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43. Collaborative studies of the authors were supported by the Human Frontiers in Science Program and the NIH. We wish to express our gratitude to our coworkers and apologize that for lack of space we were unable to present a broader coverage of the field and include more references.

REVIEW

Bacterial Biofilms: A Common Cause of Persistent Infections

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Bacteria that attach to surfaces aggregate in a hydrated polymeric matrix of their own synthesis to form biofilms. Formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections. Studies of biofilms have revealed differentiated, structured groups of cells with community properties. Recent advances in our understanding of the genetic and molecular basis of bacterial community behavior point to therapeutic targets that may provide a means for the control of biofilm infections.

For quite some time we have known that bacteria can adhere to solid surfaces and form a slimy, slippery coat. These bacterial biofilms are prevalent on most wet surfaces in nature and can cause environmental problems. Perhaps because many biofilms are sufficiently thick to be visible to the naked eye, these microbial communities were among the first to be studied by the late-developing science of microbiology. Anton van Leeuwenhoek scraped the plaque biofilm from his teeth and observed the "animalculi" that produced this microbial community with his primitive microscope. However, it was not until the 1970s that we began to appreciate that bacteria in the biofilm mode of existence, sessile bacteria, constitute a major component of the bacterial biomass in many environments (*1*), and it was not until the 1980s and 1990s that we began to appreciate that attached bacteria were organized in elaborate ways (*2*). For example, different bacterial species specifically attach to different surfaces or coaggregate with specific partners in the mouth (*3*). Often one species can coaggregate with multiple partners, which themselves can aggregate with other partners to form a dense bacterial plaque. Advances in light microscopy coupled with developments in microelectrode technology have led to an appreciation that bacterial biofilms consist of microcolonies on a surface, and that within these microcolonies the bacteria have developed into organized communities with functional heterogeneity.

Because bacterial biofilms can cause environmental problems and studies of biofilms have required the development of new analytical tools, many recent advances have resulted from collaborations between microbial ecologists, environmental engineers, and mathematicians. These efforts have led to our current definition of a bacterial biofilm as a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface.

Biofilms constitute a protected mode of growth that allows survival in a hostile environment. The structures that form in biofilms contain channels in which nutrients can circulate (*4*), and cells in different regions of a biofilm exhibit different patterns of gene expression (*5*). The complexity of biofilm structure and metabolism has led to the analogy of biofilms to tissues of higher organisms (*6*). These sessile biofilm communities can give rise to nonsessile individuals, planktonic bacteria that can rapidly multiply and disperse. The common view is that planktonic bacteria must expose themselves to deleterious agents in their environment, be they phage or amoeba in nature, biocides in industrial settings, or potent antimicrobial agents in a clinical setting. In this light, it is not surprising that

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an impressive number of chronic bacterial infections involve bacterial biofilms, which are not easily eradicated by conventional antibiotic therapy.

Bacterial Biofilm Infections

Until the relatively recent development of vaccines and antibiotics, human societies have been beset by acute epidemic infectious diseases caused by the planktonic cells of such specialized pathogens as *Vibrio cholerae* and *Yersinia pestis*. Modern-day acute infections can often be treated effectively with antibiotics (except for cases of infection by a strain that is antibiotic resistant) and are not considered to involve biofilms. However, more than half of the infectious diseases that affect mildly compromised individuals involve bacterial species that are commensal with the human body or are common in our environments. For example, the skin bacterium *Staphylococcus epidermidis* and the aquatic bacterium *Pseudomonas aeruginosa* can cause devastating chronic infections in compromised hosts (*6*). Electron microscopy of the surfaces of medical devices that have been foci of device-related infections shows the presence of large numbers of slime-encased bacteria (*7*). Tissues taken from non–device-related chronic infections also show the presence of biofilm bacteria surrounded by an exopolysaccharide matrix. These biofilm infections may be caused by a single species or by a mixture of species of bacteria or fungi (Table 1).

Biofilm infections share clinical characteristics. Biofilms develop preferentially on inert surfaces, or on dead tissue, and occur commonly on medical devices and fragments of dead tissue such as sequestra of dead bone (*8*); they can also form on living tissues, as in the case of endocarditis. Biofilms grow slowly, in one or more locations, and biofilm infections are often slow to produce overt symptoms (*9*). Sessile bacterial cells release antigens and stimulate the production of antibodies, but the antibodies are not effective in killing bacteria within biofilms (Fig. 1) and may cause immune complex damage to surrounding tissues (*10*). Even in individuals with excellent cellular and humoral immune reactions, biofilm infections are rarely resolved by the host defense mechanisms (*7*). Antibiotic therapy typically reverses the symptoms caused by planktonic cells released from the biofilm, but fails to kill the biofilm (*11*). For this reason biofilm infections typically show recurring symptoms, after cycles of antibiotic therapy, until the sessile population is surgically removed from the body (*6*). Planktonic bacterial cells are released from biofilms, and evidence supports the notion that there is a natural pattern of programmed detachment (*6*). Therefore, biofilms can act as "niduses" of acute infection if the mobilized host defenses cannot eliminate the planktonic cells that are released at any one time during the infection (*12*).

Bacterial Biofilms Are Inherently Resistant to Antimicrobial Agents

Biofilms growing in natural and industrial environments are resistant to bacteriophage, to amoebae, and to the chemically diverse biocides used to combat biofouling in industrial processes (*13*). Of importance with respect to medicine, sessile bacterial cells can withstand host immune responses, and they are much less susceptible to antibiotics than their nonattached individual planktonic counterparts (*14*). It is likely that biofilms evade antimicrobial challenges by multiple mechanisms.

One mechanism of biofilm resistance to antimicrobial agents is the failure of an agent to penetrate the full depth of the biofilm. Polymeric substances like those that make up the matrix of a biofilm are known to retard the diffusion of antibiotics (*15*), and solutes in general diffuse at slower rates within biofilms than they do in water (*16*). Antibiotics have been shown to penetrate biofilms readily in some cases and poorly in others, depending on the particular agent and biofilm (*17*). Mathematical models predict that a formidable penetration barrier should be established if the antimicrobial agent is deactivated in the outer layers of the biofilm faster than it diffuses (*18*). This is true for reactive oxidants such as hypochlorite and hydrogen peroxide (*19*). These antimicrobial oxidants are products of the oxidative burst of phagocytic cells, and poor penetration of reactive oxygen species may partially account for the inability of phagocytic cells to destroy biofilm microorganisms.

A second hypothesis to explain reduced biofilm susceptibility to antibiotics posits that at least some of the cells in a biofilm experience nutrient limitation and therefore exist in a slow-growing or starved state (*20*). Slow-growing or nongrowing cells are not very susceptible to many antimicrobial agents. Spatial heterogeneity in the physiological state of bacteria within model biofilms has been demonstrated by a variety of microslicing and microscopic techniques (*21*). Such heterogeneity of biofilms constitutes an important survival strategy because at least some of the cells, which represent a wide variety of different metabolic states, are almost certain to survive any metabolically directed attack.

A third mechanism of reduced biofilm susceptibility, which is more speculative than the preceding hypotheses, is that at least some of the cells in a biofilm adopt a distinct and protected biofilm phenotype. This phenotype is not a response to nutrient limitation; it is a biologically programmed response to growth on a surface.

Table 1. Partial list of human infections involving biofilms.

Infection or disease	Common biofilm bacterial species
Dental caries	Acidogenic Gram-positive cocci (e.g., *Streptococcus*)
Periodontitis	Gram-negative anaerobic oral bacteria
Otitis media	Nontypable strains of *Haemophilus influenzae*
Musculoskeletal infections	Gram-positive cocci (e.g., staphylococci)
Necrotizing fasciitis	Group A streptococci
Biliary tract infection	Enteric bacteria (e.g., *Escherichia coli*)
Osteomyelitis	Various bacterial and fungal species—often mixed
Bacterial prostatitis	*E. coli* and other Gram-negative bacteria
Native valve endocarditis	Viridans group streptococci
Cystic fibrosis pneumonia	*P. aeruginosa* and *Burkholderia cepacia*
Meloidosis	*Pseudomonas pseudomallei*
Nosocomial infections	
ICU pneumonia	Gram-negative rods
Sutures	*Staphylococcus epidermidis* and *S. aureus*
Exit sites	*S. epidermidis* and *S. aureus*
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Schleral buckles	Gram-positive cocci
Contact lens	*P. aeruginosa* and Gram-positive cocci
Urinary catheter cystitis	*E. coli* and other Gram-negative rods
Peritoneal dialysis (CAPD) peritonitis	A variety of bacteria and fungi
IUDs	*Actinomyces israelii* and many others
Endotracheal tubes	A variety of bacteria and fungi
Hickman catheters	*S. epidermidis* and *C. albicans*
Central venous catheters	*S. epidermidis* and others
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Vascular grafts	Gram-positive cocci
Biliary stent blockage	A variety of enteric bacteria and fungi
Orthopedic devices	*S. aureus* and *S. epidermidis*
Penile prostheses	*S. aureus* and *S. epidermidis*

A New Era in Biofilm Research: Molecular Genetic Dissection of Biofilm Development

Research in this decade has begun to reveal information about the molecular and genetic basis of biofilm development. Biofilms involving several different bacterial species have been studied (*6*) but perhaps none more intensively than biofilms of *Pseudomonas aeruginosa*. Here, we use *P. aeruginosa* and the chronic lung infections it causes in most patients afflicted with the recessive genetic disease cystic fibrosis (CF) as a model that exemplifies modern research on biofilm infections. Like other biofilms, *P. aeruginosa*-biofilms are developed communities with individual bacterial cells embedded in an extracellular polysaccharide matrix (*22*) and are inherently resistant to antimicrobial treatment. The pattern of development involves initial attachment to a solid surface, the formation of microcolonies on the surface, and finally differentiation of microcolonies into exopolysaccharide-encased, mature biofilms.

Initial attachment and microcolony formation. A recent report by O'Toole and Kolter (*23*) describes a microtiter dish screen for the isolation of *P. aeruginosa* mutants defective in the initial steps of biofilm formation. Two general classes of mutants, called *sad* (surface attachment defective) mutants, were described. One class constitutes flagella and motility mutants and does not adhere well to the plastic surface used. The other class consists of mutants defective in the biogenesis of hair-like appendages, Type IV pili. Whereas the wild-type cells cluster into microcolonies (relatively small groups of bacteria) on the plastic, the pilus biogenesis mutants form a monolayer of cells on the surface but are unable to form microcolonies. Type IV pili in *P. aeruginosa* are involved in a type of surface-associated motility called twitching, and this twitching might be required for the aggregation of cells into microcolonies.

There is evidence that during this attachment phase of biofilm development, perhaps after microcolony formation, the transcription of specific genes is activated. In particular, studies with *P. aeruginosa algC*, *algD*, and *algU::lacZ* reporter constructs show that the transcription of these genes, which are required for synthesis of the extracellular polysaccharide (alginate in this case), is activated after attachment to a solid surface (*24*). Thus, it appears that attachment itself can initiate synthesis of the extracellular matrix in which the sessile bacteria are embedded. This notion—that bacteria have a sense of touch that enables detection of a surface and the expression of specific genes—is in itself an exciting area that has been more thoroughly studied in *Vibrio parahaemolyticus*, which causes gastroenteritis in humans (*25*).

Maturation of attached bacteria into a differentiated biofilm. At an appropriate time, microcolonies differentiate into true biofilms: exopolysaccharide-encased communities that are resistant to biocides. What is the genetic program leading to biofilm development? Is there a signal that induces differentiation? The convergence of biofilm research with another research area, focused on the ability of bacteria to function in special ways when in groups, has provided some interesting clues about biofilm maturation. Research on quorum sensing in Gram-negative bacteria has shown that acylhomoserine lactone signals are produced by individual bacterial cells. At a critical cell density, these signals can accumulate and trigger the expression of specific sets of genes [for reviews see (*26*)]. Could a quorum-sensing signal be required for biofilm development? Two *P. aeruginosa* quorum-sensing systems have been characterized. One, the LasR-LasI system, controls the expression of a battery of extracellular virulence factors. It also controls the other system, RhlR-RhlI, which in turn controls genes including several required for the production of a number of secondary metabolites. RhlI catalyzes the synthesis of butyrylhomoserine lactone, and LasI directs the synthesis of 3-oxododecanoylhomoserine lactone. The wild type, a *lasI* mutant, and a *rhlI* mutant all can colonize a glass surface and form microcolonies. Microcolonies of the wild type and the *rhlI* mutant differentiate into structured, thick, biocide-resistant biofilms, whereas the *lasI* mutant microcolonies remain thin, undifferentiated, and sensitive to dispersion by a weak detergent (0.2% sodium dodecyl sulfate). Addition of the missing signal, 3-oxododecanoylhomoserine lactone, to the *lasI* mutant restores biofilm development (*27*). This indicates that one specific quorum-sensingsignal is required for biofilm differentiation, at least under the conditions of the ex-

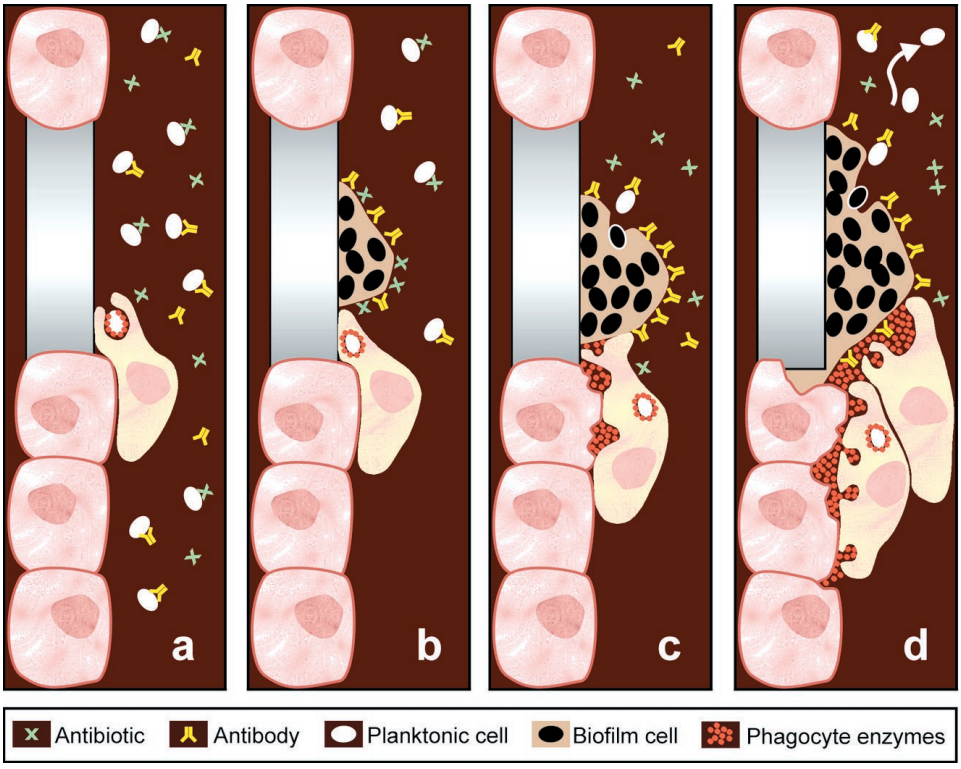

Fig. 1. Diagram of a medical biofilm. (**A**) Planktonic bacteria can be cleared by antibodies and phagocytes, and are susceptible to antibiotics. (**B**) Adherent bacterial cells form biofilms preferentially on inert surfaces, and these sessile communities are resistant to antibodies, phagocytes, and antibiotics. (**C**) Phagocytes are attracted to the biofilms. Phagocytosis is frustrated but phagocytic enzymes are released. (**D**) Phagocytic enzymes damage tissue around the biofilm, and planktonic bacteria are released from the biofilm. Release may cause dissemination and acute infection in neighboring tissue.

periments. Of some interest, acylhomoserine lactones have been reported to be produced by sessile *P. aeruginosa* communities on silicone urethral catheters (*28*).

Thus, a picture of the development of *P. aeruginosa* biofilms at a molecular level is emerging (Fig. 2). There are specific cell surface components required for adhesion to a surface and additional components required for aggregation of cells into undifferentiated microcolonies. The generation of a mature *P. aeruginosa* biofilm requires an extracellular signaling molecule that can be likened to a hormone. With regard to biofilm development, there are a number of pressing questions. Are the mechanisms of attachment and microcolony formation similar regardless of the characteristics of the surface involved? Are there conditions where biofilm differentiation can bypass the acylhomoserine lactone signaling step? What acylhomoserine lactone–regulated genes are required for biofilm maturation? Can some of these genes be linked directly to the antibiotic resistance of biofilms? Are cell-to-cell signals in biofilm formation the rule among different bacterial groups, or is this a particular characteristic of *P. aeruginosa*?

Detachment and dispersal of planktonic cells from biofilms. For bacteria in a sessile biofilm community to colonize new areas, there must be some mechanism for dispersion. Pieces of biofilms (Fig. 2) can break off in the flow and may colonize new surfaces. Furthermore, just as there are chemical cues for biofilm maturation, there may be cues for a program of events leading to the release of planktonic bacteria from a biofilm. It has been suggested that escape of *P. aeruginosa* cells from the biofilm matrix involves the action of an enzyme that digests alginate (*29*). It is worth noting that in the nonpathogenic, photosynthetic bacterium *Rhodobacter sphaeroides*, an acylhomoserine lactone quorum-sensing signal is required for dispersal of individual cells from community structures [the quorum-sensing genes in *R. sphaeroides* are called *cer* (community escape response) genes (*30*)].

Pseudomonas aeruginosa Biofilms in Cystic Fibrosis Lung Infections

The genetic defect in CF leads to the loss of the CF transmembrane regulator (CFTR) chloride channel in the apical membranes of epithelial cells (*31*). This defect leads to persistent bacterial infections of the lungs. Most CF patients are colonized with *P. aeruginosa*, and eventually they succumb to the lung damage inflicted by the persistent bacterial infection, with a median life expectancy of about 30 years. There are several explanations for CF lung pathogenesis, some of which are not mutually exclusive (*32*). One view is that the absence of a chloride channel leads to an elevated salt content in the airway surface fluid. The salt inhibits the activity of antimicrobial peptides and proteins involved in the innate immunity of the airways (*32*). This tips the balance of power just enough so that *P. aeruginosa* can colonize the epithelium as a biofilm.

The sessile *P. aeruginosa* communities release antigens while growing in microcolonies in the lung, and very high concentrations of antibodies to *Pseudomonas* are seen in the circulating blood and in the lungs. These antibodies react with their specific antigens in the outer reaches of the matrices of the infecting microcolonies, but neither the bactericidal nor the opsonizing capabilities of these defensive molecules are realized. In CF patients, a high concentration of circulating antibodies to *Pseudomonas* correlates with a negative clinical outcome. This has been ascribed to pulmonary tissue damage resulting from inflammation. Indeed, immune suppression is a part of the therapeutic arsenal of the CF clinician. Current clinical attempts to prevent the initial colonization of young patients by *P. aeruginosa* with prophylactic antibiotics are showing some promise (*33*).

Antibiotic therapy in patients colonized with *P. aeruginosa* often gives a measure of relief from symptoms but fails to cure the basic ongoing infection (*19*). Our interpretation of this is that the antibiotics act on the planktonic cells that are shed by the biofilms. This can alleviate the acute symptoms of the lung infection, but the antibiotic therapy cannot eliminate the antibiotic-resistant sessile biofilm communities.

The lifelong struggle of CF patients with *P. aeruginosa* pneumonia exemplifies most biofilm infections. The causative organisms are ubiquitous and are only pathogenic for a particular set of compromised individuals. The infection develops slowly, except for acute exacerbations, and these acute phases may be responsive to antibiotic therapy. The basic deep-seated infection cannot, however, be cured by conventional antibiotic therapy. The normal course of the infection produces an antibody response to the infecting pathogen, but the antibodies are not effective against sessile bacteria. The microcolonies of sessile bacteria in the lung act as niduses for spread of the infection (*32*).

The scientific quandary facing CF patients is that currently available antibiotics were developed against the planktonic phenotype of *P. aeruginosa*, and therapeutic agents are chosen on the basis of their efficacy against planktonic cells of this pathogen, but direct observations have shown that the bacteria actually grow in the biofilm phenotype in the lung. Thus, it should come as no surprise that current antibiotic therapies are of limited effectiveness in resolving this particular biofilm infection.

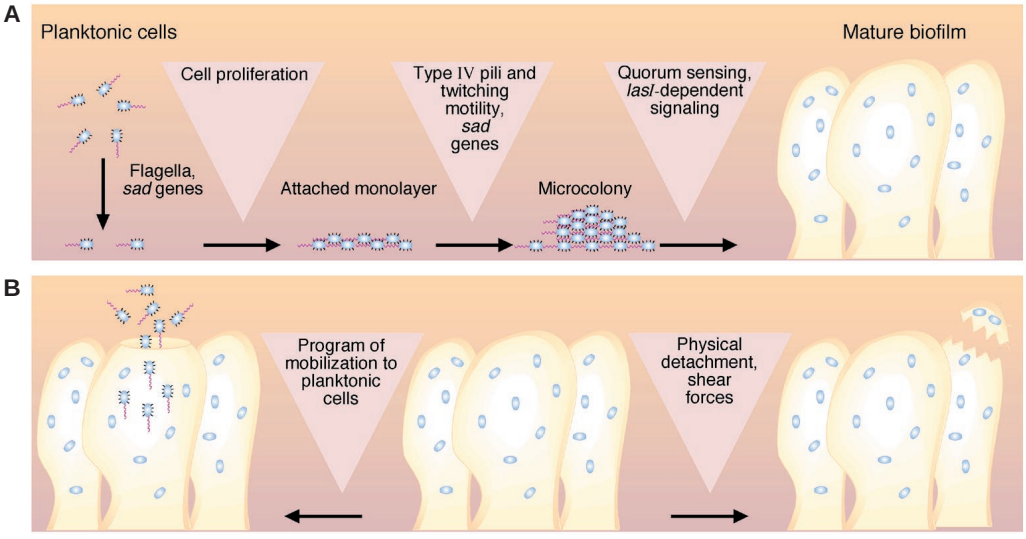

Fig. 2. (**A**) Models of the development of a mature *P. aeruginosa* biofilm from planktonic cells; (**B**) dispersal of bacteria from a biofilm. Flagella (blue) are involved in attachment, and Type IV pili (black) are required for twitching motility on a surface and the formation of microcolonies in the attached monolayer that forms on the surface. LasI-dependent quorum sensing serves as a maturation signal leading to the formation of differentiated, thick mature biofilm structures. Two proposed mechanisms for detachment and dispersal of cells from a biofilm are depicted. One pictures a programmed set of events within the biofilm leading to a local hydrolysis of the extracellular polysaccharide matrix, and conversion of a subpopulation of cells into motile planktonic cells, which leave the biofilm. The other is a physical detachment pathway in which a streamer, or some other fragment of a microcolony, simply detaches from the biofilm and is carried by the bulk fluid until it lodges in a new location and initiates a new sessile population.

The Future Challenge

Our path seems clear. We have come to understand many things about the unique biology of bacterial biofilms. Biofilms represent microbial societies with their own defense and communication systems. We have an arsenal of microscopic, physical chemical, and molecular techniques available to examine biofilms. There are many basic questions regarding the biology of biofilms that can now be answered. Our modern view of biofilm infections leads to the realization that their effective control will require a concerted effort to develop therapeutic agents that target the biofilm phenotype and community signaling–based agents that prevent the formation, or promote the detachment, of biofilms. The techniques are now available to undertake such efforts.

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34. We thank P. Dirckx, K. Lange, and N. Zear for help in preparation of figures. Supported by NSF grant MCB 9808308, NIH grant GM59026, and Cystic Fibrosis Foundation grant 97Z0 (E.P.G.) and by cooperative agreement ECD-8907039 with the Engineering Research Centers and Education Division of NSF (J.W.C. and P.S.S.).

REVIEW

Type III Secretion Machines: Bacterial Devices for Protein Delivery into Host Cells

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Several Gram-negative pathogenic bacteria have evolved a complex protein secretion system termed type III to deliver bacterial effector proteins into host cells that then modulate host cellular functions. These bacterial devices are present in both plant and animal pathogenic bacteria and are evolutionarily related to the flagellar apparatus. Although type III secretion systems are substantially conserved, the effector molecules they deliver are unique for each bacterial species. Understanding the biology of these devices may allow the development of novel prevention and therapeutic approaches for several infectious diseases.

A number of bacterial pathogens have evolved the capacity to engage their hosts in complex intimate interactions aimed not necessarily at causing disease but rather at securing the microbe's ability to multiply and move on to a new host. The relationship between bacterial pathogens and their hosts is most often a peaceful one, because it has been shaped by a coevolutionary process aimed at securing the survival of both the pathogen and the host. This is particularly the case for microbial pathogens that, through the process of host adaptation, have lost the ability to explore other niches. Sometimes, however, these pathogens cause harm to the host. In some instances, disease symptoms may simply be unpleasant manifestations of a self-limiting process that leads to the transmission of the bacteria from one host to the next. However, in other cases, fatal disease may occur when these bacterial pathogens encounter a host that has been weakened by circumstances that alter the delicate balance of the microbe-host interaction.

Recent advances in the fields of immunology and of molecular, cell, and structural biology are allowing the detailed investigation of the interactions between these highly adapted pathogens and their hosts. This close examination is not only helping in the understanding of microbial pathogenesis but is also providing

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