

## **PMB 220: Critical Thinking in Microbiology**

**Sep 19 2006**

### **Bacterial pathogenesis**

**Dan Portnoy**

Prelude: In almost every environment, there exists a microbe that has evolved a mechanism(s) to exploit that niche. The mammalian host represents a potentially rich environment for the growth of mesophilic organisms. However, the host has evolved specific innate and acquired mechanisms to prevent (or limit) growth. Pathogens have evolved to exploit this nutrient rich environment in the context of the host immune system.

#### I. Rationale for these lectures

A. The problem of infectious diseases. The big 3 (TB, AIDS, Malaria)

2. In the U.S. although many vaccines to infectious disease agents exist, e.g. DPT, there are still no vaccines for diseases such as Chlamydia, Tuberculosis, Malaria, and HIV.

3. The continuing threat of antibiotic resistant bacteria: There now exists strains of bacterial pathogens resistant to all known antibiotics.

4. Biodefense: Can we develop rapid detection systems based on our knowledge of basic mechanisms of pathogenesis?

B. Understanding of the basic mechanisms of microbial pathogenesis will lead to the rational design of vaccines, therapeutics.

C. Multidisciplinary nature of bacterial pathogenesis: bacterial physiology and genetics, Immunology/inflammation, and cell biology. Here lays the essence and challenge of bacterial pathogenesis. True understanding requires an appreciation of a wide-variety of methods, and systems.

D. Bacteria as cell biologists: Bacterial pathogens express specific determinants of pathogenesis, such as toxins, that specifically interact with the host and identify key aspects

of cell biology. The field has been recently named Cellular Microbiology. (Cossart et al., Cellular microbiology emerging. Science 271:315-316.

E. Bacteria as model systems: Long history in studies on central dogma (DNA replication, transcription, and translation); Now organisms amenable to genetics and biochemistry are models for the less tractable pathogens like HIV, Malaria and Cancer.

F. The appearance of new and potentially dangerous infections. Since I started grad school in 1978, many new infectious Diseases have emerged and re-emerged: Legionnaire's Disease, Toxic Shock, Lyme Disease, Helicobacter-induced ulcer and cancer; HIV, the re-emergence of Tuberculosis and antibiotic resistant TB.

G. Bacterial pathogens and genomics: Over 100 bacterial genomes are already sequenced or are in the process of being sequenced. Leads to studies of evolution of disease, minimal genome etc.

## II. Terminology and General Concepts.

A. Pathogen: A microorganism capable of producing pathology (disease) in a percentage of normal healthy non-immune individuals. Disease is a state where damage has occurred to the host. A pathogen will almost always cause disease in a non-immune host if administered in a sufficient dose. However, at lower doses infection may result without overt disease (sub-clinical infection). The goal of a pathogenic organism is to reproduce, not cause disease *per se*. Disease of the host is often associated with the propagation of the microorganism and/or spread of the microorganism (e.g. a cough facilitates dissemination).

B. Opportunistic pathogen. A microorganism that does not cause disease in a healthy host, but only in individuals whose normal defense mechanisms have been compromised; e.g. burn patients, recipients of organ transplants receiving immunosuppressants, Individuals with AIDS, pregnant women. Opportunistic pathogens are becoming increasingly important.

C. Virulence: Quantitative measure of pathogenesis. LD<sub>50</sub>, the lethal dose 50, is the number of microorganisms (or amount of a toxin) required to kill 50% of the test animals. ID<sub>50</sub>, infectious dose 50, is the number of organisms required to produce an infection in 50% of the test animals. LD<sub>50</sub> has historically been the gold standard to measure virulence, but it has become less prevalent due to the concerns of animal rights activists.

D. Determinant of pathogenesis or virulence factors. Components of the pathogen responsible for its ability to cause an infection. Often defined by mutations in specific genes (virulence genes) which result in the lower virulence (an increase in LD50). However, pathogenesis is multifactorial usually involving numerous determinants of pathogenicity. Loss of any of these determinants may result in lower virulence. Mutations which result in avirulence are often pleiotropic in nature often mapping to a gene involved in the regulation of multiple virulence factors. Pathogenesis can be divided into actual virulence factors, and regulation of those virulence factors.

E. Obligate vs Facultative Pathogens and Obligate vs Facultative Intracellular Pathogens. An obligate pathogen is one that cannot (or has not) been found anywhere but in association with its host. This has implications for disease eradication. A facultative pathogen is one that can grow or survive in the environment as well as its host.

An obligate intracellular pathogen can only grow inside of host cells, and cannot be cultured extracellularly; e.g. viruses. Facultative intracellular pathogens can grow both inside and outside of cells and can be cultured on an agar surface in the laboratory.

### III. Stages in Pathogenesis

#### A. Encounter:

1. Normal Flora: In terms of cell numbers, humans are 90% bacteria representing a least 1000 different bacterial species. Normal flora can protect the host, but in some cases, can also cause disease.
2. Exogenous contact: Food-borne (oral), respiratory, sexual, vector-borne etc.
3. Saprophytes
4. Zoonosis

#### B. Entry.

1. Mucosal sites: Many sites of infection occur upon mucosal surfaces such as the lung, intestinal tract, and genitourinary tract which are technically outside of the body and are normally lined with normal flora. However, many pathogens can cause disease without leaving the mucosal surface. For example, *Vibrio cholerae* attaches to the small intestine and elaborates a toxin which causes diarrhea.

The host has mechanisms for preventing access to these sites. For example, the acid pH of the stomach is a very effective disinfectant. The lungs are protected by the action of cilia. The intestine is constantly moving by peristalsis. Also, normal flora prevents access to sites.

2. Bites and wounds. The skin represent quite a barrier to microbial entry which can be bridged by an insect bite, cut etc.

C. Colonization. Pathogens must bind or stick to a host surface to avoid elimination by the ciliary escalator or peristalsis. Microorganisms usually have a predilection for a specific cell type which is often mediated by specific adhesin-receptor interaction. Adherence is often mediated by proteinacious structures on the surface of the microorganism called Pili or Fimbriae. This provides a molecular explanation for organotrophy, tissue and cellular specificity. In some cases, the specific adhesion provides a molecular explanation for host range.

Specific colonization factors provide a potential target for intervention either as vaccines or as treatment using receptor analogs. If colonization is prevented, disease is usually prevented.

D. Multiplication. The goal of a bacterium is to become bacteria while the goal of the host, in many cases is to prevent bacterial growth. Some bacterial pathogens have minimal growth requirements other than glucose, a source of nitrogen and phosphate. However, all microorganism need iron for growth. The host effectively chelates iron with transferrin (serum) or lactoferrin (mucosal surfaces). Many pathogens have mechanisms which allow them to obtain iron from host transferrin or lactoferrin. One mechanism involves secretion of low molecular weight iron binding compounds (siderophores) which then act as a source of iron for the bacteria containing the specific siderophore receptor. This system of iron acquisition is induced during growth in iron limiting conditions.

It should be noted that normally prototrophic *Salmonella typhimurium* mutants which are purine auxotrophs have an LD50 increased by one million fold.

Would you consider enzymes associated with purine metabolism as virulence factors?

E. Invasion. Depending on the source, the term invasion may have two meanings. The general meaning is to enter the host's tissues and disseminate, i.e. leave the mucosal surface. However, another meaning has arisen which is to enter host cells. Thus, *S. typhimurium* can enter intestinal epithelial cells (invade them), and cause an invasive disease by disseminating to liver and spleen.

F. Avoidance of the immune response: (Serum resistance, antiphagocytic substances like capsule, toxins, antigenic variation.

G. Transmission. The efficiency of transmission is dependent upon several factors: 1) the source of the infecting agent; 2) the number of organisms released; 3) the ability of the microorganism to

retain virulence and survive in the environment; 4) the frequency of effective contacts; 5) the susceptibility and the immune status of the population.

Pathogens have evolved with their host and transmission is critical for the survival of the pathogen. If disease does not result in transmission to another host or in the increase in the number of the pathogen, then it is likely that the infection is not relevant in an evolutionary sense. For example, *Legionella pneumophila* is an opportunistic pathogen which grows in macrophages in the lung. However, the organism is not transmitted from person-to-person. It turns out that *L. pneumophila* lives inside fresh water amoebae, the natural host. Humans are accidental hosts.

## V. Mechanisms of pathogenesis

A. Colonization Factors (adhesins). Most, but not all, bacterial adhesins are pili (fimbriae). Perhaps the most famous pilus is the sex pilus encoded by the F-plasmid. The F-pilus mediates binding of F<sup>+</sup> bacteria to F<sup>-</sup> bacteria thus initiating conjugation. Bacterial Pili involved in pathogenesis are generally rigid, rod-like appendages, composed mainly of approximately 1000 copies of a single ca. 18-kDa polypeptide (pilin), that protrude beyond the outer membrane and allow bacteria to adsorb to specific receptors on the host surface. Most *E. coli* have what is called common pili whose adherence is blocked by D-mannose. Adherence is monitored in the laboratory by hemagglutination of red blood cells. Thus adherence which is mediated by Type I pili can be examined as mannose-sensitive hemagglutination. Alternatively, there are pili which show mannose resistant hemagglutination. Pili can be classified by what species of erythrocytes they will agglutinate and by which sugars inhibit hemagglutination.

Bacterial pili have been extensively studied during the past decade. The best characterized pilus was isolated from a strain of *E. coli* which caused pyelonephritis (Pap pili, pilus associated with pyelonephritis). Pap pili-mediated hemagglutination is inhibited by digalactosides (gal-gal). There are ten genes necessary for expression of pap pili (Figure). Interestingly, the major pilus subunit (PapA) is not the adhesin. PapG, which is located at the tip of the pilus is the actual adhesin. This was found by examining mutants that still made pili, but failed to hemagglutinate. Analysis of mutations in all of the pap proteins revealed a number of interesting phenotypes including, bald mutants (no pili), longer pili, fragile pili, and non-hemagglutinating pili (Fig.).

B. Antiphagocytic substances. A number of strategies have evolved which make bacteria resistance to phagocytosis. The most famous (The Transforming Principle) is the capsule present on *Streptococcus pneumoniae*. Antibody to the capsule is protective as it promotes uptake of the bacteria by phagocytic cells. Another example is protein A of *Staphylococcus aureus*. Protein A binds to the F<sub>C</sub> protein of IgG thus covering the bacteria.

### C. Toxins

1. Endotoxin. Endotoxin is another name for bacterial lipopolysaccharide (LPS) found on all gram negative bacteria. The part of LPS which is responsible for its endotoxin activity is lipid A. LPS is released to a limited extent by growing bacteria, but is released to a large extent upon bacterial lysis which can occur during treatment with some antibiotics. LPS is extremely heat stable; it can survive autoclaving which has implications in the use of sterile solutions in the hospital (it is destroyed by dry heat; 170C for 3 hours).

The host is able to recognize gram negative bacteria by a complex response to endotoxin. Macrophages release a variety of cytokines in response to endotoxin including IL-1, tumor necrosis factor (TNF), alpha and beta interferon. Macrophages are also primed by endotoxin for enhanced release of mediators of inflammation in response to other stimuli. The worst sequelae of a gram negative infection is called endotoxic (septic) shock. Modern therapies are being considered which block endotoxic shock with non-toxic analogs of lipid A or with monoclonal antibodies to TNF.

Endotoxin is recognized by members of the Toll receptor family of which there are now 8 members. The so-called Pattern Recognition Factors.

2. Exotoxins: These are generally secreted proteins (heat-labile). One of the best studied toxins is Diphtheria toxin (DT) secreted by *Corynebacterium diphtheriae*. DT is an absolutely essential determinant of pathogenesis. The bacteria adhere in the throat, and in response to a low iron environment secrete DT. Antibody to DT is protective (The D in the DPT vaccine is inactivated DT (toxoid). During infection, the bacteria remain localized to the upper respiratory tract but the toxin spreads to all parts of the body. One molecule of DT can kill a cell.

DT is an enzyme that specifically inactivates eucaryotic protein synthesis by inactivating elongation factor 2 (EF-2). It catalyzes the reversible transfer of ADP-ribose from NAD to a specific site on EF-2. This is known as ADP-ribosylation and is the mechanism of action of some other toxins as well.



DT is synthesized as a single polypeptide chain of 560 amino acids. The N-terminal 25 amino acids serve as a leader sequence that promotes secretion resulting in a mature protein of 535 residues. However, the toxin is not active in this form and must be "nicked" and the disulfide bond joining the A and B domains must be reduced. The A fragment is the enzymatically active fragment which is not toxic by itself. The B fragment is required for binding to a host receptor.

This theme is repeated in most toxins; i.e. an enzymatic A fragment and a B fragment or subunit for binding. During intoxication of cells, the B fragment of DT binds to cells and is internalized by endocytosis. Presumably a host protease cleaves it, followed by reduction in the endosome or cytosol. The low pH of the endosome is also required for activity and is thought to activate a pore-like function of the B-subunit which facilitates the entry of the A subunit into the cytosol.

The genes encoding DT are located on a lysogenic bacteriophage called  $\beta$ -phage. Thus, a non-toxicogenic strain can be converted into a toxigenic strain by phage conversion. There are numerous other examples of toxins and other determinants of pathogenesis being encoded by either phage, plasmids, and transposons located on the plasmid. Which is the parasite? The bacterium, the phage, plasmid, or the transposon.

3. Enterotoxins: Exotoxins which act in the gastrointestinal tract. Cholera and cholera toxin will be discussed in some detail.

1. Cholera is one of the major diarrheal diseases of the world and is caused by the gram negative bacterium *Vibrio cholerae* contaminating the drinking water. The disease is characterized by diarrhea, abdominal discomfort, and vomiting, but is not usually accompanied by fever. Diarrhea can exceed one liter/hour. Of untreated severe cases, 60% will die; with adequate prompt treatment with intravenous rehydration, this figure is reduced to 1%.

2. Animal models: The most commonly used model is the rabbit ileal loop model. The small bowel of rabbits is ligated into loops of 6-8 cm in length. Inoculation of these loops with broth cultures of live vibrios, or with cell-free culture filtrates, or purified cholera toxin results in the relevant symptomatology of the disease (the outpouring of fluid into the ligated loops which become measurably distended). The second model is the infant mouse. Adult mice are not susceptible to colonization.

3. Cholera toxin. There are 5 B subunits per toxin molecule which are necessary for binding. The B subunits do not enter the cell. The host cell receptor for CT is the ganglioside GM<sub>1</sub>. There is one A subunit per toxin molecule. The A subunit is proteolytically nicked during release from the bacterial cell and an internal disulfide bond is subsequently reduced resulting in A1 and A2 subunits. The A1 peptide exhibits enzymatic activity. CT, like DT has an ADP-ribosylation activity. The substrate for CT is a host G-protein which regulates adenylate cyclase. ADP-ribosylation of the G-protein freezes it bound to GTP in an active configuration so that the activity of the cyclase is activated resulting in increased levels of cellular cAMP. Intestinal epithelial cells with elevated levels of cAMP do not absorb Na<sup>+</sup> and secrete Cl<sup>-</sup>.

Another gram negative pathogen, *Bordetella pertussis* makes a toxin (pertussis toxin) which ADP-ribosylates another regulatory G-protein controlling adenylate cyclase. Interestingly, *B.*

*pertussis* also makes a separate toxin which is itself an adenylate cyclase which requires host calmodulin for its activity.

Enterotoxigenic *E. coli* also cause diarrhea and possess a toxin genetically related to CT. However, in *V. cholerae* the toxin is chromosomally encoded while in *E. coli* it is plasmid-encoded. The gene encoding CT is surrounded by direct repeats of sequence called RS1. This facilitates genetic recombination resulting in amplification or deletion of the CT genes. Mekalanos has shown that the CT genes are amplified *in vivo*, but revert to single copy upon *in vitro* cultivation. What might this suggest?

4 Hemolysins. Dozens of different hemolysins have been described. Some of these are pore-forming proteins while some are phospholipases. The term hemolysin is somewhat misleading as the target cell(s) *in vivo* are not generally red blood cells. Thus the term cytolysin is more appropriate. However, hemolysis is a convenient read-out, and blood agar plates are useful for diagnostic purposes.

One family of hemolysins is referred to as the oxygen-labile, sulfhydryl activated cytolysins. These molecules have a conserved cysteine that renders the molecules susceptible to inactivation by oxidation. There are 15 different members of this family present in gram positive bacteria. These molecules bind to host cell cholesterol (bacteria do not contain cholesterol), oligomerize in the membrane and form large pores. The most well known is streptolysin O (SLO) secreted by Group A streptococci which are the causative agent of Strep throat. The precise role of SLO has not been established, but it has been shown to prevent neutrophil chemotaxis at sublytic doses. Another member of the family, pneumolysin, is made by *Streptococcus pneumoniae*. However, in this case, the protein lacks a signal sequence and is not secreted. Nevertheless, it has been shown to play a role in pathogenesis; How? A third member of the family, listeriolysin O, is made by *Listeria monocytogenes* and will be discussed later.

5. Type III secretion: Also called the contact-dependent secretion pathway. (For review see, Hueck, Microbiol. Mol. Biol. Rev. 62:379-433, 1998).

Pathway used by a variety of diverse gram-negative pathogens (including both plant and animal pathogens) use type III secretion as a conserved and at the same time highly adapted and essential determinant of pathogenesis. While the mechanism of secretion is conserved, the secreted proteins (effector proteins) are highly divergent.

1. Comprised of about 20 proteins that are encoded by approx. 20-kB of DNA, of low G+C content.

2. Homologous to flagella proteins involved in transport. Electron micrographs look like an injection syringe and needle.

3. Effector molecules are injected into mammalian host cell.

4. Effector molecules lack signal sequences although many of the structural proteins that make up the secretion apparatus contain signal sequences and are secreted by GSP.

F. A word about host defense:

1. Innate Immunity: In an evolutionary sense, this is the ancestral immunity present in normal individuals at all times and does not increase with repeated exposure to a given pathogen. Toll-like receptors (TLRs) are membrane bound receptors that recognize conserved microbial structures; TLR4/LipidA; TLR2/lipoproteins,LTA; TLR5/flagellin

2. Adaptive or acquired immunity: Response of antigen-specific lymphocytes to antigen including the development of immunological memory. Adaptive immune responses are generated by clonal selection of lymphocytes. This is the arm of the immune system targeted for vaccines.

3. Humoral Immunity: Traditionally, humoral immunity is acquired immunity mediated by antibodies. Immunity can be transferred from a resistant to a sensitive individual with serum.

We will refer to humoral immunity as anything in the serum that confers resistance (complement included).

4. Cellular Immunity: Traditionally, cellular immunity is specific immunity in which antigen-specific T-cells play the main role. Immunity can be transferred with T-cells.

5. How to make an antibody? Freund's Complete Adjuvant (CFA). What is it and why do you need the adjuvant?

VI. Vaccines

A. DPT vaccine. An effective vaccine of childhood. D= diphtheria toxin toxoid; P= killed *Bordetella pertussis*; T= tetanus toxin toxoid.

B. Live *Salmonella typhi* vaccine; an attenuated strain which is mutated in a galactose epimerase; The bacteria can be grown *in vitro* with galactose and they make a smooth LPS. However, *in vivo*, without galactose, the bacteria make a rough LPS and are killed by the host.

C. *N. gonorrhoea*: Nothing available; why?

D. Live bacterial carriers of foreign antigens (BCG).

#### IV. Approaches to the Study of Microbial Pathogenesis.

A. Historical Perspective: Koch's Postulates. Robert Koch (1843-1910), the father of Medical Microbiology formalized the criteria for identifying a pathogen as the causative agent of a particular disease in the 1880s (The golden age of microbiology).

##### Koch's Postulates

1. The organism is regularly found in the lesions of the disease
2. The organism can be isolated in pure culture on artificial media.
3. Inoculation of this pure culture produces a similar disease in experimental animals.
4. The organism can be recovered from the lesions in these animals.

Koch's postulates are not always feasible to perform. For example, some pathogens cannot be cultured in pure culture; for others there may be no appropriate animal model. There has been a controversy over the etiologic agent of AIDS. A minority view (guess who?) has it that since the Koch's Postulates have not been performed with HIV-1, it has yet to be the proven cause of AIDS.

B. The Molecular Version of Koch's Postulates: The 1880s were the Golden age of Microbiology as pathogens were identified and shown to cause a variety of diseases. Current research in the area of Microbiology is focused on the identification of individual virulence determinants that make a pathogen a pathogen. In 1988 Stanley Falkow formalized a set of postulates which can be used to prove that a genetic property is indeed an essential determinant of pathogenicity.

##### Molecular Koch's Postulates

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species.
2. Specific inactivation of the gene(s) associated with the suspected virulence trait should lead to a measurable loss in virulence in an appropriate model system.

3. Complementation of the mutation either on a plasmid or by allelic replacement of the mutated gene should lead to restoration of pathogenicity.

### C. Genetic Approaches to Pathogenesis.

1. Transposon mutagenesis. The elegant genetic analysis possible in a few bacteria such as *E. coli* and *B. subtilis* is not possible in most pathogens. Indeed, even natural isolates of *E. coli* are often refractile to genetic analysis such as transformation and transduction. As a consequence, methods have been developed which facilitate the introduction of transposable elements into a wide variety of both gram negative and gram positive bacteria. Transposable elements can be introduced into the genome of bacteria where they insert somewhat randomly thus causing insertion mutations. Since the average bacterial species has approximately 5000 genes, one can saturate the chromosome with ease. Furthermore, the transposon provides a molecular tag which can be subsequently used to identify the mutated gene and to clone it.

2. Cloning. The advent of recombinant DNA revolutionized the field of Microbial Pathogenesis. There are many strategies that have been developed to clone virulence factors. One of the most successful approaches was cloning the invasion protein from *Yersinia pseudotuberculosis*. At the time, it was not known how *Y. pseudotuberculosis* invaded cells, however it was clearly a very active process. Bacteria which enter mammalian cells are resistant to the bacteriocidal activity of gentamicin, while extracellular bacteria are rapidly killed by the antibiotic. Isberg and Falkow constructed a gene library from *Y. pseudotuberculosis* in *E. coli*. The entire library was added to an adherent culture of epithelial cells, followed by gentamicin. The internalized bacteria were recovered, and found to all contain plasmids with the identical DNA sequence. This sequence encoded the protein Invasin which alone facilitated the uptake of the *E. coli*

3. Find one and keep sequencing (pathogenicity islands). There are now numerous examples of genes encoding determinants of pathogenesis being genetically linked.

4. *In vivo* expression technology (IVET). Mahan et al., Science, 259:686, 1993. The idea here is that important determinants of bacterial pathogenesis that are expressed *in vivo* are not expressed *in vitro*.

Based on the observation that purine-requiring auxotrophs of *S. typhimurium* are 5-logs less virulent in the mouse model of infection. Random segments of DNA are cloned upstream of a promoter-less copy of *purA* and *lacZ*. Gene libraries are then introduced into a *purA* minus background. These strains are passed through mice and then plated on

Mackonkey plates. The goal is to find the rare *lac* minus strain that still grow in the mouse (gene is expressed in the mouse, but not on the petri plate).

Problems: What is *in vitro*? Minimal medium/rich medium, low iron, high iron, 30C, 37C? What about genes that turn on and off *in vivo*?

5. Signature -tagged mutagenesis: Hensel et al., Science: 269:400, 1995. Allows one to screen for 100 potential transposon mutants at a time for those that do not grow in the animal.

6. Fluorescence-based isolation of bacterial genes expressed in host cells. Valdivia and Falkow, Science. 277: 2007, 1997. Used the fluorescence-activated cell sorter to select cells containing bacteria expressing GFP in infected cells. You then select against bacteria that express GFP outside of cells. The beauty of this system is that you can set window of expression.

7. TrasH. Sasseti et al. PNAS98:12712. Use of a comprehensive transposon library and a bacterial microarray to identify bacterial genes necessary for growth *in vivo*.

## VIII. Intracellular Pathogens

### A. Reasons for an intracellular lifestyle

1. Escape the humoral immune system
2. Environment rich in nutrients
3. As taxis to another environment
4. Free of competition

B. Host Cells: Host cells can be divided into professional phagocytic cells such as macrophages and non-professional phagocytic cells such as epithelial cells or fibroblasts. Non-professional phagocytes do not normally ingest particles the size of a microorganism. However, many pathogens have evolved mechanisms to induce their uptake. In contrast, many intracellular pathogens take advantage of the natural phagocytic properties of macrophages and survive and multiply in the macrophages.

C. Entry into Non-Professional phagocytes (Invasion). The term invasion is not accurate as both the host and the pathogen are involved in the process; i.e., uptake is prevented by cytochalasin D which blocks actin-based processes required for conventional phagocytosis.

1. *Yersiniae*. Both *Y. enterocolitica* and *Y. pseudotuberculosis* enter mammalian cells very effectively (as many as 100 bacteria/cell). Ralph Isberg and Stanley Falkow discovered that a single gene which encoded an outer membrane protein called Inv was required. Amazingly, introduction of Inv into a common laboratory strain of *E. coli* resulted in its ability to invade just as well. The receptor for Inv are the b1 Integrins such as the fibronectin receptor. Inv-mediated uptake was shown to be mediated by binding with high affinity to its receptor.
2. *Salmonella typhimurium*. Unlike *Yersiniae*, a single gene from *S. typhimurium* cannot confer the invasive property upon *E. coli*. In fact dozens of genes appear to be required. Mutants can be isolated which still bind to host cells, but do not enter. These mutants can be rescued for entry if mixed with wild-type bacteria. Examination of entry shows that there is a marked cytoskeletal rearrangement that results in bystander bacteria being ingested. *S. typhimurium* is able to induce a process that resembles macropinocytosis induced by secretion of Type III secretion effector proteins.

#### D. The Cell Biology of Intracellular Growth.

1. Life in a vacuole. The majority of intracellular pathogens reside with a vacuole. How nutrients are obtained is not known. Many intracellular pathogens are found in a vacuole which has a pH elevated compared to a lysosome and which does not fuse with lysosomes. Again, the mechanism of vacuolar modification is not known. There are exceptions; e.g. *Coxiella burnietti* lives in a fully acidic fused compartment.

2. Life in the cytosol. Some intracellular pathogens escape from a vacuole and grow directly in the cytoplasm which is rich in nutrients.

*Listeria monocytogenes* *L. monocytogenes* has been used for decades as a model intracellular pathogen. Immunity in mice is cell-mediated; i.e. antibody plays no role. The cell biology of infection is depicted in the figure below. Subsequent to internalization, the bacteria escape from the host vacuole. It is now known that the bacteria secrete a hemolysin (listeriolysin O) related to streptolysin O which is required for disruption of the vacuole. Hemolysin minus mutants fail to enter the cytoplasm and fail to grow. Once the bacteria reach the cytoplasm, they grow rapidly with a doubling time of approximately 50 min. Next, the bacteria express a surface protein called ActA, which causes host actin to polymerize at one of the bacteria and propels the bacteria through the cytoplasm and into pseudopod-like projections. Next the bacteria are able to spread from one cell to another via the pseudopod, and thus never contact the extracellular environment.

