-cal challenge, and mice deficient in the IL-17A receptor also cannot control primary colonization (55). Sensing of pneumococci by TLR2 is required to induce Th17 responses, which lead to macrophage recruitment into the nasopharynx. These macrophages are the effector cells of pneumococcal clearance, as demonstrated by depletion experiments with clodronate liposomes (132). Th17 and macrophage responses were also dependent on intracellular signaling through Nod2, which additionally led to production of the macrophage/monocyte chemoattractant CCL2 (24). CCL2 sensing by its receptor CCR2 led to increased clearance of carriage. Pneumolysin pore formation is also required in vivo for *Ccl2* expression, suggesting a potential mechanism for access of pneumococcal products to cytosolic sensors such as Nod2. Lysozyme, which digests peptidoglycan to generate fragments for Nod2-dependent signaling, was also required for these effects on clearance (24). Pneumococci have enzymatic mechanisms to limit lysozyme's effects by modifying its peptidoglycan structure to avoid lysis (23).

Consistent with their resistance to opsonophagocytic killing in the airway, pneumococci are cleared following recognition by nonopsonic receptors. Expression of MARCO (macrophage receptor with collagenous structure), though not directly responsible for pneumococcal uptake by upper respiratory tract macrophages, contributes to monocyte and macrophage recruitment, *Ccl2* upregulation, and clearance of pneumococcal carriage (27). Macrophage migration inhibitory factor is also required during pneumococcal colonization to sustain the presence of macrophages, *Ccl2* upregulation, and clearance (22). Expression of the microRNA miR-155 also contributes to Th17 induction and macrophage recruitment during pneumococcal carriage (117). *B. pertussis* protection from whole cell vaccination is also mediated by IL-17 (36), and colonization of baboons, a model that more naturally resembles human infection than do rodents, leads to Th17 induction and downstream effector production, such as neutrophil chemoattractants (124). Acellular pertussis vaccination inducing Th1 instead of Th17 immunity did not protect against carriage in baboons (125), confirming the importance of Th17 responses in clearing *B. pertussis*.

Although Th17 responses seem to be broadly conserved in clearance of airway colonizers, the effector mechanisms by which IL-17 acts can vary by pathogen. For *S. aureus*, clearance of carriage required T cells and IL-17A, which promoted recruitment of neutrophils, not macrophages, into the nasal lumen. Neutrophils were required for clearance, though IL-23 was not needed to amplify IL-17A responses, which may have been driven by IL-1β secretion instead (4). Other work demonstrated that unencapsulated *S. aureus* is cleared faster from the nasopharynx, but only after the first week postinoculation (51). This time point coincides with the influx of neutrophils into the nasopharynx (4), suggesting that the *S. aureus* capsule resists neutrophil-mediated killing.

Paradoxically, macrophage recruitment, and eventual clearance, requires pneumococcal expression of its toxin, pneumolysin (22). It may seem counterintuitive for a colonizing organism to induce the specific host response that clears it, but it is possible that persistence in the respiratory tract is sacrificed at the expense of inducing a more robust inflammatory response, which could promote nutrient acquisition and transmission to a new host (129). Early during colonization, pneumolysin promotes higher bacterial density (44) and increased neutrophil influx (68) in the nasopharynx, supporting these possibilities.

MODIFIERS: HOST, COMPETITORS, COINFECTIONS

The balance between host and colonizer can be tipped in either direction by a range of factors that modify each of the previously described stages of attachment, growth, and immune evasion. Both congenital and acquired immunodeficiencies can predispose to disease caused by the encapsulated pathogens that colonize the respiratory tract. These conditions, such as deficiencies in signaling downstream of TLRs and IL-1 receptor (IRAK-4 deficiency) or lack of a functional spleen, lead to higher rates of invasive disease, particularly with pneumococci (16). For many of these diseases, the time of greatest susceptibility to invasive pneumococcal disease is early childhood, a period during which >50% of even healthy children are colonized with pneumococci (91). It is unclear, therefore, to what extent the pathways affected by immunodeficiencies that cause excess pneumococcal disease are also responsible for promoting bacterial colonization. In mice,

deficiencies in MyD88, a signaling adapter upstream of IRAK-4, caused increased susceptibility to both invasive disease and higher density of pneumococcal carriage in the nasopharynx (2). Less dramatic but still an important determinant of disease is the age of the host. Both colonization and invasive disease occur largely in young children, and mice colonized with pneumococci as infants were impaired in clearing the bacteria (10, 106). In aged mice, pneumococcal clearance was slower than in young adult mice and was associated with decreased influx of macrophages, the effector cells of clearance (53).

Opportunistic pathogens colonizing the respiratory tract compete with a range of other bacteria, starting with the preexisting commensal flora. Although the mechanisms remain unknown, bacteria adapted to particular hosts seem to be more capable of displacing that host's microbiota. For example, *B. bronchiseptica*, which can naturally colonize mice, could replace the culturable mouse nasopharyngeal flora after inoculation of <100 colony-forming units. In contrast, stable colonization of mice with *B. pertussis*, a human-adapted pathogen, required a more than 100-fold higher inoculum and still could not displace the indigenous flora. However, local antibiotic treatment to deplete commensal bacteria lowered the required infectious dose of *B. pertussis* to <100 colony-forming units; still, the presence of one species of mouse-commensal bacteria could inhibit *B. pertussis* colonization even in antibiotic-treated animals (131). At least one commensal bacterium has the ability to fight back against opportunistic colonizers. *Staphylococcus epidermidis* can secrete a serine protease, Esp, and doing so allows *S. epidermidis* to inhibit *S. aureus* carriage (38), possibly by inhibiting biofilm formation or cleaving adhesins. In experimental colonization of humans, preexisting *S. aureus* carriage was cleared by inoculating participants with Esp-expressing *S. epidermidis* or purified Esp protease (38).

In some instances, opportunistic colonizers pose a competitive threat to other colonizers. Pneumococcal carriage is associated with a lower risk of *S. aureus* colonization in children (98). These two species occupy different niches within the airway (the posterior nasopharynx and anterior nares, respectively), suggesting that these bacteria may not directly compete. In vivo evidence suggests that the host immune system mediates competition (54). Prior pneumococcal colonization of mice protected against secondary challenge with *S. aureus*, in an antibody-dependent manner. Pneumococcal colonization elicited antibodies that cross-react with *S. aureus*, and antibodies to a specific pneumococcal protein that could also target a homologous *S. aureus* protein were necessary and sufficient to protect against *S. aureus* carriage (54).

Another immunologic mechanism has been described as mediating the negative interaction between colonizing pathogens. Cocolonization of mice with *H. influenzae* and pneumococci led to synergistic increases in neutrophil chemoattractant production and neutrophil influxes, in a manner dependent on production of the pneumococcal pore-forming toxin pneumolysin (97). This increased recruitment of neutrophils was required, along with complement, for opsonophagocytic clearance of pneumococci during cocolonization, while *H. influenzae* persisted (58). Neutrophils had to be activated by Nod1-mediated recognition of *H. influenzae* peptidoglycan to promote pneumococcal clearance (57). One colonizing organism (*H. influenzae*) was therefore able to effectively outcompete another by inducing

an immune response that it could evade—using the host to target the other organism rather than doing so directly. This process has been referred to as within-host competition (12).

Direct interactions can occur within one species as well. For instance, pneumococci express bacteriocins in vivo and can use these antimicrobial peptides to outcompete pneumococcal strains that lack a cognate immunity protein (25). Pneumococci can also benefit from other cocolonizing strains, however, due to their natural transformability. Rates of transformation were ~10^7-fold higher during murine colonization than during planktonic growth in sepsis (67). Transfer of antibiotic resistance occurred by homologous gene transformation even without selective pressure from antibiotic exposure, suggesting that colonization provides pneumococci with ample opportunity to obtain new genetic material and benefit from cocolonization with the same or closely related species (67).

Coinfection with viral pathogens also influences bacterial colonization of the airway. The best understood of these interactions is influenza infection predisposing to pneumococcal disease (69). Multiple mechanisms have been proposed for how influenza increases susceptibility to pneumococcal pneumonia, with more limited work addressing how influenza might alter colonization, the prerequisite to pneumonia. Early work focused on influenza leading to a denuded airway epithelium, promoting increased bacterial adherence (37, 94), but increased adherence is not required for influenza to promote secondary bacterial disease (73). Nakamura et al. (78) provided an immunologic explanation for this interaction, finding that coinfection leads to synergistic increases in type I interferons that decrease CCL2 induction and prevent macrophage recruitment to the upper respiratory tract, limiting pneumococcal clearance. Influenza can also provide a nutrient source to colonizing pneumococci (107). Host recognition of influenza leads to increased secretion of Muc5ac, an airway mucin that is heavily sialylated. Pneumococci exploit this influenza-provided sialic acid from mucins to accelerate growth in vivo (107). Influenza also predisposes to murine colonization with *H. influenzae* (37) and *S. aureus* (74), the two most common pathogens of postinfluenza disease, after pneumococci (69).

PROGRESSION TO DISEASE

Potentially pathogenic bacteria that first colonize the airway can cause a range of local and systemic disease, although this is not required to establish carriage. Local and systemic disease do not promote spread to new susceptible hosts and therefore do not exert selective pressure on these organisms (45, 129). In contrast, the demands of colonization lead to the development of virulence factors that contribute to disease (129).

Local spread within the respiratory tract can extend into normally sterile sites, including the middle ear spaces, sinuses, and lungs. Bacteria colonizing the nasopharynx can be aspirated into the lower respiratory tract, and higher density of pneumococcal carriage has been associated with increased risk of pneumonia (3). It was not clear in these human studies, however, whether the increased bacterial load in the upper airway preceded or followed pneumonia. Experimental colonization of mice followed by bronchoalveolar lavage revealed correlation between higher nasopharyngeal density and increased numbers of bacteria in the

lungs (107). Influenza infection also predisposes to pneumococcal pneumonia by increasing bacterial adherence and inhibiting antibacterial innate immune defenses in the lung (69, 73).

Invasion directly from the colonizing reservoir in the nasopharynx occurs via common pathways. TLR-mediated recognition of pneumococci and *H. influenzae* leads to p38 MAPK and TGF-β activity, which decreases claudin expression and promotes epithelial opening, promoting bacterial translocation (18). The transcriptional repressor SNAIL1 is induced by TLR2 and TLR4 stimulation and downregulates claudin expression (8). LPS signaling through TLR4, even without bacterial colonization, is sufficient to promote serum leakage onto the mucosal surface (8). Host sensing of bacterial colonization promotes an inflammatory response that requires epithelial opening to allow professional phagocytes to enter the nasopharyngeal lumen. Encapsulated pathogens that survive once accessing the systemic circulation can exploit this patterned host response (18). Some pathogens have more specific invasion strategies, such as *S. pyogenes*, which transits the epithelium through M cells—specialized cells that sample the lumen for antigens (87). Invasion is not limited to pathogens, however, as viable *Lactobacillus murinus* can be found in the nasal-associated lymphoid tissue (NALT). This commensal is found at lower rates than *S. pyogenes*, however, implying either that pathogens translocate more efficiently or persist in sterile sites more readily, or both (19). In infant rats subjected to intranasal challenge, a single surviving organism of *H. influenzae* type b was sufficient to cause bacteremia (63). Accessing the intracellular space can also promote persistent colonization because the organism avoids immune-mediated clearance (109). Viable NTHi can be found in human adenoid tissue and bronchial epithelium samples (109).

TRANSMISSION

Comparatively little is known about the mechanisms underlying transmission of bacteria in the upper respiratory tract. Spread from the reservoir of bacteria in the nasopharynx to a susceptible host is a necessary aspect of the biology of those microbes that can only grow within a host. For common respiratory tract colonizers, survival on environmental surfaces can be as brief as 1 day, such as with pneumococci, or as long as 7 months, as with *S. aureus* (52). The evolution of bacteria that depend on animal hosts, therefore, is likely driven in large part by the demands and constraints of transmission. Only recently have animal models been developed to address this hypothesis.

Although rodent models have been informative about mechanisms of bacterial colonization and disease in the upper and lower airway, they have been less informative in studies of transmission. This failure in part stems from the larger bacterial inocula needed to establish colonization in some rodent models of human pathogens compared with natural infection. Mouse models of transmission of respiratory pathogens are particularly limited, however, by a lack of effective cough reflex, hampering airborne transmission that largely occurs by contact with bacteria-containing droplets (75, 77). Recent work has relied on influenza coinfection to potentiate pneumococcal transmission within litters of infant mice (26). In this model, influenza infection leads to spread of established pneumococcal colonization to new naive infant hosts, a process that occurs over a few days (26). In one set of studies, influenza increased pneumococcal titers in the directly inoculated donor, or index, mice (26), and

increasing these bacterial numbers by neutrophil depletion was sufficient to promote transmission to influenza-infected recipient, or contact, mice (105). Earlier, more observational transmission models in infant rodents for pneumococci (61) and *H. influenzae* (32) suggested that influenza was not absolutely required for transmission. More recent studies have suggested that influenza coinfection leads to increased transmission not because of higher bacterial titers in the donor mice but rather because of increased shedding from donor mice (99).

Studies in rodents have focused on transmission among litters of suckling infants, a scenario less relevant to human disease. *H. influenzae*, however, was also transmitted among weaned rats, demonstrating that the presence of the dam is not required for at least some transmission models (32). Other studies have physically separated donor and recipient animals, demonstrating that airborne transmission can occur without direct contact (11, 70). However, even for respiratory pathogens, direct contact with bacteria-containing droplets may not always be required for transmission, as desiccated pneumococci were recovered and successfully used to colonize mice up to 4 weeks after drying on an environmental surface (122).

Unlike mice, ferrets do sneeze, allowing for airborne transmission. In a ferret model of coinfection influenza promoted increased pneumococcal colonization and transmission and potentiated transmission over longer distances (70). Higher bacterial density in the donor ferrets was not sufficient for increased transmission, as influenza infection also increased the colonization density of a different pneumococcal strain that was not transmitted to recipient ferrets (70).

Host pattern recognition receptors have also been implicated in transmission, as *Tlr2*$^{-/-}$ mice had increased pneumococcal transmission during influenza coinfection and *Tlr4*$^{-/-}$ mice demonstrated increased *B. bronchiseptica* transmission (99, 100). TLR expression in donor animals limited influenza titers and bacterial shedding, and its absence was required for high levels of transmission (99, 100).

The inflammatory response to colonization and coinfection stimulates transmission, though precisely how is not yet understood. Inflammation from LPS inoculation could substitute for influenza infection in recipient, but not donor, mice to promote transmission (105). In another model, TLR2 deficiency that led to increased transmission was associated with increased bacterial shedding, a more robust neutrophil influx, and higher induction of the secreted airway mucin Muc5ac (99). Increased mucin expression suggested a potential mechanism underlying inflammation-induced transmission. In ferrets, influenza also promoted increased mucus secretions (70). Neutrophil influx to the nasopharyngeal lumen resulted in increased mucus secretion in vivo (33), which could facilitate bacterial shedding and spreading to new hosts. In *B. bronchiseptica* transmission in pigs, however, expression of the type III secretion system, which induces inflammation, was not required for transmission, suggesting that perhaps not all bacteria take advantage of inflammation for transmission (83).

Bacterial factors underlying transmission are largely unknown. Using pigs as a natural model, one study found that, surprisingly, transition between Bvg^+ and Bvg^- phases was not required for *B. bronchiseptica* transmission, as Bvg^+ phase–locked strains transmitted as well as wild-type bacteria (82). It is possible that even this natural model of transmission did not accurately reflect the time *Bordetella* must survive in the environment between hosts, a niche that would require switching to the Bvg^- phase (82). In *S. pyogenes*, inactivation of the *covR*/*S* two-component regulatory system resulted in decreased transmission, likely at the level of acquisition by the recipient mice (1).

A new model of *Bordetella* transmission by droplet employs baboons and *B. pertussis* and recapitulates the essential clinical features of human pertussis infection (123). This model was used to demonstrate that acellular pertussis immunizations protect from infection, but not colonization or transmission, a finding with important implications for vaccine design and public health (125).

CONCLUSIONS

After being shed from a prior host, colonizing opportunistic pathogens enter the nasopharynx, where they escape from mucus and attach to the epithelial lining. These bacteria must obtain a source of nutrition on the spare mucosal surface in order to replicate, and they use multiple strategies to evade host clearance. The bacterial factors elaborated during colonization can also predispose the host to invasive disease. Ultimately, colonizing organisms that can adhere, grow, and evade clearance will transmit to a new susceptible host.

Many, and sometimes all, of these respiratory colonizers share similar strategies for each stage of colonization. Phase variation of capsule influences acquisition, immune evasion, invasion, and potentially survival outside the host during transmission (59, 81, 122). Host mimicry with phosphorylcholine modifies the cell surface of many airway pathogens and is crucial for attachment and immune evasion (17, 109). The mucosal immune responses to these pathogens have commonalities, reflected by the presence of an IgA1 protease in multiple microbes, as well as the Th17 response many elicit (4, 40, 132). Bacteria often produce toxins to target the host and typically benefit from coinfection with respiratory viruses, particularly influenza (5, 45, 69). Both reflect the effects of inducing inflammation at the expense of the commensal flora. If colonizing bacteria are resistant to its effects, inflammation promotes every stage of colonization, from upregulating receptors for adherence to providing a source of nutrition, misdirecting the immune response, increasing the rate of invasion, and facilitating transmission (9, 99, 104, 129).

The relevance of inflammation to each stage of carriage suggests that potentially pathogenic colonizers may have evolved to induce inflammation to drive colonization and transmission (12). Colonization, rather than invasive disease, is the source of selective pressure on these organisms and guides development of what should be termed colonization factors rather than virulence factors. These factors, including capsule, IgA1 protease, and pore-forming toxins, can be expressed by commensal bacteria that are rarely pathogenic, such as *Streptococcus mitis* (47). Further study is required to understand which combination of

virulence activities separates the potentially pathogenic from the nonpathogenic colonizers. Future work should also focus on understanding the relationship between virulence or colonization factors and transmission, the end point of carriage. The key to understanding invasive disease due to these opportunistic pathogens lies in colonization.

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Glossary

Pneumonia	inflammation of the lung alveoli and interstitial spaces caused by viral or bacterial infection
Opportunistic pathogen	organism that does not productively infect all hosts but can take advantage of immunocompromised states to cause disease
Colonization	the presence of a microbe on a host mucosal surface that does not cause disease in the carrier host
Upper respiratory tract	the conducting airways of the respiratory tract from the nasal cavity, through the nasopharynx, to the larynx
Commensal	an organism that takes advantage of a host without adverse effects
Mucus	protective barrier produced by mucosal surfaces and composed of hydrated glycoproteins, other proteins, and small molecules
Capsule	large polysaccharide structure that is the outermost layer of some bacteria
Phase variation	rapid on-off switching of expression of different proteins to generate diversity in a population without mutation
Adhesin	bacterial protein or carbohydrate that binds host structures to promote bacterial adherence
Sialic acid	a family of nine-carbon sugars that commonly occupy the terminal ends of host glycoconjugates on cell surfaces
Nutritional immunity	preventing pathogen access to essential nutrients; first used to describe iron-limiting mechanisms
NTHi (nontypeable *Haemophilus influenzae*)	unencapsulated strains and foremost causes of *H. influenzae* disease after vaccination against encapsulated type b strains
Th17 immunity	immunity directed by the cytokine IL-17 that promotes mucosal barrier function, including phagocyte recruitment
Within-host competition	competition between bacteria that requires the presence of the host to mediate the negative interaction

Bacteriocins	bacterial antimicrobial peptides that target the same or similar species
Inflammation	patterned host responses to a range of potentially harmful stimuli; the body's attempts to return to a baseline state

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SUMMARY POINTS

1. Colonization of the upper airway is the prerequisite to invasive disease by many respiratory tract pathogens.
2. Colonization drives evolution of virulence factors that cause disease.
3. Colonizing bacteria must adhere to the mucosal surface, obtain nutrients for growth, evade host immunity, and transmit to a new host.
4. The stages of adherence are associating with mucus, forming weak interactions with host carbohydrates, and strong binding to host surface proteins.
5. Phase variation allows for rapid diversity generation within a bacterial population and promotes adherence and immune evasion.
6. Bacterial polysaccharide capsules have multiple roles in adherence and evasion of host immune responses.
7. Host inflammation can mediate bacterial competition.
8. Opportunistic pathogens exploit and induce host inflammation for nutrient sources, to evade sterilizing immunity and for transmission.

FUTURE ISSUES

1. What separates potentially virulent from nonvirulent colonizers of the respiratory tract?
2. How does the host distinguish between commensals and pathogens that colonize the respiratory tract?
3. What determines the host range for respiratory tract colonizers?
4. Why does invasive disease develop in only a small proportion of colonized individuals?
5. Are virulence factors required for transmission?
6. What selective pressure does transmission exert on respiratory tract colonizers?
7. Can inflammation benefit commensal organisms, or only potentially pathogenic ones?
8. Do all respiratory tract opportunistic colonizers take advantage of inflammation to promote colonization and/or transmission?

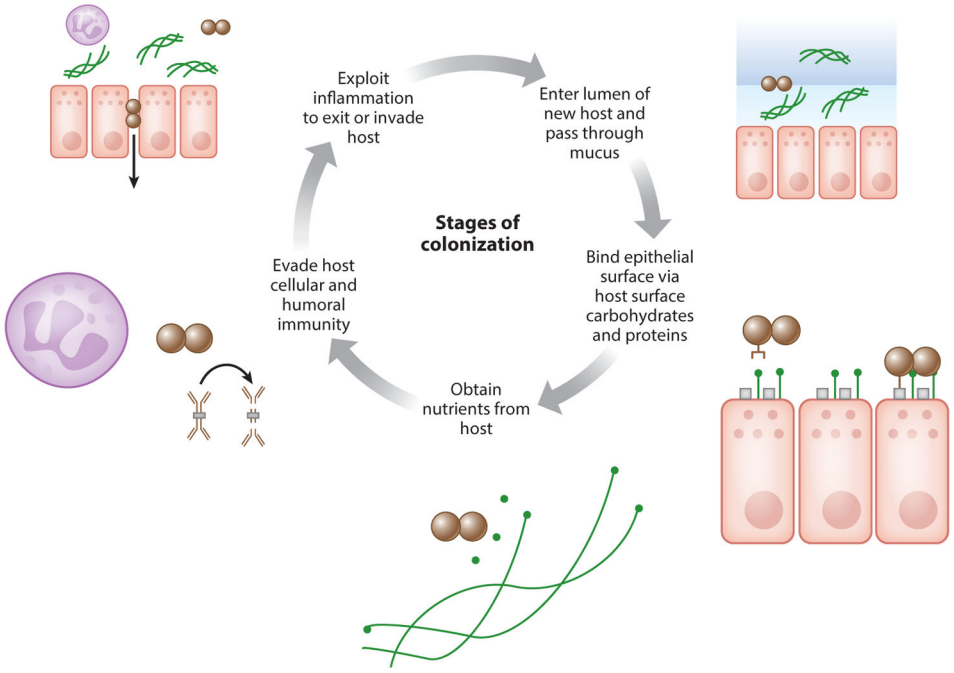

Figure 1.

The essential stages of colonization of the upper respiratory tract are presented here as a cycle. Colonizing bacteria (*brown*) enter the nasopharyngeal lumen and pass through the mucous layer (*blue*), in part facilitated by their capsule. Next, bacteria reach the epithelial surface and bind loosely and tightly to host surface carbohydrates and proteins, respectively. Bacteria obtain nutrients by exploiting host inflammation (through, for example, digestion of sialylated mucins) and evading nutritional immunity. These nutrients allow for microbial replication; persistence also involves evasion of host immune responses, both humoral (through IgA1 protease) and cellular. Opportunistic pathogens exploit these responses to drive transmission by exiting the host, and the same factors that allow for increased colonization and transmission predispose to invasion of host barriers, potentially a strategy for persistence.

Table 1

Host pressures and bacterial factors to counteract them

Host pressure	Bacterial countering factor
Anionic mucus prevents bacterial binding to epithelial surface	Polysaccharide capsule repels mucus; neuraminidases degrade mucus
Transporters limit free carbon on the mucosal surface	Glycosidases liberate carbon sources for uptake by carbon transporters; bacteria induce inflammation to promote secretion of substrates such as sialylated mucins
Lipocalin-2 binds siderophores	Nonsiderophore mechanisms obtain iron; nonenterobactin siderophores resist lipocalin-2 binding
Actively increases toxic zinc while limiting manganese	Zinc/manganese transporters
Mucociliary flow clears bacteria mechanically	Attach to host cells and tissues
Opsonophagocytosis	Antiphagocytic polysaccharide capsule
Mucosal secreted IgA1	IgA1 protease
Humoral immunity	Evade antibodies through antigenic variation
Recognizes bacterial LPS	Mask LPS with the host mimic ChoP
Neutrophil influx	Polysaccharide capsule, toxins
Inflammatory response to colonization	Exploit host responses to drive transmission
Hosts a diverse beneficial commensal flora	Displace or compete with host microbiota
TLR-mediated epithelial opening allows immune cell infiltration	Exploit epithelial opening to promote invasion and persistence
Specialized M cells sample lumen for antigens	Enter through M cells as portal for invasion
Creates microniches with opposing selective pressures	Phenotypic or phase variation generates diversity within a bacterial population

Abbreviations: ChoP, phosphorylcholine; LPS, lipopolysaccharide; TLR, Toll-like receptor.