Bacterial Metabolism in the Host Environment: Pathogen Growth and Nutrient Assimilation in the Mammalian Upper Respiratory Tract

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ABSTRACT Pathogens evolve in specific host niches and microenvironments that provide the physical and nutritional requirements conducive to their growth. In addition to using the host as a source of food, bacterial pathogens must avoid the immune response to their presence. The mammalian upper respiratory tract is a site that is exposed to the external environment, and is readily colonized by bacteria that live as resident flora or as pathogens. These bacteria can remain localized, descend to the lower respiratory tract, or traverse the epithelium to disseminate throughout the body. By virtue of their successful colonization of the respiratory epithelium, these bacteria obtain the nutrients needed for growth, either directly from host resources or from other microbes. This chapter describes the upper respiratory tract environment, including its tissue and mucosal structure, prokaryotic biota, and biochemical composition that would support microbial life. Neisseria meningitidis and the Bordetella species are discussed as examples of bacteria that have no known external reservoirs but have evolved to obligately colonize the mammalian upper respiratory tract.

INTRODUCTION
Pathogens evolve in specific host niches and microenvironments that provide the physical and nutritional requirements conducive to their growth. In addition to using the host as a source of food, bacterial pathogens must avoid the immune response to their presence. The mammalian upper respiratory tract is a site that is exposed to the external environment, and is readily colonized by bacteria that live as resident flora or as pathogens. These bacteria can remain localized, descend to the lower respiratory tract, or traverse the epithelium to disseminate throughout the body. By virtue of their successful colonization of the respiratory epithelium, these bacteria obtain the nutrients needed for growth, either directly from host resources or from other microbes. This chapter describes the upper respiratory tract environment, including its tissue and mucosal structure, prokaryotic biota, and biochemical composition that would support microbial life. Neisseria meningitidis and the Bordetella species are discussed as examples of bacteria that have no known external reservoirs but have evolved to obligately colonize the mammalian upper respiratory tract.
THE UPPER RESPIRATORY TRACT ENVIRONMENT

Structure of the Upper Airway: Tissue and Cells

Hairlike nasal vibrissae filter large particles from inhaled air that is then warmed, humidified, and conducted into the respiratory tract (Fig 1). Pathogenic microbes that enter the nasal cavity are able to bypass this filtration, and can make contact with the airway epithelium. The anterior nasal cavity has a keratinized stratified squamous epithelium that becomes less keratinized posteriorly (1, 2). Air flows through both sides of the nasal septum through scroll-shaped turbinates that have increased surface area, allowing inspired air to be humidified (to nearly 100% relative humidity) and warmed (to ~34°C). From the turbinate region, posteriorly throughout the rest of the nasal cavity, the surface consists of a pseudostratified columnar epithelium comprised of secretory goblet cells, columnar cells, and ciliated columnar cells (1, 2). The olfactory region is located in the posterior nasal cavity and is interspersed with chemoreceptive neurons. Submucosal glands produce the majority of respiratory secretions that are moved to the epithelial surface via ducts. In humans, these glands are found in the nose, trachea, and bronchi; in mice they are restricted to the nose and laryngeal region of the trachea. The nasopharynx, oropharynx, and larynx have pseudostratified columnar epithelia similar to that found in the posterior nasal cavity; the vocal cords have a squamous epithelium that lacks ciliated cells. The ciliated pseudostratified columnar epithelium extends throughout the respiratory tract, but not to the smallest bronchioles and alveoli. On regional surfaces that routinely encounter friction (e.g., food movement, swallowing), there is stratified squamous epithelium. These regions of the upper respiratory tract provide a moist, warm environment for microbial growth, but to effectively interact with those host cells, organisms attempting to colonize the airway must encounter the appropriate epithelial cells for which its adhesins and colonization factors have evolved.

The mammalian respiratory tract contains local respiratory lymphoid tissues such as the nasopharynx-associated and bronchus-associated lymphoid tissues (3). Other airway mucosal cells include macrophages and dendritic cells, some of which are resident intraepithelial dendritic cells that are positioned for luminal antigen sampling. T cells can also localize to intraepithelial spaces and to the lamina propria, along with IgA-producing plasma cells, mast cells, and B cells (3). Resident memory T cells may also be present in the mucosa after their migration from local lymphoid tissues (4), and the nasal mucosa may harbor M cells that are involved in antigen sampling at localized lymphoid tissues (4, 5). Inflammation of the airways increases vascular permeability, promoting immune-cell migration and transudation of plasma components onto the respiratory epithelial surface (6, 7).

The Mucosa

The respiratory epithelial surface is bathed in a mucus blanket that effectively traps particulates that are then moved toward the esophagus by mucociliary clearance for subsequent elimination by swallowing or expectoration (8, 9). Components of this mucus blanket contribute to its high osmolarity, but also constitute potential food sources for pathogens. Mucins abundant on mucosal surfaces are high molecular mass glycoproteins comprised of a protein core, with carbohydrate (80–90% of the mass) and sulfate (1–2%) moieties (1, 2, 10). Mucins have characteristic Pro-Thr-Ser repeats as well as Cys-rich domains that provide key desulfide-bonding capability, important for mucin multimerization and mucus function. There are two main types of mucins: membrane-bound and secreted (gel-forming). Respiratory epithelial cells produce the membrane-bound mucins MUC1, MUC4, and MUC16, which can be released from the cell surface (10) and are proposed to have important cell regulatory roles as well as key roles in mucociliary function. In the respiratory tract, the most abundant gel-forming mucins are MUC5AC and MUC5B, with MUC2 and MUC19 found in lower abundance (10, 11). In humans, epithelial goblet cells produce primarily MUC5AC, whereas MUC5B is produced by the submucosal glands and other surface secretory-cell types (1). In the mouse respiratory tract, very little Muc5ac is made, whereas secretory cells produce Muc5b (1). Within the secretory cell, newly synthesized mucin is dehydrated and then enclosed within membrane-bound secretory

![Diagram showing regions of the human upper respiratory tract](https://doi.org/10.1128/microbiolspec.MBP-0007-2014.f1)
granules. ATP and UTP are potent extracellular signals that trigger the secretion process, during which the granules fuse with the cytoplasmic membrane to allow mucin release onto the epithelium and subsequent hydration in the airway surface liquid (1, 10, 12).

The mucus layer closest to the lumen, often called the gel phase, is 0.5–5 μm thick and consists of a network (pore size, approx. 500 nm) (1, 3, 14) of gel-forming mucins. Beneath this highly viscous layer and in direct contact with the epithelium, is the periciliary layer, with a depth of approximately 7 μm. This periciliary layer, often referred to as the sol phase, has traditionally been thought to be the less viscous watery layer of the mucus blanket, allowing free movement of the cilia to achieve mucus clearance. Recent ex vivo studies using human airway tissue have provided detailed compositional analyses of the mucus layers, revealing that the periciliary layer is not “watery”, but filled with a mesh network of membrane-bound mucins and mucopolysaccharides (14). These network molecules fill the interciliary spaces and are also connected to the cilia and microvilli of airway epithelial cells. This complex periciliary layer is proposed to function as a brush and stabilize the two mucus layers, in part by prevention of soluble mucins in the luminal gel layer from extending into the periciliary layer and disrupting ciliary movement.

**Human Upper Respiratory Tract Microbiota**

Microbial inhabitants of the nasopharynx and other regions of the respiratory tract are likely to not only modify that environment, but may also produce metabolites and other factors that influence the growth of pathogenic microbes. There have been some recent efforts to map the biogeography of the respiratory bacterial population using 16S rRNA metagenomic approaches (15–22). Sampling of specific regions of the human respiratory tract can be challenging, since obtaining specimens can involve invasive procedures and certain sampling methods are prone to cross contamination of samples from differing sites. The anterior nares (nasal vestibule), where there is keratinized squamous epithelium, generally yield Staphylococci and other skin inhabitants such as Propionibacterium species (15–17). Recently, Yan and colleagues characterized the bacterial biota from three distinct regions of the human nasal cavity, comparing persistent carriers of *Staphylococcus aureus* with non-persistent carriers (22). The study sampled not only the anterior nares, but also the more posterior middle meatus and sphenoid recess (Fig. 1), both of which are covered in the ciliated, pseudostratiﬁed, columnar epithelium characteristic of the respiratory mucosa. In general, the three nasal sites of both *S. aureus* carriers and non-carriers were characterized by significant populations of members of the Actinobacteria and α-Proteobacteria (Table 1) (22). Subjects persistently colonized with *S. aureus* also appeared to be co-colonized with larger numbers of other different taxa than non-persistent carriers. Genera, including those from the Firmicutes, Actinobacteria, γ- and β- Proteobacteria, and other *Staphylococcus* species, were variably prevalent in the sample set from non-carriers. Of note, the microbiota from the middle meatus and sphenoid recess nasal sites of individuals from both subject groups exhibited more taxonomic diversity compared with the nasal vestibule site. Since the sphenoid recess nasal sites is the closest geographically of the three sampled regions to the nasopharynx, these results may inform our understanding of the nasopharyngeal and tracheal microbiota. In a different study, Charlson and colleagues performed a comprehensive biogeographical analysis of the healthy human respiratory tract, using methods designed to minimize cross-contamination of epithelial site samples (19). For the nasopharynx, they reported that *Staphylococcaceae* and *Propionibacteriaceae* were predominant, and noted signiﬁcant relative abundance of members of families listed in Table 1. Their results comparing multiple respiratory tract regions indicated more overall diversity in the bacterial populations from nasopharyngeal samples than from oral, oropharynx, and bronchoalveolar lavage ﬂuid (BALF) samples.

Hilty et al examined the bacterial populations of the human nose, oropharynx, the left upper lung lobe, and BALF from healthy controls and patients with asthma and chronic obstructive pulmonary disease (18). Populations found in the left upper lobe of all the adult donors were similar and included taxa listed in Table 1. Compared with controls, patients with chronic obstructive pulmonary disease had signiﬁcantly fewer *Prevotella* species and more *Haemophilus* and other Proteobacteria species. BALF from children with asthma also were colonized with fewer *Prevotella* species and more *Staphylococcus*, *Haemophilus*, and other Proteobacteria. Huang and colleagues examined bronchial epithelial brushings from healthy controls and patients with asthma (20). Compared with controls, asthmatics had a signiﬁcantly higher bacterial burden. Importantly, a diverse microbiota was identiﬁed in both subject groups, but several taxa, in particular, members of the Comamonadaceae, Oxalobacteraceae, and Sphingomonadaceae, were associated with asthma patients experiencing bronchial hyper-responsiveness.
Although these analyses have yielded useful information about the human respiratory microbiota, the majority of the identified bacterial taxa have not been studied to any extent that would allow predictions of their metabolites or compounds that could be produced or excreted in vivo to promote the growth of infecting pathogens. The aforementioned study of the human microbiota from three nasal sites revealed that in persistent carriers of S. aureus, there was a significant association with co-colonization by Corynebacterium accolens (22). In non-carriers, there was instead, colonization by Corynebacterium pseudodiphtheriticum. In vitro co-culture experiments showed that growth of C. accolens was enhanced by the presence of S. aureus, and conversely, S. aureus growth was stimulated by C. accolens. There was no growth enhancement when

<table>
<thead>
<tr>
<th>Location</th>
<th>Donor</th>
<th>Major taxonomic groups identified (listed in decreasing order of abundance)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal cavity: 3 sites</td>
<td>S. aureus non-carriers</td>
<td>Corynebacterium, Propionibacterium, Escherichia-Shigella, Dolosigranulum, Tomitella, Moraxella, uncultured β-proteobacteria, other Staphylococcus species</td>
<td>22</td>
</tr>
<tr>
<td>Nasal cavity: 3 sites</td>
<td>S. aureus carriers</td>
<td>Corynebacterium, Propionibacterium, Escherichia-Shigella, non-aureus Staphylococcus, Streptococcus, Prevotella, Anaerococcus, Peptoniphilus</td>
<td>22</td>
</tr>
<tr>
<td>Nasopharynx</td>
<td>healthy controls</td>
<td>Propionibacteriaceae, Staphylococcaceae, Corynebacteriaceae, Micrococcaceae, Streptococcaceae, Prevotellaceae, Veillonellaceae, Moraxellaceae, Lachnospiraceae, Acintomycetaceae, Flavobacteriaceae, Enterobacteriaceae, Flexibacteriaceae, Microbacteriaceae</td>
<td>19</td>
</tr>
<tr>
<td>Upper left lung lobe</td>
<td>healthy controls, patients with asthma or chronic obstructive pulmonary disease</td>
<td>Corynebacterium and other Actinobacteria, Prevotella and other Bacteroidetes, Staphylococcus, Streptococcus, Veillonella and other Firmicutes, Neisseria flavica/sicca, Haemophilus sp and other Proteobacteria, Fusobacterium, Megaspheara</td>
<td>18</td>
</tr>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>healthy controls, patients with asthma</td>
<td>Prevotella, other Bacteroidetes, Staphylococcus, Streptococcus, Veillonella, other Firmicutes, Haemophilus, Neisseria, other Proteobacteria, Fusobacteria</td>
<td>18</td>
</tr>
</tbody>
</table>

**TABLE 1** Human respiratory tract microbiota
C. pseudodiphtheriticum was substituted for C. accolens. Bacteria that have evolved to be obligate pathogens may be auxotrophic for vitamins and cofactors that other bacterial species can produce endogenously. For example, it is well known that some respiratory Haemophilus species are incapable of de novo production of heme and nicotinamide adenine dinucleotide (NAD), and on solid culture media these organisms will form satellite colonies surrounding bacterial species that produce these classical X (heme) and V (NAD) factors (23, 24). S. aureus was once used in clinical microbiology laboratories as the source of V factor for the satellite test for identification of Haemophilus species (25). In the host-airway environment, the growth factors needed by Haemophilus species must be provided by exogenous sources, so co-colonizing organisms, such as staphylococci, might supply needed heme, NAD, and other nutrients. Secreted enzymes such as proteases, mucinases, lipases, and nucleases from resident microflora would be predicted to degrade host resources to yield soluble nutrients. Staphylococcus species are known to excrete a number of such enzymes that also include hyaluronidase, DNase, coagulase, lipase, and staphylokinase (26–28). Streptococcus species can produce hemolysins, cytolytic toxins, and pyrogenic toxins (29). Since these enzymes and toxins may lyse host cells, making their cytosolic contents available for microbial degradation and consumption, commensal Staphylococcus and Streptococcus species may play important roles in modulating airway nutrient sources available to invading pathogens.

Nutrients in Airway Mucus

Respiratory mucus contains a number of host molecules that contribute to resistance to infection, but may also be degraded and utilized by microbes as nutrient sources. Lysozyme, immunoglobulins, complement components, cytokines, secretory leukoprotease inhibitor, defensins, cathelicidins, and lactoferrin are examples of potential sources of sugars or amino acids (30–32). Within the mucus, debris from sloughed and dead respiratory cells (1, 2) may provide additional sources of nutrition such as lipids, nucleic acids, and proteins. The respiratory microbiota, including pathogens, are also likely to use small, readily assimilated metabolites that are in the airway surface liquid. Using nuclear magnetic resonance (NMR) and mass spectrometry, metabolomic analyses of BALF from mice and humans have identified airway metabolites that may serve as nutrients for respiratory commensals and pathogens.

The BALF of healthy mice were found to contain a variety of amino acids, carbohydrates, acids, alcohols, and other metabolites (Table 2) (33). Mouse respiratory Aspergillus fumigatus infections yielded BALF that contained these metabolites but also contained increased ethanol concentrations over those in BALF from uninfected mice (34). BALF from mice exposed to airborne silica dust demonstrated higher levels of lactate, glutamate, creatine, lysine, glycogen/glucose, glycine, proline, and 4-hydroxyproline compared with BALF from unexposed mice (35).

Using BALF from healthy humans and patients with acute respiratory distress syndrome or acute lung injury, Evans and colleagues used liquid chromatography–mass spectrometry (LC-MS) to identify the metabolites listed in Table 2, all of which were found in increased concentrations in diseased patients (36). These authors also identified the metabolites palmitic acid, phosphatidylinolines, and stearic acid in both healthy and diseased subjects. A study by Rai and colleagues used NMR to identify airway metabolites in patients with acute respiratory distress syndrome and/or acute lung injury and control patients that did not have those respiratory diseases (37). In both patient populations, they identified the metabolites listed in Table 2. In patients diagnosed with acute respiratory disease, elevated levels of branched-chain amino acids arginine, glutamate, glycine, aspartate, acetate, taurine, threonine, lactate, and succinate were observed and the proline concentration was reduced, compared with control samples.

In sum, these mouse and human studies indicate that in the respiratory tract of healthy animals there exists a baseline level of metabolites available for microbial consumption. As an infection initiates and inflammation ensues, levels of certain metabolites would increase due to transudation of plasma components onto the epithelial surface. This would serve to further increase the levels of potential nutrients for pathogens. Based on these analyses and the known composition of airway fluids, a microbe’s carbon and nitrogen sources might be readily satisfied. Potential sulfur sources would include taurine, cysteine, and sulfate derived from mucins, and inorganic sulfur compounds. Phosphorus requirements could be met by host phospholipids, phosphocholine, glycerophosphocholine, nucleic acids, and other phosphate sources. Airway surface liquid is known to contain ascorbic acid (38) as well as high concentrations of glutathione that are critical for defense against oxidative damage (39); therefore, microbes may use these as carbon, nitrogen, and sulfur sources. Since bacterial auxotrophs such as Haemophilus species inhabit the respiratory tract, and excreted metabolites and the contents of dead epithelial and microbial cells exist in airway fluids, one may predict
**TABLE 2** Metabolites in airway fluids

<table>
<thead>
<tr>
<th>Airway sample</th>
<th>Donor</th>
<th>Major metabolites identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>mice: infected or treated mice, healthy controls</td>
<td>glutamate, glycine, proline, lysine, taurine, 4-hydroxyproline, glucose, glycogen, glycerol, acetate, formate, lactate, succinate, acetone, isopropanol, ethane, ethanol, glycophosphocholine, phosphocholine, choline, creatine, creatine phosphate, creatinine</td>
<td>33, 34, 35</td>
</tr>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>humans: healthy controls, patients with acute respiratory distress syndrome or acute lung injury</td>
<td>L-glutamate, L-leucine, methionine, L-phenylalanine, L-proline, L-tyrosine, L-tryptophan, L-threonate, D-glucose, alpha ketoglutarate, cis-aconitate, L-lactate, citrate, creatinine, creatine, O-acetylcarnitine, guanosine, hypoxanthine, bis[2-ethylhexyl]phthalate, uridine, hippurate, inosine, palmitoleic acid, arachidonic acid, linoleic acid</td>
<td>36</td>
</tr>
</tbody>
</table>

(continued)
that requisite vitamins such as B12, NAD precursors, and other cofactors and ions exist in airway surface liquid and their concentrations may be enhanced during inflammation or by production by commensal biota.

**Iron and Its Acquisition by Microbes**

Most living cells, including prokaryotes, require nutritional iron. In the mammalian host, there is exceedingly little iron available for microbial growth due to host iron-homeostatic mechanisms that function to limit the amount of free iron \((40–42)\). The peptide hormone hepcidin is a key regulator of erythropoiesis and it also represses the uptake of dietary iron in the gut and the liberation of iron from macrophages and hepatocytes \((43–45)\). Hepcidin inhibits iron transport to plasma and tissues by binding the cellular ferroportin iron exporter and inducing its breakdown within the lysosome. The bone morphogenetic protein pathway transcriptionally regulates hepcidin production, and iron abundance and inflammation also upregulate expression of the hepcidin gene \((45)\). Another arm of the host iron-withholding defense is that exerted by the iron-binding glycoproteins transferrin and lactoferrin, each of which can coordinate two atoms of ferric iron \((43, 44)\). Transferrin is found primarily in plasma and is the primary means by which the cells of the body receive nutritional iron. Lactoferrin exists on mucosal surfaces and is also stored within neutrophil granules that can be deployed upon encounter with microbes \((43, 46)\). The N-terminal region of lactoferrin (termed lactoferricin) has been shown in vitro to have antimicrobial activities that are distinct from its iron-scavenging functions. In human respiratory epithelial fluids, lactoferrin concentrations are approximately 100–1000 μg/ml \((46)\); transferrin is found in very low abundance here, but inflammation promotes transudation of serum molecules onto the airway epithelium, thus potentially increasing its concentration at that site.

Pathogenic bacteria must overcome host iron restriction for successful in vivo growth, and when starved for iron, they employ several general mechanisms for retrieval of iron from host sources. Under conditions of iron-replete growth (a rare circumstance in vivo), expression of iron-acquisition genes is usually repressed by Fur (or an analogous regulator) that requires ferrous iron as a corepressor \((47)\). Iron starvation leads to decreased intracellular iron corepressor concentrations and subsequent derepression of genes involved in iron uptake. For numerous pathogens, the predominant classes of in vivo-expressed genes are involved in nutrient acquisition, and of those, iron-acquisition genes dominate the transcription profile \((48)\). Therefore, microbial iron-acquisition gene expression is regarded as a transcriptional signature of the host microenvironment. In addition to regulating the systems required for iron assimilation, iron starvation is a major signal controlling the expression of bacterial virulence factor genes \((49, 50)\).

Bacteria may produce and excrete siderophores that chelate ferric iron and bind cognate surface receptors for iron uptake \((51)\). Bacteria may also have evolved transport systems allowing utilization of xenosiderophores (siderophores produced by other microbes in the

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**TABLE 2** Metabolites in airway fluids (Continued)

<table>
<thead>
<tr>
<th>Airway sample</th>
<th>Donor</th>
<th>Major metabolites identified</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchoalveolar lavage fluid</td>
<td>humans: patients with acute respiratory distress syndrome and/or acute lung injury, undiseased control patients</td>
<td>alanine arginine aspartate glutamate glycine leucine isoleucine lysine phenylalanine proline threonine valine taurine acetate lactate succinate choline creatine betaine adenine ethanol</td>
<td>37</td>
</tr>
</tbody>
</table>
vicinity). Siderophores produced by commensal organisms inhabiting the respiratory tract may provide iron to invading pathogens that can use those xenosiderophores. Corynebacterium, Burkholderia, and Actinomyces species have been identified as respiratory inhabitants (Table 1) and members of these genera have been reported to produce siderophores (52–54); however, it is not known whether the described siderophore-producing species can be found in the respiratory tract. Staphylococcus species (55), Pseudomonas aeruginosa (56, 57), and Escherichia coli (41, 51) each can produce multiple siderophores that could be used by other bacterial species, and have been identified as respiratory microbiota. The nasopharyngeal pathogens Bordetella pertussis (58), Neisseria meningitidis (59, 60), and Haemophilus species (61) are known to use E. coli siderophores; thus commensal E. coli may promote the in vivo growth of these pathogens.

Some organisms have surface receptors allowing hemin uptake or the direct, siderophore-independent uptake of host iron from transferrin and lactoferrin (42, 62). Bacteria may also have systems to transport inorganic ferrous or ferric iron sources (63, 64). Bacterial growth stimulation by host catecholamine neuroendocrine hormones such as epinephrine and norepinephrine has been described (65, 66). Studies indicate that norepinephrine can remove the iron from transferrin and lactoferrin, making it available for growth of E. coli and Salmonella enteritidis (67–69). Their use of norepinephrine required production of their endogenous siderophores enterobactin and salmochelin, respectively, or their breakdown products. Differently, the pathogens Bordetella bronchiseptica (70), S. aureus (55), and Campylobacter jejuni (71) are able to use host catecholamines to retrieve iron from transferrin and lactoferrin in the absence of a siderophore. Thus, these catecholamines function as pseudosiderophores. Although the concentrations of neuroendocrine hormones in the airway have not been well documented, sensory neurons of the airway control reflexes such as coughing, bronchodilation, and mucociliary action, as well as plasma leakage, through the function of neuroendocrine hormones (72). Cells of the human bronchus-associated lymphoid tissue have catecholamine neurotransmitter receptors (73) and rat nasal mucus contains norepinephrine and dopamine (74). Therefore, catecholamines may be available to bacterial cells in the host respiratory tract.

Members of the lipocalin family are small proteins characterized by a binding cleft for hydrophobic ligands (75–77). There are a number of lipocalin types in mammals, including lipocalin 1 found in tears and lipocalin 2 that is a biomarker of kidney damage. These two lipocalins have also been shown to be present on respiratory mucosal surfaces (77) and have been demonstrated to bind microbial siderophores. Lipocalin 1 was shown to bind to the enterobactin siderophore produced by members of the Enterobacteriaceae, as well as other structurally different siderophores (78). Lipocalin 2 (also known as siderocalin) binds the ferric and apo-forms of enterobactin in addition to other catechol siderophores (79) and norepinephrine (80). This lipocalin also interacts with an endogenous mammalian catechol that may function as a siderophore in host iron trafficking and homeostasis (81–83). Respiratory lipocalin 2 production in mice was induced after infection by Streptococcus pneumoniae, Haemophilus influenzae (84), and Klebsiella pneumoniae (85) and lipocalin 2 was identified in the nasal secretions from healthy humans (84). Pathogens such as E. coli that rely on catechol siderophores for iron acquisition in vivo may encounter significant iron restriction due to loss of that siderophore to lipocalin 2 sequestration, and to general competition for ferric iron by lactoferrin. Bacteria that produce and/or use siderophores that are not lipocalin ligands may have an in vivo growth advantage. Further, if there is a true mammalian iron-binding catechol siderophore that naturally interacts with lipocalin 2, it is plausible that microbes will have evolved mechanisms to obtain that complexed iron.

**BACTERIAL PATHOGENS OF THE NASOPHARYNX**

**Neisseria meningitidis**

Members of the genus Neisseria are Gram-negative β-proteobacteria that include the closely genetically related obligate human pathogens Neisseria gonorrhoeae, the bacterial agent of gonorrhea, and Neisseria meningitidis, a cause of fulminant meningitis, as well as a number of nonpathogenic commensal species (86–90). Although N. gonorrhoeae can colonize the pharynx and other mucosal sites in addition to the reproductive tract, the site of initial N. meningitidis colonization is the human upper respiratory tract epithelium. Most commonly, N. meningitidis lives as a commensal of the nasopharynx, oropharynx, buccal epithelium, and tonsils. Approximately 10% of healthy humans are colonized by N. meningitidis and experience no disease symptoms. Rarely, the organism can enter host respiratory epithelial cells, transit and exit those cells, and reach the submucosal tissues to disseminate via the bloodstream, causing septicemia, and pass the blood-brain barrier to...
cause meningitis. Invasive disease is associated with high mortality, even when treated with appropriate supportive and antibiotic therapy, as well as neurological sequelae and lifelong disability.

Like other Neisseriae, N. meningitidis is a diplococcus that lacks flagellar swimming motility (86). An antiphagocytic polysaccharide capsule is produced and it provides the basis for serotyping N. meningitidis strains. There are 12 capsular serogroups, but the majority of disease is caused by serogroups A, B, and C (86). Meningococcal vaccines based on capsular polysaccharides or protein conjugates are in wide use to prevent disease by serogroups A, C, Y, and W135 (86–90). Neisseria species are well known for their ability to take up exogenous DNA and undergo natural transformation (86). These organisms are capable of conjugation and resident plasmids exist in some strains. Pathogenic Neisseria species are also known for their phase variation of virulence factors such as the opacity proteins (91, 92), pili (93, 94), lipooligosaccharide (95), and heme-uptake transporters HpuAB and HmbR (86, 90, 96).

Colonization
N. meningitidis is transmitted between human hosts by airborne droplets and by respiratory secretions, and it has been hypothesized that the polysaccharide capsule may prevent desiccation during transit (90). Epithelial cell culture and ex vivo respiratory tissue models of infection have yielded useful information about meningococcal colonization that may translate to infection of the human nasopharynx (97, 98). The major adhesins are the type IV pili, also involved in twitching motility, and the opacity proteins Opa and Opc (99). Type IV pili are equipped with the PilC tip adhesin and are required for initial interaction with the host epithelial surface after which the organisms then become more intimately associated with the host cells (100). CrgA is a regulator present in the pathogenic Neisseria that is activated upon contact with epithelial cells (101, 102); in N. meningitidis, it is reported to play an important role in the controlled expression of pilus and capsule synthesis genes. The PhoP-PhoQ two component regulatory system is also induced on host cell contact and is involved in adherence of N. meningitidis to the epithelium. The Pho system is also involved in the ability of the organism to transit epithelial cells to reach the submucosa (103, 104). The outer membrane porins PorA and PorB (105), lipooligosaccharide, factor H-binding protein (106), the NadA (group B strains) (107) and TspA (108) adhesins, the antiphagocytic NhHAD adhesion/complement resistance protein (109), and the HrpA-HrpB two-partner secretion system (110) also play key roles in adherence and colonization. Opa, Opc, and PorB have been reported as important for uptake of N. meningitidis into host cells, a prerequisite to dissemination (92). Few secreted products are produced, but the organism does excrete an immunoglobulin A1 (IgA) protease that cleaves the antibody molecule at the hinge region (111), and can also degrade host lysosomal LAMP1, enabling the organism to prevent lysosome maturation (112).

Colonization of the nasopharyngeal epithelium by N. meningitidis is hypothesized to involve biofilm formation and studies of biofilm growth have used abiotic surfaces or cultured cells as experimental systems (113–115). Since there is a paucity of nonhuman animal models of meningococcal nasopharyngeal infection, there is little evidence that these organisms live in biofilm communities in the host. However, one ex vivo study of human tonsillar tissue revealed what appeared to be microcolonies of N. meningitidis present either within or beneath the epithelial surface, suggestive of subsurface biofilm formation (116). N. meningitidis strains isolated from the bloodstream during invasive disease are uniformly encapsulated but capsule production by N. meningitidis living in the nasopharynx potentially as a biofilm, is downregulated (89). Biofilm formation depends on twitching motility conferred by the type IV pili and the pilus protein PilX, and maintenance of the structure also involves HrpA-HrpB (116). Extracellular DNA is an important component of the extracellular biofilm matrix (116). When N. meningitidis contacts the host cell surface, the CrgA regulator represses capsular gene expression and the ampD and mltB genes exhibit increased expression (101). The products of ampD and mltB are involved in peptidoglycan recycling and in lytic functions, respectively, and are needed for initial biofilm formation; thus, they may provide a means for bacterial autolysis and release of DNA into the biofilm matrix (116). Experimental results indicate that a major contributor to the elaboration of extracellular DNA and biofilm structural stability is the outer membrane autolysin, phospholipase A.

Metabolism
N. meningitidis has complete Entner-Doudoroff and pentose phosphate pathways and a tricarboxylic acid (TCA) cycle (86). It can use glucose and maltose as carbon sources, but apparently does not use other commonly metabolized sugars. The meningococcus can also use pyruvate and lactate as carbon sources (117, 118). Pyruvate is oxidatively decarboxylated by pyruvate dehydrogenase, to produce acetyl CoA that...
is oxidized to CO₂ in the TCA cycle. There are no N. meningitidis genes specifying the isocitrate-lyase or malate-synthase activities characteristic of a glyoxalate shunt. Growth studies revealed that N. meningitidis can use lactate as a sole carbon source more avidly than it uses glucose, and low lactate concentrations stimulate growth on glucose (118). The N. meningitidis lactate permease is required for utilization of lactate, but importantly, it was shown to be required for ex vivo colonization of human nasopharyngeal tissues (118). N. meningitidis has three lactate dehydrogenases that yield pyruvate that can be fed into the TCA cycle (119). Since both D and L isomers of lactate can be used, it was proposed that since mammalian cells do not produce D-lactate, that which is produced by bacterial flora, such as lactic acid bacteria, may provide this carbon source to the meningococcus (119). N. meningitidis has a variety of aminopeptidases for the cleavage and liberation of N-terminal amino acids from proteins and peptides. Amino acids are assimilated to form other amino acids and can also be oxidatively deaminated and fed into the TCA cycle (120). Glutamate and proline, which are present in airway surface fluids of humans (Table 2), are two preferred amino acids catabolized by N. meningitidis. Glutamate is processed through the activities of one or both N. meningitidis glutamate dehydrogenases (121). Glutamate uptake has also been implicated in N. meningitidis internalization and survival in epithelial and other host cells (122).

**In vivo** sources of nitrogen for the meningococcus most likely include amino acids like glutamate, or other nitrogenous compounds. N. meningitidis is an aerobe, preferring an in vitro atmosphere with increased CO₂. N. gonorrhoeae growth is similar optimal with increased CO₂ but it can also grow anaerobically using nitrite as an electron acceptor (123). The Neisseria AnaA nitrite reductase yields nitric oxide (NO), which is then converted to nitrous oxide by the Nor enzyme that uses electrons from quinones (124). N. gonorrhoeae and commensal Neisseria species have the anaA and norB genes. Commensal Neisseria also have a functional nitrate reductase and can complete the denitrification process to take nitrous oxide to N₂ via a nitrous-oxide reductase (124). In many N. meningitidis isolates, the anaA gene is no longer intact, indicating loss of the nitrite-reductase function. The meningococcal nitric oxide reductase, however, is functional and predicted to provide resistance to host nitric oxide-mediated killing. N. meningitidis, but not N. gonorrhoeae, has a functional superoxide dismutase that confers additional protection from oxidative killing. N. meningitidis also produce SodB (125), SodC (126), catalase, peroxidase (127), and glutathione peroxidase (128). N. meningitidis has genes encoding a MntABC manganese transport system that, in N. gonorrhoeae, also supplies the manganese involved in protection from oxidative stress (129, 130).

**In vivo**, N. meningitidis is likely to obtain sulfur from cysteine, sulfates, or thiosulfate, although some strains have an absolute requirement for cysteine (86, 131). No reports could be found that described Neisseria mucinases or desulfurizing enzymes that could be used to liberate cysteine and sulfate from respiratory mucins. N. meningitidis Z2491 (serogroup A), MC58 (serogroup B), FAM18 (serogroup C), and other serogroup strains each have apparently intact genes encoding a sulfate-transport apparatus and the Cys enzymes required for cysteine biosynthesis from sulfate (120). Absent from the genomes was a cysC gene encoding an adenosine phosphosulfate (APS) phosphokinase activity that would produce phosphoadenosine phosphosulfate (PAPS) from APS. This activity could be provided by the product of cysN which, in Burkholderia cenocepacia, was named cysNC, the product of which was noted to be a fusion of the ATP-sulfurylase and APS-kinase domains and proposed to have dual activities in cysteine synthesis from sulfate (132).

Likely sources of phosphorus for N. meningitidis in the nasopharynx include compounds such as phospholipids, phosphocholine, and nucleic acids (Table 2). Little has been published on phosphorus metabolism in Neisseria, but N. meningitidis and N. gonorrhoeae have polyphosphate-kinase genes, the products of which promote formation of polyphosphate, and they also have the Ppx enzyme for cleavage of this stored phosphate (133, 134). For zinc acquisition, N. meningitidis uses the ZnuD outer membrane receptor-transport system for both zinc and heme (135). N. meningitidis has the genetic capability for de novo NAD synthesis as well as having genes for the Preiss-Handler salvage pathway for NAD synthesis from nicotinamide or nicotinic acid. These organisms also have the genes encoding enzymes for the synthesis of flavins and thiamine (120).

**Iron**

All strains of N. meningitidis studied to date can obtain iron from transferrin, lactoferrin, and hemoglobin (90, 96, 136–138). Both N. meningitidis and N. gonorrhoeae produce the Tbp-A-TbpB cell surface-receptor complex that is specific for human transferrin. TbpA has a structure characteristic of members of the TonB-dependent
outer membrane-transporter family (139, 140). In most Gram-negative bacteria, the TonB system is needed to supply the energy from the proton-motive force for transport of iron and other nutrient sources across the outer membrane (141). TbpA and the TbpB lipoprotein form an outer membrane-receptor complex that recognizes and binds the C-lobe of ferric transferrin, removes its iron at the cell surface, and transports that iron to the periplasm for subsequent binding by the FbpA periplasmic protein and uptake across the cytoplasmic membrane by the FbpBC transporter (142). After iron extraction, the apo-transferrin is released from the receptor. Crystallographic and structural analyses allowed the meningeococcal TbpA and TbpB interactive surfaces to be mapped onto human transferrin and provided data to support a model of iron extraction and release of spent transferrin from the receptor (139). The TbpAB system is found in all N. meningitidis and N. gonorrhoeae strains that have been examined (90). N. meningitidis and N. gonorrhoeae produce analogous dipartite-surface receptors for lactoferrin (LbpA-LbpB) and hemoglobin (HpuA-HpuB), although they are less well characterized than TbpA-TbpB (90). Recent studies implicated the LbpB protein in resistance of N. meningitidis to the N-terminal lactoferricin portion of lactoferrin that has antimicrobial activity (143). Although there are N. gonorrhoeae clinical isolates that lack lbpBA genes, all N. meningitidis strains that have been examined have them. A N. gonorrhoeae tbpBA mutant was avirulent in human volunteers, whereas an lbpBA mutant retained virulence (144, 145). Since N. gonorrhoeae initially infects human mucosa, one may surmise that lactoferrin would be a primary iron source rather than transferrin. It is unknown whether Tbp (or Lbp) is important for N. meningitidis nasopharyngeal colonization or dissemination in humans.

N. meningitidis (and N. gonorrhoeae) have a second transport system for heme. HmbR is a TonB-dependent outer membrane receptor for hemin and hemoglobin (90, 96). In the bloodstream, the amount of free heme or hemoglobin is limited due to binding by hemopexin and haptoglobin, respectively (62). The amount of available heme in the nasopharynx is unknown. However, since other nasopharyngeal bacterial pathogens such as H. influenzae (62) and Bordetella pertussis (146) possess heme-utilization systems, and damage to the respiratory epithelium would make heme more available, it is reasonable to hypothesize that heme compounds can be obtained by bacteria living in the nasopharynx.

Neisseria species do not produce siderophores; however, they are capable of using some xenosiderophores, presumably excreted by microbiota of the host. The hydroxamate siderophore aerobactin, produced by members of the Enterobacteriaceae, can be used by N. meningitidis and N. gonorrhoeae (59, 60). Both Neisseria species also use ferric enterobactin, which relies on the FetABCD transport system for uptake (147, 148). FetA is the TonB-dependent outer membrane receptor and the FetBCD proteins comprise a periplasmic binding protein-dependent transporter for iron uptake across the cytoplasmic membrane. FetA-specific antibodies have been detected in patients convalescing from disseminated meningococcal infection and it has been studied as a potential vaccine candidate (90). Sequencing the fetA genes from a number of N. meningitidis strains, both invasive and other, revealed a great deal of allelic variation and antigenic diversity in the protein, perhaps limiting its potential utility as a vaccine antigen. The fetABCD genes are Fur repressed and are also subject to genetic variation that affects transcription and therefore, Fet protein production, in both pathogenic Neisseria species (147).

In N. meningitidis, expression of fur, tpbBA, lbpBA, fbpABC, hmbR, and fetA is under control of the Fur repressor that uses ferrous iron as co-repressor to limit transcription under conditions of iron plenty (90, 149). Other N. meningitidis genes predicted to encode TonB-dependent receptors that may play a role in the acquisition of iron or other nutrients have been identified. These receptors include TdfF, TdfH, and TdfJ, although they remain uncharacterized and their substrates unknown. TdfD and TdfJ are most similar to ferric siderophore receptors and TdfH is similar to other bacterial heme receptors. A global microarray study of N. meningitidis genes that were expressed under iron-replete versus iron-limiting conditions identified a number of genes upregulated during iron starvation (149). Known iron-acquisition genes were directly repressed by Fur, as were genes encoding the FrpA virulence factor, DnaK, the ClpB chaperone, the DsbA protein disulfide oxidoreductase, fumarase C, L-lactate dehydrogenase, certain NAD biosynthesis enzymes, and the nrrF small RNA. Similar to the positive Fur regulation of E. coli sodA, bacterioferritin, and succinate dehydrogenase genes via the rybB regulatory sRNA (150), in N. meningitidis, expression of succinate dehydrogenase subunit genes sdBA and sdBC is repressed by the regulatory nrrF sRNA, that itself is iron and Fur repressed (151). Under iron-replete conditions, the TCA cycle genes sdBA and sdBC are expressed and iron limitation decreases their transcription. In N. meningitidis, Fur can directly express transcription of genes involved...
in resistance to oxidative stress including katA, sodB, norB, and aniA in addition to genes encoding a fumarate hydratase, and Nuo subunits of the NADH dehydrogenase (149). Gene expression profiling examined iron-starved N. meningitidis cultured in the presence of hemoglobin, transferrin, or lactoferrin (152). Growth on hemoglobin induced expression of genes including those encoding FrpC and surface proteins FhaB and PilE, in addition to Fur and the NorB nitric oxide reductase. Transferrin or lactoferrin utilization led to increased transcription of genes such as those encoding the PorB and Hsf surface proteins, TCA cycle enzymes, cytochrome C, and alcohol dehydrogenase. Lactoferrin also upregulated genes specifying PiIP and LgtG, which is a lipopolysaccharide-modifying enzyme that impacts serum resistance. Since hemoglobin, transferrin, and lactoferrin are primarily localized to different anatomical regions, it was proposed that because these iron compounds elicited such differences in gene expression, they essentially function as host niche indicators for N. meningitidis.

The Bordetella Respiratory Pathogens
The Bordetellae are Gram-negative β-proteobacteria of the family Alcaligenaceae. There are presently nine described Bordetella species, three of which are highly genetically related respiratory pathogens of mammals, considered to be subspecies, and often called the classical Bordetella species (153, 154). Bordetella pertussis is a strictly human-adapted species and is the agent of whooping cough or pertussis. Bordetella parapertussis causes whooping cough-type infections in humans and can also infect sheep. Bordetella bronchiseptica infects a broad range of mammalian hosts and causes respiratory diseases such as canine kennel cough and swine atrophic rhinitis. Genomic sequence analyses suggest that a B. bronchiseptica-like organism is the ancestor of B. pertussis and B. parapertussis, which have sustained significant gene loss and rearrangement compared to the genome of B. bronchiseptica (155). For example, B. bronchiseptica expresses flagellar genes and is motile, but B. pertussis and B. parapertussis are not motile and many of their flagellar genes are mutated. This type of genetic loss is thought to be the result of their evolution toward obligate and host species-specific colonization. These three classical pathogenic Bordetella species colonize the cilia of the host respiratory epithelium where they multiply and produce a variety of virulence factors, resulting in disease symptoms such as the uncontrollable coughing characteristic of pertussis. Although intracellular bacteria can be observed in infected immune-competent animals, including humans, the classical Bordetellae are thought to primarily inhabit the epithelial surface and do not disseminate throughout the body (153, 154). Despite active immunization, pertussis remains an endemic disease that has been increasing in frequency.

The respiratory Bordetellae produce a variety of virulence factors. B. pertussis is unique in its production of pertussis toxin; B. bronchiseptica and B. parapertussis have the ptx genes, but mutation has silenced them (155). Pertussis toxin is a classical A-B type toxin that enters the host cell and covalently modifies G protein-inhibitory subunits by ADP ribosylation, rendering the host protein locked in a dysregulated state, and altering normal cellular function (156). Pertussis toxin inhibits airway macrophage function, interferes with neutrophil chemotaxis, and suppresses the adaptive immune response to infection (157). All three classic Bordetella species secrete an adenylate cyclase that also has pore-forming lytic activity (158, 159). This adenylate cyclase is a member of the RTX family of toxins and targets a variety of cell types, using host calmodulin in a cytoplasmic reaction that generates supraphysiologic concentrations of cAMP. Bordetellae produce a dermonecrotic toxin that is a trans-glutaminase that acts on host Rho GTPases (160); its role during infection is not known. The classic Bordetella species have a type III secretion system that causes necrotic death of cultured cells that is independent of caspase activity (161). B. bronchiseptica type III secretion-system mutants exhibit attenuation in the lower respiratory tract of infected rodents (162) and in swine (163). The type III system BteA-secreted effector has cytotoxic activity on cultured cells, although the mechanism has yet to be determined (153, 154).

Bordetellae produce a peptidoglycan-derived disaccharide tetrapeptide that is usually recycled in bacteria during cell wall synthesis and turnover (164). In hamster tracheal explant cultures this peptidoglycan fragment, tracheal cytotoxin, synergizes with lipopolysaccharide to induce an inflammatory response and increase host nitric oxide synthase activity. As a result, host cells hyperproduce NO, ultimately resulting in the death and extrusion of ciliated cells from the epithelium (164). N. gonorrhoeae produces similar peptidoglycan fragments that cause inflammation and tissue destruction in fallopian tube cultures (165). N. meningitidis appears to recycle its peptidoglycan fragments more efficiently and those that are not recycled have only modest proinflammatory activity. Interestingly, the marine bacterium Vibrio fischeri produces a peptidoglycan-derived tracheal cytotoxin that causes remodeling of its squid host’s
ciliated tissues for the effective establishment of a host-symbiont relationship (166). Bordetella, N. gonorrhoeae, and V. fischeri interact with host ciliated epithelia and may have evolved a mechanism to modify those tissues to promote their colonization or to access nutrients released from destroyed host cells.

Most known Bordetella virulence genes are transcriptionally activated by a two-component system consisting of the BvgS membrane-sensor kinase and the DNA-binding response regulator BvgA (167–169). Bvg+ phase bacteria express BvgA-activated genes, such as those encoding pertussis toxin, adenylate cyclase, the type III secretion system, adhesins, and certain metabolic functions. If the NaCl in Bordetella growth medium is replaced with sulfate salts, there is a reversible loss of virulence-associated traits; upon return to standard medium lacking sulfate, production of the virulence traits resumes (170). This phenomenon is known as antigenic (or phenotypic) modulation. Millimolar concentrations of nicotinic acid in the growth medium cause similar reversible modulation (171), as does growth at low temperature (e.g., 25°C) (170). The transcriptional response to modulating stimuli requires the BvgAS sensory system (172). bvg mutants and modulated wild-type cells are considered Bvg− phase organisms and do not express BvgAS-activated genes, but instead express genes (such as motility genes) that are often denoted as Bvg-repressed. A B. bronchiseptica bvg mutant can survive remarkably better in nutrient-free phosphate-buffered saline compared with the Bvg+ parent strain and provision of a modulating concentration of sulfate rescues the survival of the wild-type strain (173). The metabolism underlying these experimental observations is not known. Bacteria grown at intermediate modulator concentrations are characterized by expression of the BipA surface protein and adhesin genes but not genes encoding secreted virulence factors (154, 169). It is unknown whether the sulfate, 25°C growth, or nicotinic acid modulators are natural in vivo signals detected by BvgS. The Bvg+ phase is required for infection in experimental hosts, but no in vivo role for the Bvg− phase has been demonstrated (169, 174, 175). However, it was shown that B. bronchiseptica can encounter modulating conditions while living in the mouse respiratory tract (176).

Colonization

Pertussis is an acute disease during which the organisms are effectively cleared from the host, although coughing can persist for months (153), whereas infection of nonhuman mammals by B. bronchiseptica can be chronic (177). A study of the contribution of the mouse nasal microbiota to Bordetella colonization demonstrated that at a very low inoculum, B. bronchiseptica could readily displace the resident flora and establish nasal colonization. For B. pertussis, even very high inocula could not compete with the nasal biota, but pretreatment of mice with antibiotics significantly reduced their infectious dose to levels similar to those of B. bronchiseptica (177). Pertussis patients occasionally present with primary or secondary pneumonia, but B. pertussis is generally localized to the upper respiratory tract (153). Histologic studies examining respiratory tissues from patients who died from pertussis, specimens from necropsy Bordetella-infected experimental animals, and from infected tissue explants, show that Bordetella cells adhere preferentially to cilia (178–180). These observations suggest that all ciliated respiratory epithelial surfaces are targets of Bordetella colonization. The ciliary receptors to which Bordetella cells adhere are not known. B. bronchiseptica infection of dog tracheal explants demonstrated remarkably rapid induction of ciliostasis upon bacterial contact (181). Microarray studies of cultured BEAS-2B human bronchial epithelial cells infected with B. pertussis showed upregulated proinflammatory cytokine responses and increased transcription of the MUC2 and MUC5AC genes (182). In addition, B. pertussis was shown to bind these respiratory mucins, which is a trait that may be highly relevant to airway colonization. Ciliostasis, along with mucus accumulation and damage to the epithelium, may interfere with effective clearing of the organisms and may provide nutrients that promote continued bacterial growth.

B. pertussis experimental studies have used a number of animal models, with the mouse being the most often used, although it does not display symptoms typical of human whooping cough (153, 154). Recently, a baboon model of pertussis was developed that recapitulates many of the symptoms of this coughing disease (183). Experimental models of natural B. bronchiseptica respiratory infection include the mouse, rat, rabbit, guinea pig, and swine. Bordetella surface molecules implicated in colonization include lipopolysaccharide, type I fimbriae, the autotransporter protein pertactin, and filamentous hemagglutinin (FHA) (153, 154). Currently used acellular pertussis vaccines consist of detoxified pertussis toxin, FHA, pertactin and fimbrial proteins, depending on the manufacturer (153). On rabbit tracheal explants, B. bronchiseptica mutants lacking pertactin, fimbriae, or FHA exhibited decreased binding to cilia, suggesting that all three collectively function as adhesins (179). The fimbrial subunits can undergo

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genetic-phase variation, and fimbriae were shown to be important in colonizing the rodent lower respiratory tract (184, 185). A two-partner secretion system processes and transports FHA to the bacterial surface where it is tethered, although a significant amount is also released (186). FHA has heparin-binding and carbohydrate-recognition domains needed for Bordetella adherence to cells in culture. B. bronchiseptica FHA-deficient mutants colonized only the nose but not the trachea or lower respiratory tract of rats (187) and swine (188). In mouse lower respiratory tract infections, B. bronchiseptica mutants lacking FHA elicited a stronger inflammatory response than did an isogenic FHA+ strain, suggesting that this adhesin may have a role in influencing the inflammatory response that may promote persistence of the infection (187, 189). In vitro biofilm formation by B. bronchiseptica was reported to involve FHA and fimbriae, and both Bvg+ and Bvg intermediate-phase cells optimally produced biofilms (190). The Bps exopolysaccharide (191) and extracellular DNA (192) have been identified as components of Bordetella biofilms. Importantly, the Bps polysaccharide was required for B. bronchiseptica biofilm formation in the nasal cavities of mice (191). B. pertussis also formed nasal biofilms in infected mice and bps mutants poorly colonized the nose and trachea compared with biofilm-proficient strains (193). The Bps polysaccharide has a role in complement resistance (194), as do the BrkA and Vag8 complement-resistance proteins (195, 196). Flagellar motility is required for the early stages of in vitro B. bronchiseptica biofilm formation, which is also characterized by expression of Bvg− phase genes (197). As the biofilm structure matures, the Bvg-regulated gene-expression program shifts to a Bvg+ response, with decreased flagellar gene transcription. The stringent response signal ppGpp was shown to be involved in biofilm formation in vitro (198) and cyclic-di-GMP and a putative diguanylate cyclase were reported to affect B. bronchiseptica motility and biofilm formation (199). It has long been thought that Bordetella species produced a capsule, which has been infrequently observed and not well characterized (200). Capsule production has not been reported to be involved in biofilm development or maintenance.

Metabolism
The classic Bordetella subspecies are obligate aerobes, and based on growth experiments and analysis of the Bordetella genomic databases, they do not have a glycolytic pathway for utilization of carbohydrates (155, 201). Their main mode of metabolism is the oxidative deamination of amino acids. These Bordetellae have a functional TCA cycle as well as an apparently intact glyoxalate bypass, consistent with the reported metabolism of acetate (202). B. pertussis cultured in standard minimal media having imbalanced nitrogen: carbon levels was shown to excrete ammonia as well as acetate, acetoacetate, and β-hydroxybutyrate, and intracellular poly-β-hydroxybutyrate storage granules were observed (203). Glutamate was identified in early research as a critical nutrient for B. pertussis (204) and can serve as the main amino acid for growth. B. parapertussis and B. bronchiseptica have genes encoding a urease, but in B. pertussis several of these genes are mutated (155). The urease has no apparent role in virulence (205) and the genes are repressed by BvgAS (206). Of the three classic subspecies, only B. bronchiseptica has an intact set of genes encoding components of a periplasmic nitrate reductase. No other putative denitrification gene candidates are present in the genome databases of the three classic subspecies.

Cysteine or cystine are preferred sulfur sources for the Bordetellae, and glutathione can also be used. For B. pertussis, neither methionine nor inorganic sulfate can serve as a substitute for cysteine (207, 208). Concordantly, B. pertussis has mutations in the cys genes involved in sulfate transport and cysteine biosynthesis from sulfate (120, 201). B. parapertussis and B. bronchiseptica have intact cys genes, but not a cysC homolog encoding an APS kinase. Similar to N. meningitidis that also lacks a cysC, Bordetella spp. have a CysN that is a predicted bifunctional sulfate adenyllyltransferase-adenylsulfate kinase protein (CysNC), as described for B. cenocepacia (132), that demonstrates high similarity with N. meningitidis CysN (53% identity; 9 e-148). In vitro, sulfate is a known inducer of the Bordetella-modulation response, abrogating expression of Bvg-activated genes (170). It was demonstrated that in B. pertussis stationary-phase cultures, metabolism of cysteine in the medium resulted in production and excretion of sulfate and pyruvate (209). The accumulated sulfate resulted in modulation of the bacteria, with decreased production of pertussis toxin. In the nasopharynx, one main host source of sulfur for B. pertussis is likely cysteine. The Bordetellae produce no known mucinases. That B. pertussis has lost the capacity to synthesize cysteine indicates it has evolved to utilize the cysteine reliably available in the host, obviating the need to take up sulfate for cysteine synthesis. In the host, B. parapertussis and B. bronchiseptica likely use cysteine as the preferred sulfur source, but may also utilize sulfate and perhaps related sulfates. For sulfur acquisition, all three
subspecies have genes predicted to allow the use of taurine (120, 155), which is found in airway surface fluids (Table 2).

Genomic-sequence analyses predict the Bordetellae can synthesize their required vitamins and cofactors including heme, thiamine, flavins, biotin, and folate. The Bordetella also have genes predicted to be involved in magnesium and zinc uptake (155, 201). The classical Bordetellae require NAD precursors such as nicotinic acid or nicotinamide and lack obvious nadA and nadB genes encoding NAD-biosynthesis enzymes, indicating the absence of a de novo NAD-biosynthesis pathway (155, 201, 210). In vitro, millimolar concentrations of nicotinic acid cause the Bvg-modulation response, but similar concentrations of nicotinamide have no modulating effect. That Bordetellae and pathogens such as H. influenzae lack de novo NAD-biosynthesis pathways, yet still colonize the respiratory tract, implies that NAD precursors are in sufficient abundance at this site to support microbial growth. Host sources of phosphorus in the upper respiratory tract likely include nucleic acids, phospholipids, phosphocholine, and inorganic phosphate. The classic Bordetella strains have a gene locus encoding putative phosphate transporter proteins (pstSCAB) as well as a polyphosphate kinase and exopolyphosphatase. These strains also have a gene predicted to encode a low-affinity inorganic phosphate transporter.

Iron
To obtain iron, B. pertussis, B. parapertussis, and B. bronchiseptica produce and utilize the alcaligin siderophore (211, 212, 213), and B. pertussis and B. bronchiseptica are also capable of using the xenosiderophores enterobactin (58), ferrichrome, and desferrioxamine B (214). B. bronchiseptica was also reported to use aerobactin, ferrichrys, ferricrocin, ferrirubin, protocelin, schizokinen, vicibactin, and pyoverdins (215). B. pertussis, B. parapertussis, and B. bronchiseptica each have at least 13 genes predicted to encode TonB-dependent outer-membrane receptors for iron uptake. The systems for four iron sources have been characterized to date: alcaligin, enterobactin, heme, and catecholamines.

Alcaligin is a dihydroxamate siderophore produced by the classical Bordetellae for iron uptake via the TonB-dependent outer-membrane receptor, FauA (216). Upon iron depletion and Fur derepression, the alcaligin-system genes are positively regulated by the AraC family transcriptional regulator, AlcR, in a process that requires activation by alcaligin (217). Interestingly, the alcaligin gene cluster of the opportunistic pathogen Bordetella holmesii was identified on a pathogenicity island apparently acquired by horizontal transfer, a process hypothesized to have contributed to its emergence and adaptation to the human host (218). Bordetellae do not produce the catechol siderophores enterobactin, salmochelin, and corynecubactin, but can use them to obtain iron via the BfeA outer-membrane receptor (58, 219). After Fur derepression, transcription of bfeA is induced by the BfeR AraC-type regulator that requires enterobactin or other catechols for its function (211, 219). The classical Bordetella subspecies use heme, presumably both as an iron and a heme source. Hemin is transported to the periplasm by the BhuR TonB-dependent receptor and subsequent uptake occurs via the ABC family BhuTUV transporter; BhuS bears similarity to known heme degrading or utilization proteins (146). The Bordetella bhuRSTUV heme genes are positively regulated by a surface-signaling mechanism that involves the adjacent Fur- and iron-repressible hurI and hurR genes. Under iron-depleted conditions, hurIR-bhuRSTUV are derepressed, resulting in basal levels of protein production (220, 221). When extracellular hemin binds the BhuR receptor, the receptor-occupancy signal initiates a regulatory cascade. The periplasmic N-terminal extension of BhuR transmits the signal to the cytoplasmic membrane-bound HurR anti-sigma factor with its bound HurI sigma factor (221). This activates the HurP protease, resulting in HurR cleavage, release of HurL, and dramatically increased bhuRSTUV transcription and Bhu protein production (222).

Infection studies have demonstrated the importance of Bordetella iron acquisition systems to growth in a host. A B. pertussis tonB mutant was attenuated in mouse infections (223) and a B. bronchiseptica alcaligin-biosynthesis mutant exhibited significantly reduced respiratory tract colonization in neonatal swine (224). Studies using a B. pertussis alcaligin-receptor mutant showed that alcaligin utilization was critical for colonization in a mouse model of respiratory infection (225) and the BfeA enterobactin receptor was important for B. pertussis growth in the early stages of mouse infection (226). In contrast, a B. pertussis bhuR heme-receptor mutant showed an in vivo growth defect only in the later stages of infection, suggesting that persistence in the host depends on heme utilization (227). During late infection, with increased inflammation and tissue damage, heme may become available to Bordetella cells on the epithelial surface. The ability of the heme-receptor mutant to produce and use its alcaligin siderophore could not compensate for its growth defect during late infection, suggesting that alcaligin may not effectively scavenge
iron once extensive tissue damage by *B. pertussis* has occurred. *In vivo* gene-expression studies demonstrated that *B. pertussis* increases expression of the alcaligin, enterobactin, and heme utilization genes during mouse respiratory infection (226). Since these iron-uptake systems are positively regulated in a manner requiring the presence of the cognate iron source, these results indicate these iron sources are present in the host during infection. Furthermore, the temporal patterns of gene expression were correlated with the infection stage during which the iron systems have their greatest impact on *in vivo* fitness: alcaligin gene expression was high throughout infection, the enterobactin-receptor gene was most highly expressed during early infection, and heme-receptor gene transcription was low during initial infection and significantly increased after the peak of infection (226). These mutant and gene-expression studies confirmed that Bordetella can discriminate between iron sources in the changing host environment and this ability contributes to its success as a pathogen.

The iron-acquisition systems used by *B. pertussis* during human infection are unknown. However, a study of human sera from uninfected controls and *B. pertussis* culture-positive patients showed antiserum responses to the receptors for alcaligin, enterobactin, and heme (226). This indicates that *B. pertussis* produces these proteins during natural human infection of the upper respiratory tract. These results also underscore the concept that *B. pertussis* is iron-starved *in vivo*, and implies that the cognate iron sources that induce expression of the alcaligin, enterobactin, and heme-system genes are available for consumption and perceived by *B. pertussis* in the human host environment.

Transcriptional-profiling studies of *B. pertussis* identified genes that were upregulated in response to iron starvation (228). In addition to identifying alcaligin, heme, and enterobactin-system genes, increased transcription of the type III secretion-system genes was observed as well as increased type III secretion-protein production. Genes encoding the previously unknown *fbpABC* periplasmic-binding protein-dependent transporter were also identified. Importantly, the Fbp ferric iron-uptake system was not specific for a single iron source but was required for utilization of inorganic ferric iron and ferric complexes of alcaligin, enterobactin, ferrichrome, and desferrioxamine B in both *B. pertussis* and *B. bronchiseptica*. Since the prototypic iron-uptake system often has a dedicated periplasmic and cytoplasmic membrane-transport apparatus, the finding that the Fbp system was needed for utilization of four structurally distinct siderophores is novel. The *B. pertussis* cDNA microarray study (228) also identified *ftrA*, encoding a cytoplasmic membrane permease similar to the *Saccharomyces* *Ftr1* (230) and *E. coli* *EfeU* (231) proteins required for ferrous iron utilization. *Brucella abortus* has a *FtrABCD* ferrous iron-utilization system that is very similar to the *Bordetella* system (232). In *B. pertussis* and *B. bronchiseptica*, *ftrABCD* are needed for ferrous iron utilization during growth at pH conditions of <7.0. Siderophores bind ferric iron, but pH values less than 7.0 favor ferrous iron stability, and siderophores like alcaligin lose their effectiveness. In the mammalian respiratory tract, the pH values range from ~5.5 to 7.9 (233, 234), suggesting that iron acquisition by pathogens in the airways requires the flexibility to utilize not only a variety of iron sources, but both oxidized and reduced forms of iron.

Host catecholamine neuroendocrine hormones such as epinephrine, norepinephrine, and dopamine have overall structural similarity to enterobactin and can remove iron from transferrin and lactoferrin, making it available to bacterial pathogens (65–69). *B. bronchiseptica* can obtain iron bound to transferrin or lactoferrin by a mechanism that uses these catecholamines (and L-DOPA) and any of three TonB-dependent outer membrane receptors, BfrA, BfrD, and BfrE (70). This iron-retrieval mechanism functions in the absence of any siderophore, indicating the receptors bind ferric complexes of the catecholamines. With ferric transferrin as the only source of iron, norepinephrine can synergize with alcaligin or enterobactin and significantly stimulate *B. bronchiseptica* growth. On infecting a host, *B. bronchiseptica* may not yet produce useful amounts of alcaligin and may rely on xenosiderophores or catecholamine hormones for iron uptake from lactoferrin or transferrin. That *Bordetella* cells can use host catecholamines to obtain iron from lactoferrin and transferrin in the absence of a siderophore suggests that these organisms have evolved to exploit host stress hormones for nutritional gain.

**CONCLUDING REMARKS**

*N. meningitidis* is thought to have evolved from nonpathogenic *Neisseria* mucosal-commensal species and itself grows as a commensal most of the time. Despite its paucity of toxins and other obvious virulence factors, *N. meningitidis* cells that colonize the nasopharynx can enter the epithelial cells and gain access to the submucosa where they can disseminate, leading to catastrophic septicemia and meningitis. *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*,
and *B. bronchiseptica* likely evolved from *Achromobacter* species that live in the external environment. Bordetellae such as *Bordetella petrii* (235) and *Bordetella* strain 10d (236) are also inhabitants of the outside world, living in soil and river sediments. In contrast to *N. meningitidis*, the classical *Bordetella* species evolved to produce a variety of secreted toxins that modify or damage host cells. Unlike *N. meningitidis*, these pathogenic *Bordetella* species remain localized to the upper respiratory tract of immune-competent hosts. Despite these differences, both *N. meningitidis* and *B. pertussis* have evolved in their hosts to become obligate human pathogens. They are sensitive to desiccation, are similarly transmitted between hosts, and share metabolic traits such as an obligately aerobic respiration, and the ability to deaminate amino acids for use as carbon sources. Microorganisms that live in the nasopharynx have a close relationship with mucus, and infecting pathogens must penetrate the mucus layers to colonize respiratory epithelial cells. Flagellar motility is not necessary for transit through the mucus blanket since *N. meningitidis* and *B. pertussis* do not produce flagella. These bacteria may be able to interact uniquely with airway mucin networks, and effectively surfe the mucus layers to reach the epithelium. For *N. meningitidis*, its twitching motility may have a role in this process.

Components of mucus are sources of nutrition for *N. meningitidis* and the classical *Bordetella* species. Whiteley and colleagues' review of the host as a growth medium (237) noted Louis Pasteur's early model of how the host, in effect, serves as a culture vessel for microbial pathogens (238). Edward D. Garber was a plant geneticist who also studied fungal and bacterial plant pathogens. His studies led him to further crystallize the fundamental concept that microbes are successful are those that can obtain required nutrients from the host (239, 240). A host niche that has those nutrients and also lacks inhibitory factors will be effectively colonized. Recent advances in genetic and analytical technologies have provided critical knowledge of host-niche environments. Determination of the contributions of resident airway microbiota to pathogen colonization and nutrient acquisition would be especially informative. It is also important to know what specific mucosal metabolites are taken up by pathogens and metabolized in different airway microenvironments, during different stages of infection, and in healthy, chronically diseased, and stressed hosts. Identification of the molecules that bacterial pathogens assimilate while living in the respiratory tract will allow development of more biologically relevant growth media for their *in vitro* cultivation and study. These studies will provide a more comprehensive understanding of the connections between bacterial metabolism and virulence.

**ACKNOWLEDGMENTS**

I thank Timothy Brickman and Vaiva Vezys for helpful discussions and critical review of sections of this chapter. Research in my laboratory has been supported by Public Health Service Grant AI-31088 from the National Institute of Allergy and Infectious Diseases.

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