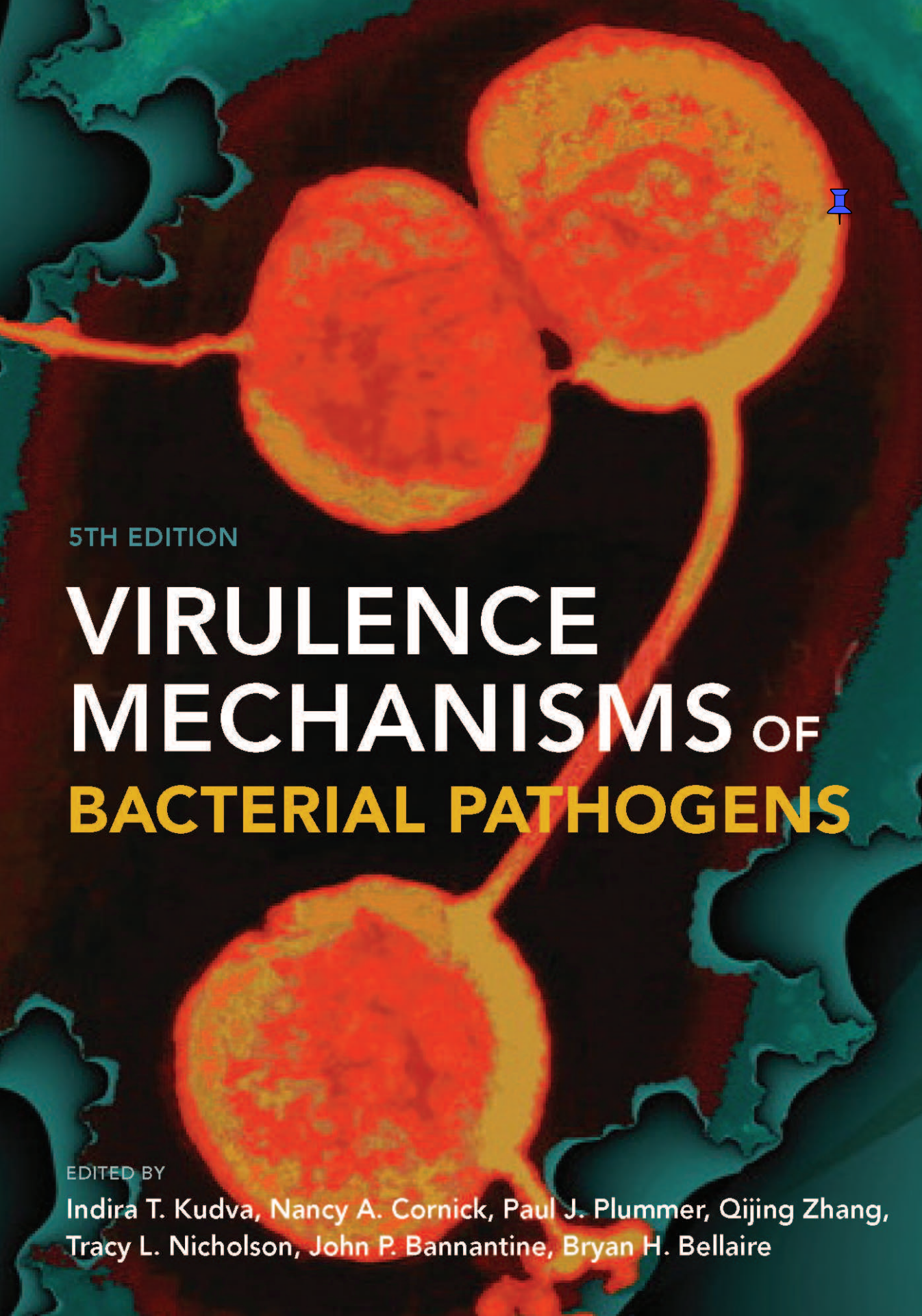
5TH EDITION
VIRULENCE MECHANISMS OF BACTERIAL PATHOGENS
EDITED BY
Indira T. Kudva, Nancy A. Cornick, Paul J. Plummer, Qijing Zhang, Tracy L. Nicholson, John P. Bannantine, Bryan H. Bellaire

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Preface

"Generation of new ideas and refinement or extension of established concepts are the essence of advances in knowledge": Dr. Carlton Gyles, Preface, Virulence Mechanisms of Bacterial Pathogens, 1st Edition, 1988, ASM Press. This was the driving force behind the current fifth edition of this monograph, which essentially is a compilation of bacterial virulence strategies and cutting-edge therapies (targeting these strategies) that have been unraveled in recent years and/or provide new insights into established dogmas.

Previous editions of this book always provided interesting and timely information on topics not always covered in textbooks making them reliable reference sources. Traditionally these were published as follow-up to a series of International Symposia on Virulence Mechanisms of Bacterial Pathogens, held in Ames, Iowa in 1987, 1994, 1999, and 2006. Hence, the last edition was published in 2007. With all the scientific advancements made in the area of bacterial pathogenesis since then, there was a pressing need for a more recent, updated version of this book. To make up for the lapsed time and the inevitable financial constraints, a general consensus was reached to initiate the publication sans a symposium. This turned out to be quite an insightful decision as it enabled the editors and all contributors to provide their undivided attention to weaving together a harmonious and comprehensive monograph.

Sections in this edition have been organized in a systematic manner keeping in sync with the journey a pathogen undertakes in its host. Therefore, these sections discuss, key events occurring at the bacterial-host interface (section I) that enable colonization, bacterial reliance on communication (section II) and secretion (section III) to initiate/enhance virulence, bacterial defense (section IV), persistence (section V), and host-exploitation strategies (section VI) that allow for extended survival in the host. The concluding section (section VII) discusses novel therapeutic approaches being developed to target some of these virulence mechanisms.

It was our intent to deliver the science through this monograph and allow our savvy readers the luxury of philosophizing. As such, we sought contributions from distinguished experts, whether as authors or reviewers, making this monograph a one-stop learning tool for recent advances made in the field of bacterial virulence while stepping away from being just another "textbook". The contents were selected to be beneficial to diverse readership (students, faculty, scientists in academic, clinical, corporate and/or government settings) while promoting discussion, extrapolation, exploration and multi-dimensional thinking.

Indira T. Kudva
Executive Editor

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BACTERIAL-HOST INTERFACE

1

Evolution of Bacterial Pathogens Within the Human Host

KIMBERLY A. BLIVEN[1] and ANTHONY T. MAURELLI[1]

INTRODUCTION

The success or failure of a pathogen is entirely dependent on its ability to survive, reproduce, and spread to a new host or environment. Host immune systems, predators, microbial competitors, parasites, and environmental resource limitations all exert selective pressures that shape the genomes of microbial populations (1). Host fitness, meanwhile, is determined by the ability of the host to survive and reproduce; the host must therefore effectively curtail diseases that impair either of these abilities.

Dawkins and Krebs suggest that the conflicting drives between host and pathogen have led to an evolutionary "arms race," where an asymmetric "attack-defense" strategy has come into play (2). At the basic level, this concept suggests that when the host evolves new defenses to thwart the pathogen's attack, the pathogen is forced to adapt a more effective attack strategy to penetrate the heightened defenses. In response, the host must once again evolve to cope with the new attack mechanism, and the cycle continues. Evolutionarily fit pathogens, which are able to survive, replicate, and spread effectively within the host, have the most likely chance

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of passing their genes on to the next generation. Similarly, host genotypes are more likely to persist within the population if those particular individuals are more capable of controlling or resisting infection. Evolution, therefore, is driven by positive directional selection in the arms race model; eventually, the most beneficial alleles will become fixed in a population. Another model favors frequency-dependent (balancing) selection, a process that maintains rare alleles and therefore preserves polymorphic diversity within a population (3). Simply put, allele fixation is prevented in certain instances because different bacterial alleles confer distinct advantages to the pathogen in the presence of different host alleles (i.e., different environments). Supporting evidence for both directional and frequency-dependent selection can be found within nature, and both types probably occur in bacterial populations.

In this chapter, we explore the host-pathogen interface and offer examples of pathogen adaptation in response to common host selective pressures (Table 1). Although we will focus exclusively on bacterial pathogens within the human host, many of the concepts discussed in this review are readily applicable to other organisms, such as viruses, parasites, and fungi, which can infect a wide range of hosts including plants, animals, and amoeba (4–6).

As a final note, much of the evidence presented here to support presumed evolutionary events is either speculation based on what is currently known or suspected about host and microbial biology or is the result of artificial laboratory-induced evolution during serial passaging of bacterial strains. Due to the sheer enormity of evolutionary timescales, defining the precise origins of and factors driving natural evolutionary events is often a difficult undertaking.

ANTAGONISTIC PLEIOTROPY AND THE FITNESS COST-BENEFIT ANALYSIS

At the most basic level, the theory of natural selection stipulates that within a bacterial population, beneficial traits will be conserved (selected for) and deleterious traits eventually discarded (selected against). The actual evolutionary process is considerably more complex, however, due to the existence of genetic drift (the change in genetic diversity of a population due to random chance) and antagonistic pleiotropy.

Antagonistic pleiotropy is the concept that a single gene may control more than one phenotype, some of which may be beneficial to the organism and some deleterious (7). Therefore, a gene may confer a selective advantage within one particular environment, but its expression could be detrimental within a different environment. Conservation of this gene ultimately is determined by the overall necessity of the gene to the organism's fitness and the timing of selection. Bacterial pathogens may evolve mechanisms to neutralize the deleterious effects arising

TABLE 1 Examples of pathogenic mechanisms to evade or overcome selective pressures within the human host

Selective pressures	Pathogenic mechanisms to evade or overcome these pressures
Physical barriers in host (i.e., mucosal epithelium)	Mucinases Enterotoxins Exfoliative toxins Transcytosis through M cells
Host complement	Complement inhibitor protein C3 protease
Sequestration of host resources (e.g., iron)	Enterobactin/aerobactin systems
Host B and T cell lymphocytes	Cytotoxins T3SS-mediated apoptosis
Antibiotics, antimicrobial peptides	Efflux pumps Mutations in antimicrobial targets Enzymes to inactivate antibiotics (e.g., beta-lactamases)
Bacterial colicins	Colicin immunity proteins
Bacterial T6SSs	T6S immunity proteins

from antagonistic pleiotropy, while at the same time conserving the beneficial ones. Temporal regulation is a powerful tool to ensure that specific genes are only turned on when required and are turned off to prevent detrimental expression within a particular environment. Certain outer membrane proteins or systems are temporally regulated within the host, because they may provide a marker for recognition by the host immune system. Flagellar expression, for example, is downregulated by *Salmonella enterica* serovar Typhi *in vivo* to prevent activation of the host inflammatory response; however, outside the host, motility is likely important for the bacterium to seek out and scavenge nutrients from the environment (8).

Other bacteria avoid the deleterious effects of a gene through gene inactivation; mutants that lose functionality of the gene once it becomes deleterious can out-compete the wild-type parent strain, and eventually these mutants will dominate the population. *Pseudomonas aeruginosa*, an opportunistic pathogen of cystic fibrosis patients, often switches to a mucoid phenotype *in vivo* as a result of overproduction of the exopolysaccharide alginate, which allows for the production of a bacterial biofilm in the lung (9, 10). MucA is a *P. aeruginosa* transmembrane protein that binds to and represses the sigma factor AlgU, which acts as the transcriptional activator of the alginate synthesis operon. AlgU activates AlgR, a suppressor of type III secretion system (T3SS) expression; when *mucA* is expressed, therefore, so are the T3SS genes. During acute infection, the T3SS plays an essential role in establishment of the bacterium within the respiratory tract. Once infection has been established, however, chronic infection appears to favor loss of T3SS and a switch to biofilm production (11). Both of these phenotypes are at least partially driven by various mutations in *mucA* which lead to derepression of AlgU, subsequent production of alginate, and suppression of the T3SS (9). Hauser speculates that loss of the T3SS protects the bacterium from eventual recognition by the host, because patients infected with *P. aeruginosa* develop antibodies against T3SS effector proteins; conversely, biofilm production likely allows for the persistence of the organism in the respiratory tract (11).

Finally, certain bacteria simply tolerate deleterious fitness costs if the benefits of expressing the gene outweigh the negative effects. Antibiotic-resistance mutations that allow bacteria to survive exposure to antimicrobials often come with a significant fitness disadvantage, for example, and secondary compensatory mutations in these strains may eventually arise to restore fitness rather than lose resistance (12).

THE IMPACT OF HOST-PATHOGEN INTERACTIONS ON MICROBIAL EVOLUTION

Inside the host, a successful pathogen will pilfer resources to survive, replicate, and eventually escape; concomitantly, the host will attempt to recognize and subsequently rid the body of the intruder. Coevolution between host and pathogen naturally occurs as a result of these interactions (13). For practical purposes, we restrict our discussion to bacterial adaptation within the human host, but it is important to recognize that many of these concepts are applicable to pathogens of other hosts as well, such as plants and amoeba (14–16). As novel genetic variants within the human population emerge which prove more successful at preventing or overcoming infection, only pathogen variants that allow the bacteria to surmount or avoid this new response will be successful. Within the last century, these natural host defenses, which take much longer to evolve than their microbial counterparts, have been supplemented by man-made developments, such as antibiotics and modern medical interventions, which place added pressures on microbes to adapt (17). Host innate and adaptive immune responses and modern medical interventions are all selective pressures that

contribute to pathogen evolution within the human host. Furthermore, microbial competition, against either other pathogens or commensal bacteria, also shapes pathogen genomes.

Bacteria have several advantages over the human host when it comes to evolution: first, their generation times are significantly shorter, leading to more rapid selection within a population. In conjunction with a shorter generation time, bacterial populations are typically larger, which may allow for greater genetic diversity from which to select. Lastly, many bacteria utilize horizontal gene transfer (HGT), which accounts for the rapid spread of advantageous alleles between strains or even species (18). Virulence genes are commonly located on transferred pathogenicity islands (PAIs), which are segments of the genome associated with mobility elements, such as integrase genes or transposons. PAIs can often be distinguished from the remainder of the genome by a disparate G+C content (19).

Host Selective Pressures: The Innate and Adaptive Immune Systems

The innate immune system is one of the first challenges encountered by the incoming pathogen following host contact. These diverse host defenses include physical barriers such as the mucosal epithelium, activation of the complement cascade, circulating antimicrobial peptides and cytokines, leukocytes, activation of the adaptive immune system, and sequestration of host nutrients away from pathogenic bacteria. In addition to effective evasion of innate immune mechanisms, bacteria must also prevent or avoid adaptive immune responses, which include B cell antibody production and T cell–mediated cytotoxicity. Pathogenic bacteria have evolved different approaches to overcome these host defenses.

In the human colon alone, intestinal microbiota concentrations average 10^{11} microorganisms per gram gut content, while 3×10^{8} prokaryotes are thought to colonize the entire skin surface of the human adult (20). Consequently, bacteria that exploit more hostile and less frequently occupied niches may gain a selective edge in survival by avoiding sites of high competition. Natural structural barriers, however, typically prevent pathogens from engaging deeper host tissues. Physical blocks to infection include the intestinal and respiratory mucosa, the blood-brain barrier, the blood–cerebral spinal fluid barrier, and the placental barrier (21). Most of these structures consist of a single layer of epithelial or endothelial cells bound closely together by tight junctions, adherens junctions, and desmosomes, which preclude bacteria from passively crossing (21, 22). Gastric and respiratory epithelia support an additional protective coating of mucus, which consists primarily of mucin glycoproteins and antimicrobial molecules (23). Mucin glycoproteins, produced by epithelial goblet cells and submucosal glands, can either remain cell-associated or undergo secretion into the mucosa, where they contribute to the viscous layer of mucus that can effectively trap microbes (24). Additionally, nonspecific antimicrobials, such as defensins and lysozymes, and specific antimicrobials, such as IgG and secretory IgA, also limit the growth of microbes within the mucosa (23). Bacterial pathogens have developed numerous mechanisms to counteract these defenses.

The mucosal barrier can be broken down by mucinases such as the Pic enzyme of *Shigella* and enteroaggregative *Escherichia coli* (EAEC) (25, 26). The *pic* gene is located on a chromosomal pathogenicity island in *Shigella* and flanked by insertion-like elements in EAEC, indicating a history of horizontal gene transfer in these pathogens (26). This potential gene transfer is intriguing because mucin degradation is also important for certain gastrointestinal commensals, which metabolize mucin glycoproteins for energy (27). It is tempting to speculate that these enzymes first evolved within human commensal bacteria as a means of nutrient acquisition and

only later spread to emerging pathogens to confer passage through the mucosal surface. Such a concept would support the hypothesis proposed by Rasko et al., who suggest that commensal *E. coli* acts as a "genetic sink" for pathogenic *E. coli* isolates (28). Other pathogens, such as *Yersinia enterocolitica* and *Vibrio cholerae*, avoid the thickest layers of the mucosal layer by targeting microfold cells within the small intestine for uptake (23, 29). These specialized epithelial cells sample microorganisms residing in the intestinal lumen and present them to immune cells in the underlying lymphoid tissue. Microfold cells are situated in the region of the epithelium known as the dome, which lacks mucin-secreting goblet cells (23).

Next, to breach the epithelial/endothelial barrier, pathogens must either actively cross using microbial-mediated processes or opportunistically cross following disruption of barrier integrity. Some pathogens, such as *Bacteroides fragilis* and *Staphylococcus aureus*, directly break cell-cell junctions (30, 31). *B. fragilis*, an opportunistic pathogen, encodes a zinc-dependent metalloprotease toxin, BFT (*B. fragilis* enterotoxin), which cleaves the extracellular domain of E-cadherin, a host zonula adherens protein (30). Like the *pic* genes of *Shigella* and EAEC, the *bft* gene is carried on a PAI present in all enterotoxigenic *B. fragilis* strains (32). *S. aureus* induces bullous impetigo and staphylococcal scalded skin syndrome through the actions of three exfoliative toxins (ETs): ETA, ETB, and ETD (31). The ETs act as serine proteases which cleave human desmoglein 1, a transmembrane protein of desmosomes. The genes encoding these toxins are carried on different mobile genetic elements: the ETA gene is carried by a family of Sa1int phages; the ETB gene is plasmid-encoded; and the ETD gene localizes to a 9-kB PAI (33, 34). Other pathogens, such as *Shigella*, *Salmonella*, and *Listeria*, transcytose through microfold cells in the gut to gain access to the basolateral surface of the intestinal epithelium (35). Because these specialized host cells overlay Peyer's patches (or gut-associated lymphoid tissue), enteric bacteria transcytosed through microfold cells must then contend with macrophages, T lymphocytes, B lymphocytes, and dendritic cells.

As a putative example of counterevolution, the human host may have developed mechanisms to avoid bacterial-mediated adhesion processes. *Helicobacter pylori* binds to the adhesion decoy Muc1, a mucin expressed on the surface of epithelial cells in the gastrointestinal tract (36). Muc1 is subsequently shed from the epithelial surface along with coupled bacteria, precluding long-term adhesion. Consequently, wild type mice have a 5-fold lower *H. pylori* colonization burden than *Muc1*$^{-/-}$ mice. Furthermore, human epidemiological studies have linked shorter Muc1 alleles to a higher probability of chronic gastritis progression, indicating that longer Muc1 alleles may confer a protective advantage to the host (37). Polymorphisms between human Muc1 alleles are largely restricted to the extracellular domain, which consists of a region of 30 to 90 tandem repeat units rich in serine and threonine. A study by Costa et al., demonstrated a significant positive association between the number of Muc1 tandem repeats and bacterial adherence for two strains of *H. pylori in vitro* (38). Longer Muc1 alleles probably evolved from shorter alleles via duplication events and may have emerged to protect against pathogens such as *H. pylori* (39).

Complement cascade activation via the classical, lectin, and alternative pathways precedes the cleavage of C3 convertase into C3a, an anaphylatoxin, and C3b, which binds to the surface of microbes (otherwise known as opsonization) to promote the eventual clearance of bacteria through phagocytosis. Additionally, C3 convertase may convert to the lytic C5 convertase through addition of a C3b molecule. Pathogens have evolved mechanisms to evade or block these processes (40). The *S. aureus* staphylococcal complement inhibitor protein stabilizes C3 convertase, preventing its cleavage into the active C3a and

C3b fragments and attenuating anaphylatoxin activity and bacterial opsonization (41). Like many of the previously described pathogenicity factors, the gene encoding staphylococcal complement inhibitor (*scn*) is located on a PAI (42). Rather than preventing C3 cleavage, the *Neisseria meningitidis* serine protease NalP splits C3 at a unique site, generating shorter C3a-like and longer C3b-like fragments (43). The C3b-like fragments are capable of binding *N. meningitidis* but are rapidly degraded by host complement factors H (fH) and I (fI). Although the activity of the C3a-like fragment has not been determined, this fragment lacks the conserved C-terminal arginine residue found in wild type C3a that is essential for activity, and therefore this truncated version is likely inactive.

A final example of an innate host selective pressure is the sequestration of host resources or nutrients away from colonizing bacteria. Iron, an essential nutrient, is in short supply within the host, either sequestered away in host cells or stored as a complex in hemoglobin, which is inaccessible to most microbes (44). Correspondingly, pathogens have been forced to develop numerous mechanisms to scavenge host iron. Predictably, these systems are often iron-regulated, and their genes are expressed following bacterial exposure to the low-iron environment of the human host. Certain surface-bound receptors can recognize iron-bound complexes, such as heme, transferrin, or lactoferrin. Additionally, secreted bacterial siderophores (aerobactin and enterobactin) steal iron away from host transferrin and lactoferrin. *E. coli* strains can encode for both of these systems (45). Another putative example of arms race coevolution is the mammalian neutrophil gelatinase-associated lipoprotein (NGAL). NGAL directly binds the catecholate-type ferric siderophore complexed to iron, preventing bacterial iron sequestration and eventually exerting a bacteriostatic effect upon microbial populations (46). Some bacteria can even bypass this defense mechanism, however. Uropathogenic *E. coli* strains express the siderophore salmochelin, a glycosylated form of enterobactin resistant to the effects of NGAL (47).

Finally, if a pathogen manages to evade the innate immune system and can successfully compete with commensal bacteria, it must then elude host adaptive immune responses, including B- and T-cell lymphocytes (48). One bacterial strategy employed in this evasion process inhibits lymphocyte proliferation. The VacA cytotoxin of *H. pylori* blocks the activity of host calcineurin, leading to downstream attenuation of interleukin-2 (IL-2) transcription, a key mediator of T cell proliferation (49). Alternatively, bacteria can avoid the adaptive immune response altogether by mediating lymphocyte cell death. For example, *Shigella* induces B-cell apoptosis through the actions of its T3SS (50).

Host Selective Pressures: Antibiotic Resistance

The rise of adaptive antibiotic resistance in bacteria is perhaps one of the most intensely studied examples of pathogen evolution in response to a specific selective pressure(s) (51). Blair et al. separated adaptive resistance mechanisms into three primary categories: reduced drug permeability through alterations in the bacterial membrane or the development of efflux pumps that quickly expel antimicrobials; prevention of binding through mutation of antimicrobial targets; and the direct inactivation of antimicrobial agents by specific enzymes (51). Well-characterized efflux pumps include the multidrug exporters discovered in the common food-borne pathogens *E. coli* (ArcAB-TolC), *S. enterica* (EmrAB), and *S. aureus* (QacA/B, NorA) (52). Linezolid, an oxazolidinone class antibiotic, binds the 23S rRNA subunit and blocks tRNA interactions with the A site to prevent peptide bond formation (53). Unsurprisingly, linezolid resistance in a number of bacterial species has been linked to a G2576T mutation in the 23S rRNA gene,

precluding linezolid binding at this site and providing an example of Blair's second category of adaptive drug resistance (54, 55). Finally, inactivating enzymes such as beta-lactamases, aminoglycoside acyltransferases, and monooxygenases are responsible for the hydrolysis, group transfer, or oxidation of their respective antibiotics (56, 57).

The rapid spread of antimicrobial resistance, and the rise of multidrug resistance, is often linked to the HGT dissemination of genes encoding these enzymes, because many PAIs and plasmids have been shown to carry one or more drug-resistance genes (58). Resistance adaptations often come with a fitness cost, however, which has been demonstrated both *in vivo* and *in vitro* (59).

Microbial Competition

Competition between microbes undoubtedly plays a role in driving pathogen evolution, although this aspect of microbial evolution has not been widely studied and, except for a few examples, is still only very poorly understood. Bacteria can directly eliminate potential rivals through use of toxic peptides (bacteriocins) or through the utilization of type six secretion systems (T6SSs) (60, 61).

Bacteriocins are toxic peptides produced by bacteria that can target and kill neighboring microbes. Colicins, the most well-known members of this category, are produced by strains of *E. coli*, although bacteriocins have been described in a wide variety of bacteria, including *S. aureus*, *Pseudomonas pyogenes*, *Yersinia pestis*, and *Serratia marcescens* (61, 62). In *E. coli*, colicins exhibit a number of different modes of action. Pore-forming colicins, such as colicin A, can insert into the inner membranes of susceptible bacteria to create ion channels (63). Nuclease colicins, such as colicins E9 and E3, translocate across the outer and inner membranes of a susceptible bacterium to the cytoplasm, where they function as DNases (E9) or RNases (E3) (64, 65). Lastly, colicin M, a unique member of the colicin family, blocks peptidoglycan biosynthesis by degrading undecaprenyl phosphate-linked peptidoglycan precursors. These lipid-anchored intermediates are critical for the transport of peptidoglycan subunits across the cytoplasmic membrane (66, 67). To protect their own population against the harmful effects of these toxic peptides, the producers of colicins must concomitantly express immunity proteins, which block the action of their respective colicins. Immunity proteins of pore-forming colicins sit in the inner membrane and block colicin insertion. Nuclease colicin immunity proteins bind to DNase or RNase colicins to prevent their enzymatic activity, and the immunity protein Cmi binds colicin M to render it catalytically inactive (61, 68). Competing bacteria can acquire these immunity proteins via HGT, providing protection against *E. coli* colicin toxicity. For example, *Shigella*, which does not produce the pore-forming colicin V, nevertheless encodes an immunity protein on its SHI-2 PAI, which protects against colicin V produced by strains of *E. coli* (69, 70).

The recently discovered T6SSs of Gram-negative bacteria are responsible for the direct delivery of effector proteins into neighboring eukaryotic or bacterial cells, resulting in the death of host cells or the lysis of potential microbial competitors (71). VgrG1, an ADP-ribosyltransferase, is secreted from the *Aeromonas hydrophila* T6SS into host cells, where it disrupts the actin cytoskeleton and induces host cell apoptosis (72). Most of the described T6SS effectors, however, have been shown to target other microbes. The T6SS-exported proteins 1 and 3 (Tse1 and Tse3) of *P. aeruginosa* exhibit amidase and muramidase activity, respectively, against bacterial peptidoglycan (73). *P. aeruginosa* also encodes type VI lipase effector (Tle) proteins, which degrade the bacterial phospholipid phosphatidylethanolamine (74). In *Dickeya dadantii*, the Rhs (rearrangement hotspots) proteins RhsA and RhsB are secreted through

the T6SS and function as toxic endonucleases in susceptible bacteria. While *D. dadantii* is a plant pathogen, the human pathogen *S. marcescens* also expresses a T6SS-secreted Rhs-family protein, although its function is unknown (75, 76). Similar to the colicin proteins, pathogens which encode a T6SS must also express immunity proteins to prevent self-killing. *P. aeruginosa* encodes T6SS immunity 1 and 3 (Tsi1 and Tsi3) proteins, which interact with and inactivate Tse1 and Tse3 through mechanisms that are not yet understood (73).

Intriguingly, T6SSs may also be effective tools for gene acquisition via HGT. In *V. cholerae*, the T6SS is coregulated with competence genes by the regulator TfoX, and transformation events are dependent upon the presence of an active T6SS (77). Borgeaud et al., suggest that following activation of TfoX, both competence and T6SS systems are expressed and assembled. After T6SS-mediated lysis of neighboring cells, DNA is released to the extracellular space, where it can then transform the competent bacterium (77).

CONCLUDING REMARKS

Bacterial pathogens within the human host are exposed to a vast variety of selective pressures which shape bacterial genomes and drive the evolution of novel virulence factors. Concomitantly, human genomes also evolve as a result of these interactions, leading to a genetic arms race between pathogens and their hosts. In bacteria, HGT can enhance this process by allowing for the rapid dissemination of potentially beneficial alleles across strains or even species.

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