Virulence Factors in Anaerobes

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Among the broad spectrum of species of anaerobic bacteria in the normal flora of humans, a few exhibit marked pathogenic potential and are responsible for the majority of infections. The factors that determine the virulence of particular species are varied and probably interrelated. Just as most anaerobic infections are polymicrobial and depend on interactions of a combination of species, the virulence of a species probably depends on a combination of properties, including surface structures, metabolic functions, ability to avoid the host's defenses, and capacity to damage tissues. Thus, the production of each virulence factor—adhesins that attach to epithelial and red blood cells and to other bacteria, producing metabolically interdependent ecosystems; capsules that protect against phagocytosis and induce abscess formation; lipopolysaccharide; proteases, including those that degrade immunoglobulins and complement components; and other hydrolytic enzymes—represents only a component of virulence, but a consideration of these factors in combination begins to clarify the mechanisms by which anaerobes cause disease.

The term *pathogenicity* describes the general capacity of a microorganism to cause disease. A wide range of anaerobic bacteria have been isolated at one time or another from infections of humans, with a high proportion of these infections caused by only a few anaerobic species. The quantitative pathogenic capability of these species defines their virulence, and the specific properties that enable them to cause disease are their virulence factors.

Except in the classic clostridial diseases, in which specific exotoxins are responsible for characteristic clinical effects, three main features are common to most anaerobic infections. First, the source of the infecting microorganism is the endogenous flora of the patient's own gastrointestinal, oropharyngeal, or genitourinary mucosa. Second, alterations of the host's tissues due, for example, to trauma and/or hypoxia provide suitable conditions for the development of secondary or opportunistic anaerobic infections. Third, anaerobic infections are generally polymicrobial, often involving mixtures of several anaerobic and aerobic species acting synergically to cause damage.

Although anaerobic bacteria can cause severe and even fatal infections, the initiation of infection generally depends on host factors. Even in such opportunistic situations, some species show a particularly strong pathogenic potential not evident for the majority of related species that reside in the same normal habitat and have many of the same metabolic and phenotypic characteristics. Thus, most of the various species of gram-negative anaerobic bacilli found in the normal flora of humans have some pathogenic capability in mixed infections derived from their normal habitat, but a few species display much greater potential than the others for causing opportunistic infections. The majority of serious anaerobic infections are caused by this small number of more virulent species, which are listed in table 1 [1, 2].

In the clostridia, virulence is related directly to the production of specific and highly potent exotoxins. In the nonspore-forming anaerobes, the properties that determine virulence are less well defined, although studies of several of the more common pathogenic species have begun to elucidate some possible mechanisms. The gram-negative anaerobic bacilli responsible for the majority of anaerobic infections have been studied in most detail during the last 20 years: *Bacteroides fragilis* in abdominal infections; *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Fusobacterium nucleatum* in periodontal disease and other infections related to the mouth; *Porphyromonas asaccharolytica* and *Bacteroides ureolyticus* in superficial necrotizing infections; and *Fusobacterium necrophorum* in necrobacillosis (in both humans and other animals). None of these species represents a major component of the normal flora at the relevant infection sites. *B. fragilis* accounts for fewer than 10% of fecal isolates of *Bacteroides* species but for ~75% of isolates from abdominal wounds. It is also isolated from sites of uterine and pelvic sepsis and from brain and lung abscesses but is not part of the normal flora of the vagina or the mouth. *P. intermedia* is isolated from the normal gingival flora but represents a much smaller proportion of the total count of *Prevotella* than do other species; *P. gingivalis* is rarely isolated from healthy gingiva. Both of the latter species are predominant components of the gingival flora in patients with periodontal disease [3]. *P. asaccharolytica* and *B. ureolyticus* are found in small numbers in the normal flora of the gastrointestinal and genitouri-

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Table 1. Species of gram-negative anaerobic bacilli present in the normal flora of humans and isolated most commonly from infections.

Site of isolation	Species
Normal flora	
Feces	*Bacteroides vulgatus*
	Bacteroides distasonis
	Bacteroides thetaiotaomicron
	Bacteroides uniformis
Vagina	*Prevotella bivia*
	Prevotella disiens
	Prevotella melaninogenica
Mouth	*P. melaninogenica*
	Prevotella intermedia
	Prevotella oralis
	Fusobacterium nucleatum
Infections	*Bacteroides fragilis*
	B. thetaiotaomicron
	Porphyromonas asaccharolytica
	Porphyromonas gingivalis
	P. intermedia
	Bacteroides ureolyticus
	Fusobacterium necrophorum

nary tracts but are associated particularly often with necrotizing, ulcerative, and gangrenous infections (e.g., genital, decubitus, varicose, and diabetic ulcers and gangrene) and with pilonidal, sebaceous, and breast abscesses [4]. Compared with other anaerobic species, *F. necrophorum* has more of the characteristics of a virulent primary pathogen; it is rarely isolated from the normal human flora but most typically causes severe purulent tonsillitis with pseudomembrane formation and lymph node involvement, septicemia, and metastatic abscess formation that may prove fatal for a previously healthy person [5].

The Infective Process

Several key stages are common to all infective processes, although their relative significance differs with the type of infection. Virulence factors enable species to elicit these stages of infection (table 2). Specifically, to establish an infection, bacteria must attach to target cells (often mucosal or epithelial cells), invade the tissues, establish themselves by multiplying at the site of infection and avoiding elimination by the host's defense mechanisms, and cause damage both to local tissues and—in systemic infections—to the whole patient.

Attachment and Adhesion

Several pathogenic species of gram-negative anaerobic bacilli can adhere to various types of cells. The target cells examined in greatest detail thus far are epithelial cells, which reflect the organisms' normal habitat, and erythrocytes, which may be agglutinated by the more pathogenic species. Among the intestinal *Bacteroides* species, *B. fragilis*—the most virulent—shows a particularly pronounced ability to adhere to epithelial cells and to cause hemagglutination [6, 7]. *B. fragilis* produces fimbriae and a capsule, both of which may be involved in attachment. The role of the fimbriae is not clear; they are present on isolates from abscesses and on those from the normal fecal flora, and their contribution may be related to their ability to adhere to the mucosal surface [8]. The capsule is an established virulence factor that protects against phagocytosis and induces abscess formation; however, it is also important in adhesion to mucosal epithelial cells and peritoneal mesothelium [9] and in hemagglutination by means of the capsular carbohydrate residues on the bacterial surface. These properties are independent of fimbria production [6, 7]. *B. fragilis* also manifests adherence to fibronectin and laminin, but the degree of adherence varies among strains and its relation to virulence is not yet clear [10]. The role of mucosal adhesion in the pathogenesis of

Table 2. Potential virulence factors in gram-negative anaerobic bacilli.

Infective stage, factor	Species
Adhesion	
Capsular polysaccharide	*B. fragilis*
Fimbriae	*B. fragilis*
Hemagglutinin	*P. gingivalis*
Lectin	*F. nucleatum*
Coaggregation	Many oral species
Invasion	
Phospholipase C; protease	*F. necrophorum*
Establishment of infection/tissue damage	
Exoenzymes	
Hemolysin	Many species
Proteases	*Porphyromonas* species
Collagenase	*P. gingivalis*
Fibrinolysin, Neuraminidase, Heparinase, Chondroitin sulfatase, Glucuronidases, *N*-Acetylglucosaminidase	*B. fragilis*, *Porphyromonas* species, and others
Antiphagocytic and antilytic factors	
Capsule	*B. fragilis*, *P. gingivalis*
Metabolic products	*P. gingivalis*
Lipopolysaccharide	*B. fragilis*
IgA (IgM, IgG) protease	*Porphyromonas* species, *Prevotella* species
Metabolic products	
Volatile fatty acids	Most anaerobes
Sulfur compounds	Most anaerobes
Amines	Most anaerobes
Stimulation of host-mediated damage	
Lipopolysaccharide	*F. necrophorum*, *P. gingivalis*
Surface-mediated material	*P. gingivalis*

intraabdominal infection by *B. fragilis* is not entirely clear; however, such adhesion may increase the proportion of *B. fragilis* organisms present at the mucosal surface (as opposed to counts in the luminal or fecal flora), thereby enhancing the likelihood of infection once the mucosa is breached.

Most other species studied in relation to adhesive mechanisms have been oral periodontal pathogens. *F. nucleatum* adheres via a lectin-mediated mechanism [11] through an arginine bridge [12] to a galactose-containing receptor on crevicular epithelial cells and erythrocytes, causing hemagglutination [13]. Among the pigmented oral species, *P. gingivalis* and *P. intermedia* adhere to crevicular epithelium and cause hemagglutination [6]. *P. gingivalis* has a hemagglutinin on its surface that is not part of the capsule or lipopolysaccharide (LPS) [14]. Protease produced by this species may also enhance its adhesive properties by degrading immunoglobulins and complement components that might themselves block receptors on the bacterial surface [15].

Another aspect of adhesion that may be important in polymicrobial infections is the ability of bacterial species to coaggregrate—i.e., to adhere to one another [11]. *F. nucleatum* coaggregates with other periodontal pathogens (e.g., *P. gingivalis*) via outer-membrane proteins, differences in which may be related to differences in virulence among strains of *P. gingivalis*, *Porphyromonas endodontalis*, and *P. intermedia* [15]. *Prevotella denticola* and *P. intermedia* coaggregate strongly with *Streptococcus sanguis*, and *Prevotella loescheii* has a fimbrial adhesin that is composed of 77-kD hexamers rich in aspartate and glutamate and that mediates adhesion to *Actinomyces*, *Capnocytophaga*, and *Selenomonas* species [11]. These coaggregates may be important in providing the appropriate conditions for the symbiotic metabolism and pathogenic interaction characteristic in these infections.

Although it is not relevant to the classic clostridial diseases, adhesion may be important in infections caused by *Clostridium difficile*, leading to antibiotic-associated diarrhea and pseudomembranous colitis. *C. difficile* has two surface proteins that act as adhesins, enabling the organism to attach to the intestinal mucosa in the initial stages of infection [16].

Invasion

Most anaerobic pathogens are not primarily invasive. *Clostridium tetani*, *Clostridium perfringens*, and most of the gram-negative anaerobic bacilli do not invade across intact, healthy epithelial or mucosal surfaces. Rather, the initiation of infection depends on initial damage due to trauma, hypoxia, neoplasia, or some other alteration to provide the route of entry for the anaerobes. *F. necrophorum* is an exception to this rule; it is a primary pathogen that can cause systemic infection in a previously healthy person. Initial infection of the tonsils and pharynx can evolve rapidly into invasive, potentially fatal disease. The specific properties that enable *F. necrophorum* to invade are not clear but may include the range of toxins and aggressins that are the hallmark of its virulence.

Establishment of Infection

Once the initial damage has allowed the anaerobes to penetrate the tissues, they must establish a focus of infection by multiplying and avoiding elimination by the host's defense mechanisms.

Metabolic interactions and nutrition. Most anaerobic infections are polymicrobial, and the metabolic interdependency of the bacterial mixtures involved—which generally include both obligate anaerobic and facultative species (e.g., *B. fragilis* and *Escherichia coli* in intraabdominal infections)—is important to their establishment in the tissues, to the satisfaction of their nutritional requirements, and to the expression of their synergic pathogenicity. For optimal growth, most anaerobic pathogens require complex nutrients released from the damaged host tissues. Host factors are significant in the establishment of anaerobic infection; from this perspective, the virulence of anaerobic species is a reflection of their ability to exploit the compromised host environment. In particular, tissue damage and necrosis, a reduction in blood supply leading to hypoxia, and the presence of a blood clot or a foreign body or substance (especially $CaCl_2$) create conditions appropriate for anaerobic growth [17]. The capacity of facultative species such as *E. coli* to consume oxygen, thereby creating reduced conditions favorable to the growth of anaerobes such as *B. fragilis*, may be a contributory—but not necessarily essential—factor in abscess formation. The alleged role of catalase and superoxide dismutase in the establishment of anaerobic bacteria at more oxygenated sites has not been substantiated; there appears to be little relation between the level of production of these enzymes by a given strain and the virulence of that strain [18].

In the development of clostridial myonecrosis, carbohydrate breakdown in anoxic muscles reduces the redox potential; this effect is enhanced by the fermentation of pyruvate to lactate, which also lowers the pH. Muscle proteinases are activated, releasing amino acids that further reduce the pH and provide essential nutrients for the clostridia. Bacterial growth and enzyme production then take over and enhance the degradative process [19]. Nonclostridial anaerobes require several growth factors and nutrients produced by damaged host tissue or by other bacteria acting in synergy. In other words, factors produced by one organism may enhance the growth of another. For instance, *B. fragilis* uses hemoglobin and hemoglobin-haptoglobin complexes as sources of iron and of the porphyrin component [20]. In mixed infections, such as intraabdominal abscesses, it can use the heme-binding protein from *E. coli* to overcome iron-limiting conditions [21]. *B. fragilis* also produces heparinase and chondroitin sulfatase, which hydrolyze heparin and chondroitin sulfate, allowing their use as nutrients [22]. Similarly,

P. gingivalis lyses erythrocytes by the action of its cysteine proteinase, gingivain, to release heme and demetallates protoheme to protoporphyrin, providing for its iron needs [14]. Hydrolytic enzymes, neuraminidase, and proteases produced by black-pigmented anaerobic bacilli such as *P. gingivalis*, *P. asaccharolytica*, *P. intermedia*, and *P. denticola* release nutrients and growth factors for these species themselves and for the interdependent members of the polymicrobial ecosystem in which the infection takes place [23–25]. The roles of some of these enzymes in tissue damage are considered below. Other growth factors, such as vitamin K and protoheme, are produced by one species and used by others in this symbiotic environment [11].

Resistance to host defenses. The host responds to bacterial infection, including anaerobic infection, both with phagocytosis and with opsonization and killing by serum immunoglobulin and complement. The most virulent species of anaerobic bacteria have various means of avoiding and resisting these defense mechanisms. Several of the pathogenic species—particularly *B. fragilis* and the black-pigmented, gram-negative anaerobes—produce a capsule in vivo. The polysaccharide capsule of *B. fragilis* has been most widely studied; in addition to promoting adhesion and providing protection against phagocytosis [26], the capsule provokes abscess formation [27]. The formation of intraabdominal abscesses in humans is generally due to synergy between obligate anaerobes (usually *B. fragilis*) and facultative species [28]. However, in an experimental model, capsulated strains of *B. fragilis* alone, heat-killed capsulated strains, and even purified capsular material each induced abscess formation [27]. Furthermore, immunization of rats with the purified capsular polysaccharide of *B. fragilis* induced T cell–mediated immunity that protected against abscess development [29]. The capsular polysaccharide is a complex of two distinct polysaccharides—A and B—that have different antigenic and immunoelectrophoretic properties and are held together by ionic interactions [30].

B. fragilis inhibits macrophage migration and impairs the phagocytosis of other species involved in polymicrobial infection. Thus, for example, enterobacteria at a site of mixed infection provide suitable growth conditions for *B. fragilis* and are themselves protected from phagocytosis. The presence of capsular material [31] and the depletion of serum opsonins by *B. fragilis* [32] contribute to this synergic protection, but soluble products have also been shown to inhibit the activity of phagocytic cells. Culture filtrates of *B. fragilis* inhibit both the polymorphonuclear leukocyte (PMN) chemotactic response to *E. coli* components and the phagocytosis and killing of this organism by PMNs and macrophages [33]. The low-molecular-weight factors responsible for this inhibition are succinic and other short-chain fatty acid metabolic products of the anaerobes [32, 34].

P. gingivalis produces a capsule that provides protection against phagocytosis and intracellular killing [35, 36]. It also generates metabolic products that compete with chemotactic peptides, heat-labile opsonins, and complement components to block chemotactic receptors on PMNs [37]. The *P. gingivalis* capsule masks the LPS, thus preventing the activation of complement [38].

Several virulent anaerobic species generate products that inhibit or destroy the humoral components of the host's defenses. The LPS of *B. fragilis* has little endotoxic activity but reduces the opsonic activity of complement [39]; the synergic antiphagocytic effects of *B. fragilis* may be due in part to depletion of serum opsonins [32, 40]. The black-pigmented species produce a range of proteolytic enzymes active against immunoglobulins and complement. Enzymes of *Porphyromonas* species (*P. gingivalis*, *P. asaccharolytica*, and *P. endodontalis*) degrade IgA, IgM, IgG, and plasma proteins such as C5 and the bacteriolytic component C3 [41]. IgA proteases are also produced by *Prevotella melaninogenica* and *P. intermedia* [42]. Most anaerobic bacteria produce various soluble metabolites that exert leukotoxic effects, inhibit leukocyte chemotaxis, and damage mucosal cells [32]. Even species for which no specific antiphagocytic factors have been demonstrated—e.g., the periodontal pathogens *P. intermedia*, *Prevotella oralis*, and *F. nucleatum*—have been shown to be less readily phagocytosed than the α-hemolytic streptococci and anaerobic cocci commonly found at the same infected sites [43].

Tissue Damage

As has been described, several virulence factors contribute to tissue damage as an infection becomes established; other virulence factors make a major contribution later in the disease process (table 2). For example, specific exotoxins produced by the infecting bacteria cause the particular damage associated with tetanus and *C. difficile* colitis. Myonecrosis due to *C. perfringens* is also considered to be mediated by exotoxins, but in this case several toxins are actually potent exoenzymes: phospholipase C (α toxin) destroys cell membranes and lyses erythrocytes, while collagenase and hyaluronidase break down tissue components; the role of other toxins and enzymes is less clear [19]. In this infection, the pathogenic mechanisms of *C. perfringens* are similar to the less specific mechanisms of the gram-negative anaerobes but are especially potent; as a result, *C. perfringens* is potentially highly virulent.

In infections in which gram-negative bacilli play a substantial role, several virulence factors appear to act in combination to produce damage that is manifest as tissue necrosis and abscess formation. These factors include soluble metabolic products, hydrolytic enzymes, proteases, and LPS or other surface-associated material.

(*1*) *Metabolic products.* Several products of anaerobic metabolism are toxic to mammalian cells: volatile fatty acids, sulfur compounds (including H_2S), indole, and (especially) amines. These substances are produced by most anaerobes;

amines have been linked particularly strongly to tissue damage in infections with *F. nucleatum*, a species that exhibits little hydrolytic activity and no unusual LPS-related features [18]. These compounds are also implicated in the production of the foul-smelling exudate of bacterial vaginosis, in which amines generated by the metabolic interaction of *Prevotella* species (predominantly *Prevotella bivia*), *Gardnerella vaginalis*, and *Mobiluncus* species may stimulate excessive secretion by the vaginal mucosa without inducing inflammation [44].

(*2*) *Hydrolytic enzymes.* Most pathogenic anaerobes produce extracellular enzymes that hydrolyze tissue components and are thought to play a significant role in the development of disease. *B. fragilis*, *P. gingivalis*, *P. asaccharolytica*, *P. intermedia*, *P. melaninogenica*, and *P. denticola* produce hyaluronidase, chondroitin sulfatase, heparinase, and a range of enzymes that hydrolyze carbohydrates (e.g., α- and β-glucosidases, *N*-acetylglucosaminidase). All of these enzymes play a dual role, causing tissue damage and providing nutrients for the infecting microorganisms. In addition to such hydrolytic enzymes, both a lipase that damages cell membranes and a separate hemolysin are produced by *F. necrophorum* [14, 18, 24, 25].

(*3*) *Proteases.* Proteolytic activity has been most widely studied in the asaccharolytic and strongly proteolytic *Porphyromonas* species and in *Prevotella* species. Proteases are thought to be important in the destruction of gingival tissue and of the collagen bridges in the gingival crevice in periodontal disease [45]. *P. gingivalis* produces proteases that degrade immunoglobulins and complement components as well as tissue proteins such as collagen [18]. In addition, as has been mentioned, *P. gingivalis* produces a cell surface–associated cysteine proteinase, gingivain, that disrupts basement membrane proteins, lyses cell membranes, and hydrolyzes a wide range of proteins [14, 24]; the observation that mutants not producing this proteinase are avirulent confirms its role as a virulence factor [46]. Proteases may be important in the contribution of the asaccharolytic species *P. asaccharolytica* and *B. ureolyticus* to tissue damage in ulcerative and gangrenous lesions such as genital and perineal ulcers, decubitus and varicose lesions, and diabetic gangrene [4]. Several species produce fibrinolysin, which breaks down the fibrin deposited in and around infected sites as the host's defense mechanisms attempt to circumscribe the infection.

(*4*) *LPS.* The LPS of most gram-negative anaerobes exhibits much less endotoxic activity than the LPS of enterobacteria. Also of interest is that the LPS of *B. fragilis* may inhibit enterobacterial endotoxic activity. Until recently, it was thought that LPS from *Bacteroides*, *Porphyromonas*, and *Prevotella* species did not contain ketodeoxyoctonate (KDO) and thus was less likely to cause classic manifestations of endotoxic damage, such as disseminated intravascular coagulation [47, 48]. These species are now known to produce KDO that is detectable only after acid extraction, and other differences [48a] are known to be related to the reduced toxicity.

However, the LPS of *P. gingivalis* appears to play a significant role in the pathogenesis of periodontal disease, in which (like the LPS of *B. fragilis*) it reduces the opsonic activity of serum and induces host-mediated damage by stimulating gingival inflammation, increasing the secretion of collagenase from host cells and reducing collagen formation, and inducing localized bone resorption around the tooth root—a process that greatly increases the likelihood of tooth loss [49–51]. These effects are attributable to the release of biologically active agents, including cytokines, from host cells exposed to bacterial products. Whole cells and purified LPS of *P. gingivalis* cause the release of interleukin 1 and tumor necrosis factor from macrophages and monocytes [49], and LPS from several of the more virulent species of gram-negative anaerobes induces DNA replication and polyclonal immunoglobulin production in B lymphocytes [51]. Intensive studies of *P. gingivalis* in relation to periodontal disease have yielded evidence that the production of multiple virulence factors by this species is controlled by a common regulator ("regulon"), which is itself regulated by environmental factors such as temperature, osmolality, and iron and magnesium levels that cause variation in the expression of virulence [49].

F. necrophorum is an exception to the general rule that LPS from gram-negative anaerobes is less toxic than that from enterobacteria. Its LPS contains KDO and displays endotoxic activity similar to that of enterobacterial LPS, particularly during the septicemic phase of necrobacillosis, in which the patient is clearly septic and may develop endotoxic shock and disseminated intravascular coagulation [6]. The purified porin fraction of *F. nucleatum* induces B cell mitogenesis and activation and stimulates macrophage activity [52].

(*5*) *Surface-associated material.* Cell wall components other than LPS may be virulence factors in gram-negative anaerobes. A surface-associated material from *P. gingivalis*, distinct from LPS and gingivain, has been shown to stimulate bone resorption through the release of interleukin 1 and tumor necrosis factor from host cells and also to inhibit synthesis of DNA and collagen [53].

Conclusion

The factors that determine the virulence of particular species of anaerobic bacteria are varied and probably interrelated. Just as most anaerobic infections are polymicrobial and depend on interactions of a combination of species, the virulence of a species probably depends on a combination of properties, including surface structures, metabolic functions, ability to avoid the host's defenses, and capacity to damage tissues. Thus studies on the production of individual factors —adhesins, capsules, LPS, and proteases and other hydroly-

tic enzymes—focus on only a portion of the mechanisms of anaerobic virulence; however, taken in combination, the results of these studies have begun to clarify the mechanisms by which anaerobic microorganisms cause disease.

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