

Virulence factors and their mechanisms of action: the view from a damage–response framework

Arturo Casadevall and Liise-anne Pirofski

ABSTRACT

The virulence factor concept has been a powerful engine in driving research and the intellectual flow in the fields of microbial pathogenesis and infectious diseases. This review analyzes virulence factors from the viewpoint of the damage–response framework of microbial pathogenesis, which defines virulence factor as microbial components that can damage a susceptible host. At a practical level, the finding that effective immune responses often target virulence factors provides a roadmap for future vaccine design. However, there are significant limitations to this concept, which are rooted in the inability to define virulence and virulence factors in the absence of host factors and the host response. In fact, this concept appears to work best for certain types of bacterial pathogens, being less well suited for viruses and commensal organisms with pathogenic potential.

Key words | damage–response framework, microbe, pathogen, pathogenicity, virulence, virulence factor

Arturo Casadevall (corresponding author)
Liise-anne Pirofski
Division of Infectious Diseases,
Department of Medicine and the Department of
Microbiology and Immunology of the Albert
Einstein College of Medicine,
1300 Morris Park Avenue,
Bronx NY 10461,
USA
Tel.: +1 718 430 2215
Fax: +1 718 430 8968
E-mail: casadeva@aecom.yu.edu

INTRODUCTION

The idea that pathogenic microbes are endowed with certain components that confer upon them the capacity for virulence is the central theme of the virulence factor concept. Although the definition of what constitutes a virulence factor is varied and controversial (Casadevall & Pirofski 1999), and this idea has been undermined by the finding that commensal organisms cause disease (Casadevall & Pirofski 2001), the concept maintains a powerful grip in the imagination of investigators and continues to drive much of the intellectual and experimental energy in the field of microbial pathogenesis. The virulence factor concept has unquestionably led to the identification of important microbial attributes of virulence that have greatly furthered our understanding of microbial pathogenesis. Furthermore, the approach of defining virulence factors by the use of the molecular postulates (Falkow 1988, 2004) has provided an experimentally rigorous approach to the study of virulence in certain microbes. Nevertheless, the virulence factor concept has significant limitations for a global

understanding of microbial virulence. In this paper, we review the historical context for the emergence of the virulence factor concept and then consider it from the viewpoint of the damage–response of microbial pathogenesis.

THE HISTORICAL CONTEXT

The development and proof of the germ theory of disease in the second half of the 19th century brought about a revolution in the history of medicine because it associated microbes with diseases. Once it was clear that some microbes caused disease, the next challenge was proving that the presence of certain microbes in a host led to the development of a certain disease. Koch's postulates, formulated at the time the germ theory was proven, provided a rigorous framework for assigning disease causality. However, the postulates had significant

limitations, including that they applied primarily to bacterial diseases and implied that the ability to cause disease was a transferable property that could be expressed in another host. The subsequent recognition that human hosts were inhabited by large numbers of microbes, although only a few had been associated with disease, contributed to the view that the microbes that caused disease were unique. The concept that there were pathogenic and non-pathogenic microbes raised the question of whether or not pathogenic microbes differed from non-pathogenic microbes, and if so, how they differed.

To understand the differences between pathogenic and non-pathogenic microbes early investigators tried to identify characteristics that allowed pathogenic microbes to cause disease. The majority of these early studies involved bacteria, since they caused most lethal infectious diseases for which an etiology could be determined at the turn of the 20th century. Studies on many of the most devastating infectious diseases of the time led to the discovery that the microbes that caused them expressed disease-conferring determinants. The discovery that the diseases caused by toxin-producing (diphtheria, tetanus, anthrax) and encapsulated microbes (pneumococcal pneumonia, meningococcal meningitis and *Haemophilus influenzae* meningitis) required the presence of specific microbial determinants led to the view that there were intrinsic differences between pathogens and non-pathogens and the concept that disease-associated microbes were endowed with certain characteristics that enabled them to cause disease. Kolmer viewed microbial pathogenicity as arising from two microbial factors that he called toxicity and aggressiveness, with the latter being a measure of invasive power (Kolmer 1924). Microbial poisons and toxins that damaged the host caused toxicity, whereby aggressiveness was a complex trait that included the ability of a microbe to survive and multiply in tissue (Kolmer 1924). Toxicity could result from the action of microbial exotoxins or endotoxins. The pathogenesis of diseases with certain organisms suggested that aggressiveness and toxicity were separable. For example, *Streptococcus pneumoniae* was viewed as highly aggressive, since it was endowed with a polysaccharide capsule that facilitated survival in tissue yet made little or no toxin, whereas *Corynebacterium diphtheriae* was highly toxic but displayed relatively little aggressiveness with regards to tissue

invasion. Although the characteristics of toxicity and aggressiveness were put forth as separate traits, the discovery that toxin-mediated cell damage could facilitate tissue invasion revealed that the interplay between microbial effects, the host response and the subsequent behavior of the microbe led to convergence, rather than separability. This phenomenon was illustrated by the 'leukocidins' produced by *Staphylococcus aureus* and *Streptococcus pyogenes* (Stewart 1968; Wilson & Miles 1975), which were exotoxins that facilitated invasion by killing leukocytes.

In the intellectual milieu that viewed pathogenic and non-pathogenic microbes as fundamentally different, Bail proposed his *aggressin* theory, which held that pathogenic microbes produced compounds known as aggressins that interfered with host defense mechanisms and allowed the microbe to establish itself in the host (for a review of Bail's work in English, see Zinsser (1914)). Although the compounds on which Bail based his theory were probably bacterial endotoxins, his ideas were highly influential and planted the conceptual seed that would eventually evolve into the view that pathogenic microbes had virulence factors. However, even in the early days of medical microbiology, there were indications that microbe-centric views of microbial pathogenesis could not be explained by the view that pathogenic and non-pathogenic microbes were intrinsically different. First, the discovery of the phenomenon of virulence attenuation implied that virulence was not a stable phenotype. The fact a pathogenic microbe could lose virulence suggested that there was a distinction between microbial virulence and pathogenicity. Although many authorities consider pathogenicity and virulence as synonymous, we make a distinction between these terms. We define pathogenicity as the capacity of a microbe to cause damage in a host (Casadevall & Pirofski 1999). In contrast, we consider virulence to be a relative quality and define it as the relative capacity of a microbe to cause damage in a host (Casadevall & Pirofski 1999). The need for a relative term is apparent from the fact that there are no absolute measures of virulence. The fact that virulence can be attenuated, or enhanced for a pathogenic microbe, highlights the relative quality of this term. Early investigators noted that attenuated microbes could sometimes be restored to their pathogenic phenotype by passage

through animal hosts. *Neisseria meningitidis* was found to rapidly lose virulence when isolated from patients with meningococcal meningitis, though it could be restored by passage in mucin-treated mice (Miller 1933). Second, landmark studies in immunity had shown that pathogenic microbes did not cause disease in immunized hosts and that passive antibody administration could completely protect certain naive hosts against lethal infection. The ability of the immune system to neutralize the pathogenicity of a microbe, and thus render it non-pathogenic, also argued against an essential difference between pathogenic and non-pathogenic microbes based on microbial characteristics alone. Consequently, some authorities professed the view that there was no fundamental difference between pathogenic and non-pathogenic microbes.

By the mid-20th century, the occurrence of diseases that were attributed to microbes previously considered to be non-pathogenic was linked to medical advances that disrupted host immune function. For example, by the 1950s the introduction of broad spectrum antimicrobial therapy and corticosteroids was associated with candidiasis, a disease that was extremely rare earlier in the century (Jawetz 1956), and the widespread use of intravenous catheters was associated with *Staphylococcus epidermidis* bacteremia. The emergence of diseases caused by commensal microbes in hosts with immune impairment and/or altered skin and mucosal surfaces posed a direct challenge to microbe-centric views of microbial pathogenesis, while illustrating the critical role played by the host in the development of disease. However, microbial diseases that occurred in immunocompromised hosts were often viewed as a separate entity from the infectious diseases known since antiquity, as exemplified by the concepts of microbial opportunism and opportunistic infection (Poindexter & Washington 1974; Lauter 1975; von Graevenitz 1977 Armstrong 1993). Opportunistic microbes were often considered different from the pathogenic microbes, which were the subject of classical studies and referred to as primary pathogens. However, the distinction between opportunistic and primary pathogens was problematic, because microbes that caused disease in apparently normal hosts, such as *M. tuberculosis* and *S. pneumoniae*, caused disease more frequently in the setting of immune impairment and could be labeled as opportunists.

The emergence of the immunocompromised host as a distinct clinical group that was predictably at risk for diseases caused by certain microbes provided compelling evidence that virulence could not be an invariant microbial trait and challenged the view that pathogenic microbes possessed special characteristics that distinguished them from non-pathogenic microbes. *Candida albicans* and *Staphylococcus aureus* isolates recovered from the bloodstream of patients with and without catheters were indistinguishable when analyzed by molecular typing techniques. Despite a significant effort, virulence factors that are essential for virulence, such as those found in bacteria, have yet to be discovered in *Candida albicans*. In contrast, *Cryptococcus neoformans*, a yeast that causes disease predominantly in immunocompromised hosts, has a polysaccharide capsule and the ability to synthesize melanin pigments which are required for virulence in normal, as well as immunocompromised, hosts. However, the fact that acapsular *C. neoformans* strains can cause disease in hosts with severely impaired immunity illustrates that even the ability of classical virulence factors to cause disease can be a function of the immunological status of the host.

The absence of distinguishing characteristics between human commensal organisms and those associated with disease and the increasing reliance on concepts such as opportunism to explain the ability of a microbe to cause disease in one host, but not another, suggest that the view that virulence reflects the action of unique microbial determinants on the host is untenable. This has introduced uncertainty into the universality of the concept and definition of virulence factors. Since virulence factors are often targets of the immune response and the response to virulence factors can neutralize their action and provide protection, the quest to identify virulence factors has also led to studies of host defense and immunity. Such studies have served to validate the importance of virulence factors in disease pathogenesis. For example, antibody responses to microbial capsules and toxigenic proteins often render the host immune from disease with those microbes. In fact, the level of antibody to the capsular polysaccharide to *Haemophilus influenzae* can be used to ascertain the immune state of the host. However, studies of the immune response to microbial determinants have also revealed that determinants that elicit responses that benefit the host may

not occur or predominate during natural infection. Efforts to alleviate uncertainty regarding the nature and dispensability of virulence factors by striving for a more rigorous, but universal definition of virulence factors could bring faster progress in the development of vaccines and therapies for the most prevalent infectious diseases of our times, which are notable for their ability to cause disease in some, but not all, hosts and to occur in the setting of immune impairment.

THE PROBLEM OF DEFINING MICROBIAL VIRULENCE

The concept of virulence factors cannot be separated from that of microbial virulence, thereby defining one of the thorniest problems in microbial pathogenesis. The problem in defining virulence arises because virulence is a microbial property that can only be expressed in a susceptible host. Hence, virulence is not an independent microbial property, because it cannot be defined independently of a host. As a consequence of the dependence of virulence on the presence of a susceptible host, microbial determinants that contribute to virulence cannot be independent microbial attributes of virulence. Logically, the dependence of virulence factors on virulence, which in turn is dependent upon the host, implies that the definition of a virulence factor requires a functional definition for microbial virulence.

Historically, a precise definition for virulence has been elusive because virulence is only one outcome resulting from the interaction between a microbe and a host. Consequently, there are numerous definitions for virulence in the literature that have been formulated from microbe- and host-centric views of microbial pathogenesis. In 1999 we proposed a new general theory of host-microbe interactions (Casadevall & Pirofski 1999) that has been developed in subsequent papers into the 'damage-response framework' of microbial pathogenesis (Casadevall & Pirofski 2000, 2001, 2003; Pirofski & Casadevall 2002). In contrast to prior microbe- or host-centric views of microbial pathogenesis, the damage-response framework incorporates the contributions of both the host and microbe to microbial pathogenesis and virulence. The damage-response framework provides a new approach to defining

virulence and virulence factors that sidesteps the conundrum caused by the limitations of prior definitions.

VIRULENCE AND VIRULENCE FACTORS IN THE CONTEXT OF THE DAMAGE-RESPONSE FRAMEWORK

The damage-response framework is based on three tenets that are both self-evident and incontrovertible: (1) microbial pathogenesis is the outcome of the interaction between two entities, namely a host and a microbe; (2) the relevant outcome of host-microbe interaction in a given host is damage in the host and (3) host damage can reflect the action of microbial factors, the host response, or both. Damage is not a static outcome, but can change as a function of the immune response or time. When damage is plotted as a function of the host response, host-microbe interaction can be represented by a parabola, whereby maximal damage occurs in situations of either weak or strong responses (Figure 1). For example, consider human infection with *Mycobacterium tuberculosis*. In individuals with weak immune responses such as patients with AIDS the infection disseminates to various organs while in apparently immunocompetent individuals the infection

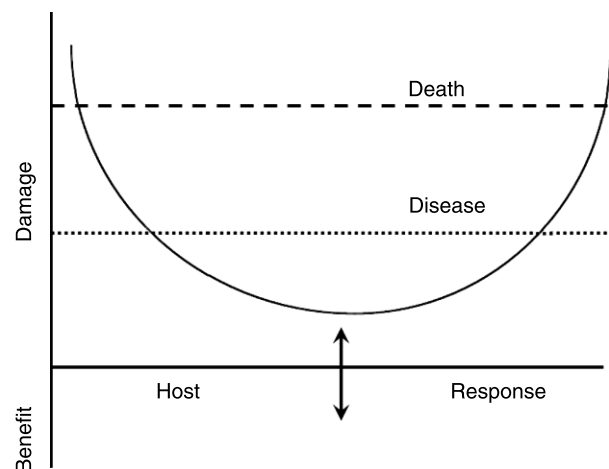


Figure 1 | Basic curve of damage-response framework of microbial pathogenesis. The damage-response framework posits that the outcome of the interaction between a host and microbe depends on the extent of damage (or its inverse, which is a benefit) sustained by the host. The damage-response framework proposes that the basic curve is a parabola whereby host damage is maximized at the extremes of the host response. For a detailed discussion of the damage-response framework see Casadevall & Pirofski (1999, 2000, 2003).

usually localizes to the lungs where a florid immune response can result in caseous necrosis. Use of this strategy to characterize damage as a function of the host response for different microbial agents led to the description of six types of host–microbe interactions, whereby the shape of the curve is a variation of the classical parabola (Casadevall & Pirofski 1999). In contrast, plotting host damage as a function of time simplified microbial pathogenesis into four outcomes of microbial infection, namely commensalism, colonization, persistence (chronicity) and disease (Casadevall & Pirofski 2000). The states of commensalism and colonization become indistinguishable as host damage approaches zero, as these states are continuous and differ only in the amount of damage incurred by the host as a function of time.

The damage–response framework provides a new way to look at host–microbe interactions. For example, consider the case of the host-associated microbial flora. Since humans are born sterile, the acquisition of the host-associated microbial flora is an infection in the sense that numerous microbes find residence in the body of the host. For some individuals the initial acquisition of certain microbes that are later considered commensal, such as *C. albicans*, can be associated with the development of a disease, neonatal candididasis. However, with increasing age, neonatal diseases usually resolve, possibly due to microbial control by the maturing immune system and the presence of other microbes. Hence, the acquisition of host-associated flora is viewed from the damage–response framework as an infection that leads to a state of colonization, which in this case is synonymous with commensalism since there is no apparent damage from this interaction. Furthermore, it is noteworthy that a single infection event can lead simultaneously to the states of colonization, persistence and disease in the same host. Consider a case of reactivation of latent infection with *C. neoformans* resulting in meningoencephalitis. In that individual the initial site of infection may remain as a pleural granuloma (persistence) with reactivation resulting in yeast cells spreading to airways without apparent inflammation or disease at that site (colonization) and meninges resulting in life-threatening hydrocephalus (disease).

We believe that the damage–response framework is a significant conceptual advance, because it provides a

robust, yet flexible, system that is based on simple accepted assumptions in microbial pathogenesis. The focus of the damage–response framework on host damage provides a new set of definitions that permit a different approach to the problem of virulence and, by extension, to virulence factors. In the damage–response framework, a pathogen is a microbe capable of causing host damage, virulence is the relative capacity of a microbe to cause damage in a susceptible host and a virulence factor is a microbial component that can damage a susceptible host. It is noteworthy that this definition of a pathogen is so broad that it could eventually lose its meaning since practically any microbe could cause damage in some host. However, this conundrum is precisely our point since we do not feel that making a distinction between pathogenic and non-pathogenic microbes is a useful way to approach microbial pathogenesis (Casadevall & Pirofski 2002). In fact, calling a microbe a pathogen has the inherent logical flaw that confers to the microbe an attribute that is independent of the host and consequently one might question the essential usefulness of this term. We have argued that, instead of making distinctions between pathogens and non-pathogens, a more productive avenue is to focus on the outcome of the host–microbe interaction (Casadevall & Pirofski 2002). It is noteworthy that others have challenged the validity of limiting the word ‘pathogen’ to microbes as a corruption of the original meaning of the term (Cunliffe 2008).

Despite the definitional issues, controversies and limitations that arise as attempts are made to put complex phenomena into simpler terms, we need words to communicate and the definitions proposed in the damage–response framework are simple, functional and free from limitations imposed by formulations based on microbial- or host-centered constraints. For example, one of the problems with defining a virulence factor as a microbial component that is needed for virulence but not viability is that it excludes many cell wall compounds that can contribute to pathogenesis by virtue of their effects on the host (e.g. bacterial endotoxins). Bacterial endotoxins are essential components of the outer membrane of Gram-negative organisms, and as such can be required for viability; however, endotoxins were recognized as aggressins by investigators in the early 20th century as ‘endogenous bacterial toxins’ and are widely accepted as ‘virulence factors’ today. This problem does not

arise in the damage–response framework, because it defines a virulence factor in the context of host damage, which allows the inclusion of microbial components that are either necessary and/or dispensable for microbial survival *in vitro* as virulence factors.

VIRULENCE FACTORS AFFECT THE SHAPE OF THE ‘DAMAGE–RESPONSE’ RELATIONSHIP

In the context of the damage–response framework, virulence factors can also be viewed as microbial components that alter host–microbe interaction by increasing the degree of damage relative to the degree of the host response and time. In terms of the basic parabola representing host–microbe interactions, the curve could be shifted upwards to reflect an increased amount of damage, up and to the left to reflect increased damage in the setting of a weak immune response, or up and to the right to reflect increased damage in the setting of a strong immune response. Consider the interaction of *Streptococcus pyogenes* with a host. In the setting of weak host responses, *S. pyogenes* can cause pharyngitis, whereas in hosts that mount inappropriately strong responses the outcome can be rheumatic fever and glomerulonephritis. In recent years, many toxins have been described for *S. pyogenes*, which can mediate such diverse diseases as myonecrosis or toxic-shock syndrome. The expression of these toxins is required for the specific disease syndromes but not for viability, and consequently the toxigenic phenotypes meet the more restrictive virulence factor definition of a component needed for virulence but not viability. In the damage–response framework, the presence of genes coding for exotoxin in *S. pyogenes* is also considered a virulence factor, because these proteins have the capacity to inflict damage on the host. In fact, an infection with a toxigenic strain has the effect of altering the damage–host response function relative to what would be expected with a non-toxigenic strain, such that the host suffers increased amounts of damage, irrespective of the immune response.

TYPES OF VIRULENCE FACTORS

The microbial attributes that confer the potential for virulence fall primarily within several categories, including

the ability to enter a host; the ability to evade host defenses; the ability to grow in a host environment; the ability to counteract host immune responses; the ability to acquire iron and nutrients from the environment and the ability to sense environmental change. However, attempting to fit virulence factors within neat categories of function is probably a futile exercise since some categories overlap and some attributes can be assigned to more than one group. For example, enzymes that digest host tissue damage the host, generate nutrients and can promote entry, and mechanisms that permit a microbe to evade phagocytosis enable survival in a host. When the outcome of these adaptations causes host damage, the microbe is a pathogen and its virulence is a relative measure of the damage it can induce. The microbial determinants that mediate damage in the context of microbial pathogenicity and virulence are the virulence factors that are the subject of this review. When assessing the contribution of virulence factors to virulence, it is important to consider the following themes: (1) very few virulence factors function as all-or-none determinants of virulence; (2) host damage can result from both direct microbial damage, the interaction of microbial components with the host or the immune response to microbial components and (3) immune responses, and in particular specific antibody responses, can neutralize many, if not most, virulence factors.

In the subsections below, we list a sampling of the types of virulence factors found in the literature. Our goal is neither to be exhaustive nor complete, but rather to give a feel for how different types of virulence factors work and damage the host. Furthermore, we make no attempt to group them into functional categories since most virulence factors have multiple effects in their interaction with the host and any attempt at neat categorization is largely a self-defeating exercise.

Exotoxins

Toxins were recognized as virulence factors at the dawn of the medical microbiology age when they were associated with disease with several toxigenic bacteria, including *Corynebacterium diphtheria*, *Vibrio cholera* and *Clostridium tetani*, the causes of diphtheria, cholera and tetanus, respectively. The toxins produced by these bacteria

are exotoxins, which are necessary for causing disease, but not required for the viability of the cell. Genes carried in phages, plasmids or pathogenicity islands usually encode these toxins and abolition of toxin production is usually accompanied by abrogation of virulence. In addition, toxins of *C. diphtheriae* and *C. tetani* can be denatured to produce toxoids which elicit protective immune responses that neutralize the toxin and prevent disease. Bacterial toxins contribute to virulence by interfering with cellular homeostasis, and for the toxigenic bacteria the disease can be usually attributed entirely to the action of the toxin on the host. The toxins of *Bacillus anthracis*, edema factor and lethal factor are enzymes that inactivate calcium- and calmodulin-dependent adenylate cyclase and mitogen-activated protein kinase, respectively (Collier & Young 2003). For *B. anthracis* both enzymes contribute to virulence by interfering with macrophage function and inhibiting an effective immune response (Collier & Young 2003). Similarly, *Bordetella pertussis* produces a calmodulin-activated adenylate cyclase toxin. In general, bacterial toxins mediate damage irrespective of the immune response because these proteins seldom elicit neutralizing responses in the context of natural infection, possibly because they are produced in too small amounts to trigger an immune response. Consequently, toxin-mediated diseases such as tetanus and diphtheria do not induce immunity and affected individuals can experience recurrent diseases. However, administration of preformed neutralizing antibody or vaccination with toxoids can induce a toxin-neutralizing antibody response that prevents disease.

Modulins

A large group of microbial compounds can damage a host by eliciting inflammatory responses. These compounds often do not meet the classical criteria for virulence factor definition because they are necessary components of bacterial cells. Bacterial lipopolysaccharide is a well-known example of a microbial compound that can cause massive host damage by interacting with Toll-like receptors and triggering an inflammatory cascade. Microbial products that elicit detrimental cytokine responses, such as lipopolysaccharide, have been called modulins (Henderson *et al.* 1996). Although many types of components that elicit

cytokine responses such as toxins and adhesins also have other functions in the pathogenic process, their ability to cause damage through a common pathway mediated by host inflammatory components has led to the suggestion that are also modulins (Henderson *et al.* 1996).

Enzymes

Numerous enzymes have been implicated in microbial virulence. Although the number of enzymes in this category is vast we will discuss several examples to illustrate their mechanism of action. Enzymes that are considered virulence factors are generally active against host components and contribute to virulence by damaging host tissues. Tissue damage makes the host permissive for microbial infection. Enzyme virulence factors that damage tissue include proteases, neurominidases and phospholipases. These enzymes damage cells and provide nutrients by digesting substrates into smaller components that can be assimilated by microbes. However, they also alter host cellular receptors in a manner that can subvert the binding of their usual ligands, such as complement, and alter microbial behavior to promote invasiveness, serum resistance and evasion of host immune mechanisms. Other enzymes, such as urease, contribute to virulence by facilitating survival inside phagocytic cells (Cox *et al.* 2000).

Adhesins

Adhesins are microbial components that enable a microbe to attach to host tissues. Since it is widely accepted that attachment is required for most microbes to infect and grow in a host, adhesins are considered virulence factors. Adhesins are chemically diverse molecules that include proteins, polysaccharides and bacterial cell wall components. For *Entamoeba histolytica*, attachment to colonic cells is mediated by the Gal/GalNAc lectin. Some organisms like *Streptococcus pyogenes* have multiple adhesions, including lipoteichoic acids and M protein. Flagellae are adhesins for several bacterial strains, including *Aeromonas* spp. (Kirov *et al.* 2004) and *E. coli* (Pratt & Kolter 1998). Microbial surface-component-recognizing adhesive matrix molecules (MSCRAMM) are a diverse family of proteins that mediate attachment to host surfaces

for several bacteria such as *Staphylococcus aureus* (Wann *et al.* 2000) and *Enterococcus faecalis* (Sillanpaa *et al.* 2004). *Candida albicans* has a complex system of adhesion molecules that includes polysaccharides, cell surface glycoproteins of the adhesion-like family (ALS) (Hoyer 2001), a hypha-specific surface protein (Hwp1) (Staab *et al.* 1999) and integrin-like proteins (Gale *et al.* 1998). Like other virulence factors, adhesins are surface-exposed molecules that can elicit protective immune responses. Hence, in the case of *E. histolytica*, induction of an IgA response to Gal/GalBAc can make the host more resistant to amoebiasis (Houpt *et al.* 2004).

Motility

Motility is a complex trait that has been associated with virulence in both bacteria and parasites. Motility is manifested by approximately 80% of known bacterial species and is critical for the adaptation of mobile microbes to new environments (Soutourina & Bertin 2003). Bacterial cells can move by the action of specialized organelles called flagella. For movement in intracellular spaces, many microbes exploit host actin to propel themselves forward (Goldberg 2001). Actin-based motility is used by several intracellular pathogens including *Shigella* spp., *Listeria monocytogenes* and *Rickettsiae* for cell-to-cell spread (Goldberg 2001). Like bacteria, some protozoa use flagellae for motion, whereas amoebae employ pseudopodia to crawl. Other protozoa, like *Toxoplasma gondii*, manifest a specialized form of movement called gliding motility, which results from the action of a myosin-actin motor coupled to the translocation of surface adhesins (Sibley 2003). Fungi do not have specialized motility, but the organisms are capable of hyphal growth, which permits movement through cellular elongation. Although viruses are not generally thought of as capable of self-initiated movement, actin-based motility has been described for vaccinia virus (Goldberg 2001). Like other virulence attributes, the ability to move is intimately linked with other traits that are associated with virulence. For example, many bacteria are mobile by virtue of flagella that also function in attachment, biofilm formation and colonization of host tissues, and the flagellar apparatus is used for the export of substances associated with virulence (Soutourina & Bertin 2003). Flagellar synthesis is often

coordinately regulated with other virulence factors within a common genetic regulatory network (Soutourina & Bertin 2003). Furthermore, flagella often induce strong immune responses and manifest antigenic variation. Flagella-dependent mobility in *Legionella pneumophila* and *Yersinia enterocolica* contributes to virulence by facilitating the encounter of bacteria with host cells and enhancing cell-invasive capacity (Young *et al.* 2000; Dietrich *et al.* 2001). For *Burkholderia cepacia*, flagellar movement has been shown to be important for penetration of epithelial barriers and may contribute to the establishment of systemic infections (Tomich *et al.* 2002). For *T. gondii*, gliding motility allows penetration of intestinal barriers and initiates systemic infection (Sibley 2003). Trypanosomes have a single flagellum in a position that allows for both movement and the segregation of mitochondrial genome, events that are critically important for replication and interaction with mammalian hosts (Gull 2003). In summary, motility is a common characteristic in pathogenic organisms that contributes to virulence by allowing the microbe to migrate to favorable niches, encounter host cells, penetrate cell membranes and escape from the intracellular environment. For practically every motile microbe the ability to move is dependent on multiple genes under complex regulatory control and mutations that impair motility often result in virulence attenuation.

Capsules

Many pathogenic bacteria possess polysaccharide capsules, which are required for virulence in mammalian hosts. Encapsulated bacteria with polysaccharide capsules include *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae*. Among the eukaryotes, only *C. neoformans* has a polysaccharide capsule. Most capsules function in microbial pathogenesis by protecting the microbe against host immune mechanisms, although for some the capsular structures can serve as adhesins. For example, polysaccharide capsules are usually poorly immunogenic and anti-phagocytic and thus protect microbes from phagocytosis and intracellular killing. However, there is increasing evidence that soluble capsular polysaccharide released from encapsulated microbes can also contribute to virulence through immunomodulatory effects. For example,

the capsular polysaccharide of *C. neoformans* has been documented to mediate numerous untoward effects on immune cells from causing alterations in cytokine production to interfering with leukocyte migration (Vecchiarelli 2000). Like toxins, antibody responses to capsular polysaccharides, when they occur or are induced by immunization, often render the host immune to disease by the relevant organism. In this regard, effective polysaccharide-based vaccines have been generated against *S. pneumoniae*, *N. meningitides*, *H. influenzae* and *C. neoformans*, among other microbes. Like toxins, capsules are not necessary for viability *in vitro* and can be shown to be important for virulence by generating mutants and comparing the virulence of non-encapsulated and wild-type strains. Although many microbial capsules are composed of polysaccharides, some are composed of cross-linked amino acids. In this regard, the capsule of *Bacillus anthracis* is composed of poly-gamma-D-glutamic acid and functions to interfere with phagocytosis. However, like the experience with polysaccharide capsules, antibodies to gamma-D-glutamic acid are opsonic and protective against *B. anthracis* in murine models of anthrax (Kozel *et al.* 2004).

Complement evasion

The complement system is a central component of innate immunity and host defense against microbial agents. Complement proteins serve diverse host functions, including having direct antimicrobial activity, mediating opsonization, which promotes phagocytosis, and promoting the release of inflammatory mediators. Many microbial agents express determinants that enable them to evade the deleterious effects of complement activation in the host. Microbial complement inhibition results in myriad effects, including serum resistance of Gram-negative organisms and inhibition of opsonophagocytosis and leukocyte chemotaxis. An important mechanism of serum resistance involves the ability of the O-polysaccharide side chains of Gram-negative lipopolysaccharides (LOS) to bind complement components, thereby preventing their membrane binding and inhibiting complement-mediated cell lysis (Rautemaa & Meri 1999). Pneumococci can degrade C3b without host proteins (Angel *et al.* 1994), and Group A and B streptococci

express a C5a peptidase, which inhibits leukocyte recruitment (Hill *et al.* 1988; Ji *et al.* 1996). Other strategies for complement inhibition involve microbial determinants that bind or mimic ligands of human regulators of complement activation (RCA), such as Factor H, CD55 (decay accelerating factor, DAF), CD21 (CR2) and CD46 (MCP) (Lindahl *et al.* 2000). The nature of these determinants is diverse, but they have in common that they are surface-exposed molecules. Examples of microbial components that affect complement system activation and regulation are: sialic acid residues on gonococci and in the capsule of type III group B streptococci that promote Factor H-mediated inactivation of C3b (Ram *et al.* 1999); pneumococcal surface protein C (PspC) that binds Factor H; pneumococcal surface protein A (PspA) that inhibits the activation of the alternative complement pathway; and the streptococcal M protein that binds C4bp, a decay-accelerating factor (Jarva *et al.* 2003). Viruses also employ complement inhibition strategies that involve accelerating decay of C3b and C4b. Vaccinia virus complement-control protein (VCP) and smallpox inhibitor of complement enzymes (SPICE) are complement inhibitory proteins, which are structural and functional mimics of RCAs, though SPICE is more specific for human complement than VCP (Dunlop *et al.* 2003; Favoreel *et al.* 2003a). Other mechanisms by which microbes avoid or inhibit complement include expression of Fc receptor-like glycoproteins by herpesviruses (Favoreel *et al.* 2003b) and the use of CR3 for cell entry by *M. tuberculosis* (Velasco-Velazquez *et al.* 2003), CR2 for cell entry by Epstein-Barr virus and complement-mediated enhancement of cell entry for HIV and other viruses (Wurzner 1999; Kacani *et al.* 2001). In addition, viruses that bud through the cell membrane, such as HIV, and some parasites can incorporate lipid rafts expressing GPI-anchored ligands for RCAs (Wurzner 1999; Nguyen & Hildreth 2000). Given the critical role of the complement system as the humoral arm of innate immunity, microbial interference with complement function can have major effects on the host-microbe interaction. As a virulence strategy, microbe-mediated complement inhibition alters the host-microbe interaction such that the potential for damage from the host inflammatory response may be reduced, but this can be counterbalanced by the potential for greater damage from microbial invasion and serum resistance.

Pigments

Pigment production, and specifically melanin-like pigments, have been associated with virulence in several microbes (Nosanchuk & Casadevall 2003). Melanin in melanotic organisms can protect against a variety of host defense mechanisms that include free radical fluxes, defensins and phagocytosis (Nosanchuk & Casadevall 2003). The prototypical organism for which the contribution of melanin to virulence has been most extensively studied is *Cryptococcus neoformans* where melanization is catalyzed by a laccase. Mutants deficient in laccase are less virulent and exhibit impaired dissemination from primary pulmonary infection (Salas *et al.* 1996; Noverr *et al.* 2004). Interference with melanization *in vivo* can prolong survival (Nosanchuk *et al.* 2001) and antibodies to melanin have been shown to be protective in animal models of infection (Rosas *et al.* 2001). Other pigments associated with virulence in diverse microbes include pyocyanin in *P. aeruginosa* (Lau *et al.* 2004) and malarial pigment in *Plasmodium falciparum* (Lyke *et al.* 2003).

Pro-apoptotic mechanisms

Apoptosis is a non-inflammatory form of cell death that contributes to the maintenance of normal host tissue (Weinrauch & Zychlinsky 1999). Microbial inhibition of apoptosis has the potential to enhance virulence by preventing downregulation of the inflammatory response, whereas enhancement of apoptosis has the potential to promote microbial persistence by killing antimicrobial effector inflammatory cells. However, microbial regulation of apoptosis also has the potential to reduce virulence by dampening or increasing the inflammatory response to the benefit of the host. Pro-apoptotic effects have been demonstrated for toxins, such as the alpha toxin of *S. aureus*, *L. monocytogenes* listerolysin O, *E. coli* alpha hemolysin, diphtheria toxin, *P. aeruginosa* exotoxin A, shiga-like toxins and exotoxins of *B. pertussis* and *H. pylori*; proteins produced by type III secretion systems, such as those found in *Shigella*, *Salmonella* and *Yersinia* spp. and superantigens of *S. aureus* and *S. pyogenes*, although the mechanisms by which these determinants mediate apoptosis differ (reviewed in Weinrauch & Zychlinsky

(1999)). The effect of pro-apoptotic mechanisms on the host-microbe interaction is complex and incompletely understood. *P. aeruginosa* exotoxin-mediated neutrophil apoptosis has been proposed to promote the *Pseudomonas* persistence by allowing the organism to evade neutrophil uptake (Usher *et al.* 2002), whereas *Yersinia* YopJ-mediated induction of macrophage apoptosis has been implicated in the evasion of host immune mechanisms (Monack *et al.* 1997). In contrast, pneumococcal induction of alveolar macrophage apoptosis, which can be increased by opsonized organisms (Dockrell *et al.* 2001), has been proposed to enhance host defense through control of the inflammatory response (Ali *et al.* 2003; Dockrell *et al.* 2001). Although apoptosis of *M. tuberculosis*-infected macrophages has been implicated in mycobacterial killing and host defense in clinical tuberculosis (Keane *et al.* 1997; Rojas *et al.* 1997; Danelishvili *et al.* 2003), virulent strains have been found to inhibit and/or induce less apoptosis than attenuated or avirulent mycobacterial strains (Keane *et al.* 1997). These observations are consistent with the concept that intracellular and/or persistent bacteria may benefit, whereas extracellular bacteria may be harmed by pro-apoptotic mechanisms. Hence, the effect of pro- or anti-apoptotic mechanisms on microbial virulence as well as on host defense is a function of the host-microbe interaction. Virus-induced apoptosis has been proposed to benefit the host by destroying cells in which viral replication would take place, with the caveat that the death of host cells could be detrimental, as described for Sinbis virus, whereas virus-induced anti-apoptotic mechanisms can enhance viral replication and survival (Griffin & Hardwick 1997).

Biofilm formation

Biofilms are dense aggregates of microorganisms embedded in an exopolysaccharide matrix (Cvitkovitch *et al.* 2003). Biofilms are ubiquitous in nature and biofilm formation is acknowledged to be a critical component of the pathogenesis of certain infectious diseases (Donlan 2001, 2002; Parsek & Singh 2003). Microbes in biofilms manifest different gene expression than microbes suspended in solution (planktonic forms), which translates into differences in cell surface properties, biosynthetic capacity, etc. The phenomenon of biofilm formation is closely linked to other processes

involved in microbial pathogenesis, including quorum sensing, attachment and signaling, and consequently attempts to consider these processes in isolation necessarily involve a degree of simplification and reductionism that is artificial. For some diseases, such as bacterial endocarditis, some types of nephrolithiasis, cystic fibrosis, and dental caries, biofilm formation is an essential component of the pathogenic process. In these diseases, biofilm are composed of both bacterial and host components, which serve to isolate the microbes from host defense mechanisms and antimicrobial therapy. For example, in bacterial endocarditis the organisms are encased in fibrin strands forming vegetations, which is the anatomical term given to inflammatory growths on heart valves that are resistant to host immune mechanisms and can only be eradicated by lengthy therapy with antimicrobial drugs (Parsek & Singh 2003). Medically relevant biofilms can be composed of a single microbe (e.g. in endocarditis) or constitute a diverse community of microbial organisms (e.g. dental plaque). Biofilm formation on medical prosthetic devices such as catheters is responsible for persistent infections, which invariably leads to catheter loss since the instruments cannot be sterilized with antimicrobial therapy (Donlan 2002). The ability of certain commensal microbes such as coagulase-negative *Staphylococcus* and *Candida albicans* to infect prosthetic devices and catheters is associated with biofilm formation. For coagulase-negative staphylococci biofilm formation in intravascular catheters is a two-step, multigene-determined process whereby the bacteria adhere first and then proliferate to form multi-layered colonies encased in microbial polysaccharide and host components, such as fibrin (reviewed in von Eiff *et al.* (2002)). Similarly, *Candida* spp. catheter-related infections are associated with the formation of a tough biofilm, which differs from bacterial biofilms in consisting of a dense network of yeast, hyphal and pseudohyphal structures (Douglas 2003). For *C. albicans*, attachment to plastic surfaces triggers hyphal transition and mutants deficient in filamentous growth are poor biofilm formers (Douglas 2003). However, the importance of biofilm formation to the pathogenic process appears to depend on the organism and the system used to study virulence. For various microbes such as *Listeria monocytogenes* (Borucki *et al.* 2003) and *Staphylococcus aureus* (Kristian *et al.* 2004) no correlation has been

established between their tendency for biofilm formation *in vitro* and virulence in animal models. In fact, the interaction between biofilm formation and other virulence factors can be complex and sometimes antagonistic. For *E. coli* and other Gram-negative bacteria, capsule induction blocks the function of self-recognizing adhesion proteins that are important for biofilm formation (Schembri *et al.* 2004). Hence, the contribution of biofilm formation to virulence is microbe- and setting-dependent, being of particular importance in prosthetic device infections and certain pathogenic processes such as bacterial endocarditis.

Two-component systems, histidine kinases and quorum sensing

Microbes sense the environment and respond to environmental stimuli by the initiation of signal transduction events. Since infection resulting in disease almost always involves establishing a life in a new environmental niche, it is no surprise that environmental sensing systems have been associated with the virulence of a multitude of pathogenic microbes. Prokaryotes have various types of two-component systems, which achieve signaling by transferring a phosphoryl group from a phosphohistidine moiety in the sensor kinase component to an aspartate in the response regulator. Various prokaryotic two-component systems have been shown to be global regulators of virulence factors. For example, in *Bordetella pertussis* the products of the *bvgAS* locus *BvgA* and *BvgS* comprise a two-component system that regulates expression of filamentous hemagglutinin, fimbria, toxins and type III secretion proteins, each of which has been implicated in virulence (Mattoo *et al.* 2001). *Staphylococcus aureus* has a complex sensing apparatus that includes several two-component systems which regulate various characteristics associated with virulence (reviewed in Bronner *et al.* (2004)). Eukaryotic microbes also sense the environment and signal through a phospho-relay mechanism that involves histidine kinases (Santos & Shiozaki 2001). *Candida albicans* undergoes yeast to hyphal transition after sensing a variety of stimuli that include mammalian serum. In *C. albicans*, morphogenic changes associated with virulence are mediated through signal transduction mechanisms that include histidine kinases (Dhillon *et al.* 2003) and cAMP signaling cascades (D'Souza & Heitman 2001).

Quorum sensing is a cell-to-cell communication mechanism by which bacteria can sense their population density by the production of small molecules. Quorum sensing regulation has three distinct phases: production of the signaling small molecules by bacteria, accumulation of the signaling molecules as a function of bacterial density and the response by bacteria when a threshold concentration is reached (Podbielski & Kreikemeyer 2004). Bacterial responses to quorum-sensing molecules have global regulatory changes in microbial physiology and can affect virulence. Quorum-sensing-related regulation mechanisms have been associated with virulence in many microbes including *S. aureus* (Yarwood & Schlievert 2003), *P. aeruginosa* (Smith & Iglewski 2003) and *Streptococcus* spp. (Cvitkovitch *et al.* 2003). The relationship between quorum sensing and the response to two-component systems is often intimately linked. For example, in *S. aureus* quorum sensing via the accessory gene regulator (*agr*) two-component system has been linked to the virulence of this organism (Yarwood & Schlievert 2003). Quorum sensing affects the expression of many microbial traits associated with virulence, including biofilm formation and toxin production. Quorum-sensing molecules may actively participate in pathogenesis through effects on the host and some promote apoptosis of macrophages and neutrophils (Tateda *et al.* 2003). There is increasing evidence that quorum-sensing mechanisms are targeted by the innate and adaptive immune responses. Human airway epithelial cells have been shown to inactivate one of the two quorum-sensing molecules of *P. aeruginosa* (Chun *et al.* 2004). Conversely, quorum sensing may affect the type of immune response, and differences in immunoglobulin production have been described in rats infected with wild-type and quorum-signal-deficient mutants of *P. aeruginosa* (Wu *et al.* 2004), possibly as a consequence of direct effects by quorum-sensing molecules on the antibody response (Ritchie *et al.* 2003).

Secretion systems

Bacterial secretion systems export microbial effector proteins that are essential for virulence. There are at least four types of secretion systems that have been implicated in virulence known as Types I–IV. The Type I secretion system is a protein-mediated secretion system which is used in the

export of certain toxins and in drug efflux (reviewed in Remaut & Waksman (2004)). The Type II secretion system, also known as the general secretion system, is widely distributed in bacteria and is responsible for the export of certain toxins and enzymes (Sandkvist 2001). The Type II secretion system is composed of a multi-subunit protein assembly that spans the periplasmic space and it functions to export proteins to the extracellular compartment (Sandkvist 2001). In addition to the general secretion pathway, some bacterial pathogens have specialized systems for secreting proteins into host cells. Several well-known Gram-negative bacterial pathogens have Type III secretion systems that consist of a syringe-like structure that functions to inject microbial effector proteins directly into the host cell cytoplasm (Buttner & Bonas 2002). In *Salmonella* spp. the Type III needle complex is a sophisticated structure composed of as many as 20 proteins that may have an evolutionary relationship to flagella (Kimbrough & Miller 2002). The payload delivered through the Type III secretion system includes a variety of effector proteins that have detrimental effects on host cells. In *Salmonella enterica* the effector molecules SopE, SopE2 and SopB cause actin rearrangements whereas the *Salmonella*-actin binding proteins SipA and SipC alter host actin dynamics in a concerted process that promotes bacterial uptake (Zhou & Galan 2001). For pathogenic *Yersinia* spp., the ability to resist phagocytosis resides in the use of a Type III secretion system to inject the tyrosine phosphatase YopH into the cytoplasm of phagocytic cells which disrupts cellular function and causes rounding up of the cell (Fallman *et al.* 2001). Hence, the *Yersinia* Type III secretion system illustrates there is continuity and overlap between attributes that enable survival in a host and those that facilitate inducible resistance to phagocytosis. Type IV secretion systems constitute another type of protein delivery system to eukaryotic cells that are evolutionarily related to bacterial conjugation systems (Christie 2001). Many of the bacterial effector molecules delivered by Type IV secretion pathways interfere or co-opt host cellular pathways (Nagai & Roy 2003).

Iron acquisition

Iron is essential for microbial growth and metabolism. While obtaining iron is a major challenge for prokaryotic

and eukaryotic microbes that infect humans, the restriction of iron availability is a central aspect of host defense against many Gram-negative and Gram-positive organisms, protozoa and fungi. The availability of free iron is limited in humans by iron-binding proteins, such as transferrin, lactoferrin and ferritin. The close relationship between iron acquisition and virulence is illustrated by associations between iron overload states and infectious diseases (Weinberg 1999) and experimental models showing that iron administration enhances lethality for microbes such as *Neisseria meningitidis* (Holbein 1980). In contrast, iron uptake mutants can be avirulent (Genco *et al.* 1991) and iron deficiency is associated with increased resistance to infection (Litwin & Calderwood 1993). In host–microbe interactions, iron-withholding mechanisms of the host and/or impaired iron uptake mechanisms of the microbe can reduce, and iron acquisition mechanisms of the microbe and/or increased host iron can enhance, microbial virulence.

There are several mechanisms by which bacteria obtain iron from human tissues: the expression of siderophores, low molecular weight chelators of iron and/or surface receptor proteins that bind transferrin, lactoferrin, ferritin, hemoglobin, ferrous iron transporters, heme or haptoglobin–hemoglobin complexes. In general, bacteria that can survive either within or outside of a host use siderophores to obtain iron, whereas species-specific organisms that do not survive in the environment acquire iron from the host through surface receptors. The expression of siderophores is transcriptionally regulated by the level of iron in microbial cells by negative repressor molecules, principally Fur (ferric uptake regulation), first described in *E. coli*, and DtxR (diphtheria toxin regulator), first described in *C. diphtheriae* (Litwin & Calderwood 1993; Hantke 2001; Ratledge 2004). Mycobacterial siderophores are regulated by a functional homolog of DtxR, IdeR (iron-dependent transcriptional repressor) (Ratledge 2004). The impact of mycobacterial siderophores on virulence is exemplified by the reversal of the bacteriostatic effect of serum on *M. tuberculosis* by carboxymycobactin (Rodriguez & Smith 2003). Siderophore expression is coordinately regulated by iron and other virulence determinants such as the oxidative stress response (Ratledge 2004) and toxins (Litwin & Calderwood 1993). Organisms that do not express siderophores obtain iron

through species-specific surface-exposed receptors for transferrin, lactoferrin and other iron-containing molecules (Genco *et al.* 1991; Litwin & Calderwood 1993). *Neisseriae* spp. can utilize diverse human iron sources, including transferrin, lactoferrin, hemoglobin and haptoglobin–hemoglobin complexes (Genco *et al.* 1991). *Yersinia pestis* has a complex iron acquisition system that is essential for virulence and includes heme transport (Perry 1993). Expression of Neisserial transferrin receptors (tfr), which can undergo phase variation, is negatively regulated by Fur (Perkins-Balding *et al.* 2004). Tfrs elicit host immune responses and tfrs have shown promise as vaccine antigens (Ala'Aldeen 1996; Perkins-Balding *et al.* 2004). Iron translocation from the cell surface to the cytoplasm is mediated by mechanisms involving ABC transporter and homologous genes (Modun *et al.* 2000; Perkins-Balding *et al.* 2004).

Intracellular survival

A subset of pathogenic microbes has the capacity for surviving inside phagocytic cells and mechanisms that ensure intracellular survival. Some microbes have become so specialized that they are obligate intracellular pathogens in a process that is associated with genome reduction and a complete dependence on the host cell for replication. Other microbes retain the capacity for survival and replication independent of their hosts and these are known as facultative intracellular pathogens. Each intracellular pathogen has a unique approach for ensuring intracellular survival, with the caveat that all variations function to undermine phagocytic cell microbial-killing mechanisms. Since phagocytic cells kill ingested microbes through a well-choreographed mechanism that involves phagosome formation, maturation and acidification, it makes sense that the so-called intracellular pathogens use only a few general strategies for avoiding intracellular killing. For example, *Listeria monocytogenes* avoids phagosomal killing by producing a toxin known as Lysteriolysin that allows escape into the cytosol where it replicates and spreads through other cells through actin-tail-based motility. Other microbes interfere with phagosome maturation and function and examples include *Legionella pneumophila*, a Gram-negative bacterium that interferes with phagosome maturation, and *Histoplasma capsulatum*, a

soil fungus that interferes with phagosomal acidification. Interestingly, other facultative intracellular microbes such as *C. neoformans* are able to survive in mature acidic phagosomes because they are endowed with a set of attributes that can interfere with microbicidal mechanisms such as powerful antioxidants that include enzymes, a polysaccharide capsule and melanin pigment. Intracellular pathogens survive inside phagocytic cells by damaging cellular homeostasis and antimicrobial mechanisms. The ability for intracellular survival should be considered a specialized phenotype that is enabled by numerous microbial attributes, each of which can be considered a virulence factor.

Other virulence determinants

Because of space limitations, we cannot provide an exhaustive summary of all virulence determinants. Nevertheless, special mention should be made of phenomena associated with virulence that constitute major fields of study in certain fields. In fungi the yeast to hyphal transition is associated with virulence in several pathogenic organisms (Gow *et al.* 2002; Romani *et al.* 2002). Bacteria interfere with cytokine secretion and inflammatory cascades through numerous mechanisms that range from adhesion to direct cellular injury mediated by secretion systems (Wilson *et al.* 1998). Bacteria require Mn and Zn and transporters for these metal ions have been associated with virulence (Claverys 2001; Papp-Wallace & Maguire 2006). The ability for antigenic variation is widespread among pathogenic microbes and may represent a fundamental mechanism for evading immune responses (Deitsch *et al.* 1997). Numerous mechanisms for antigenic variation are found in bacterial, fungal, protozoal and viral pathogens (Deitsch *et al.* 1997). Thermotolerance to mammalian temperatures is considered a necessary characteristic of mammalian pathogens.

SUMMARY

The virulence factor concept has been a powerful engine in driving research and the intellectual flow in the fields of microbial pathogenesis and infectious diseases. At a practical level the finding that effective immune responses often target virulence factors provides a roadmap for future

vaccine design. However, there are significant limitations to this concept, which are rooted in the inability to define virulence and virulence factors in the absence of host factors and the host response. In fact, this concept appears to work best for bacterial pathogens, being less well suited for viruses and commensal organisms with pathogenic potential.

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