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## ADVANCES IN PATHOGEN REDUCTION

# *Bacteria Pathogen Virulence Primer*

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### Introduction

The manner in which bacterial pathogens caused human disease has been and is currently the subject of much medical and scientific research. The medical community is largely responsible for research centering on clinical and pathological data and observations. The world's community of microbiologists and biochemists have focused on microbial biology to elucidate exact mechanisms by which bacteria cause disease and how to inhibit microbes with drugs. Underlying these efforts is a realization that bacterial pathogens are not static in terms of their own evolution and are

fully capable of an adaptation response to new environments which present opportunities to survive and multiply. Whether these environments are mediated by new antibiotics, drugs, new foods, or new food processing techniques; in time, bacteria are readily able to adapt to most new environments (Smith, 1995).

Within the last two decades, several foodborne pathogens have been labeled "new" or "newly emerging." While they were only recently recognized by clinical microbiology labs, it is possible that these pathogens have most likely been part of the natural microbiota. They may have been poised to live within the human host for some time, but only recently were given the opportunity to survive and multiply by either a newly-opened means of transmission or a new ecological niche.

Regardless of the source of bacterial pathogens, professionals in the food and meat industry must be continually aware of these microbial hazards. Not only do food pro-

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cessing techniques change (hence presenting new places for bacteria to thrive) but our national population demographics are shifting to an older mean age and a larger proportion of our people being categorized as immunocompromised or "at greater risk" for foodborne diseases (CAST, 1994). Knowledge is the basis to understanding how to control introduction of pathogens and prevent their transmission through the food supply.

This primer will attempt to provide that knowledge and update members of the food production community on well studied themes of pathogen virulence. Enterovirulent *Escherichia coli* (EEC), especially the enterohemorrhagic *E. coli* (EHEC, including serotype O157:H7), will be used as examples of the means by which a highly pathogenic bacterium actually causes human disease. A current hypothesis on its origin will be presented. Throughout this paper, pathogenicity and virulence are in reference to bacterial agents and human disease.

### Pathogenicity and Virulence

Pathogenicity is defined as the ability of an organism to cause disease. Although often used synonymously, virulence and pathogenicity do not have the same meaning. Virulence is best described as the degree of pathogenicity within a group of strains and is usually dose-related (Smith, 1995; Isenberg, 1988; Finlay and Falkow, 1989).

Since pathogenicity is most easily detected from disease manifestations (morbidity and mortality), dose response curves have been the traditional means to assess pathogen virulence. Commonly, such curves are used to calculate values including LD<sub>50</sub>, or the lethal dosage for 50% of a test population of animals. Steep dose response slopes would indicate that small increases in dosages of a pathogen resulted in a relatively large increase in either morbidity or mortality (high virulence), the opposite—low slope over a large dosage increase, would indicate low virulence. In the case of non-typhoid *Salmonella typhimurium*, infectious doses can range from less than 100 cells to as many as 10<sup>9</sup> bacterial cells to cause disease. This wide range serves as a good illustration of differences in virulence of a class of bacteria—salmonellae, which when originating from nature, are all considered to be pathogenic. Although overly simplified, this example is the basis for much of our understanding of the gross manifestations of pathogen virulence. Any such lethality curve must be interpreted with the knowledge that very few, if any, animal models (largely rodent based) are a particularly realistic model for the human organism, but they do serve to offer an estimate of infectious dosages. When available, either by design or circumstantial, human based dosage data is more desirable for this purpose. In the case of *E. coli* O157:H7 (the major serotype of enterohemorrhagic *E. coli*), the infectious dose has been estimated to be as few as 10 cells. This estimate was obtained from plate counts of samples of incriminated food products and not from actual dose-response assays.

### The Host-Parasite Relationship is Changing as Does Our Population

Pathogenicity and virulence are an interaction of the parasite (bacterial pathogen) and the host (in this discussion the human). Host factors play a critical role in virulence and the host condition will respond to varying degrees upon ingesting a pathogen—from no response at all to mortality from the same strain of bacterium. An example of this phenomenon is the case of virulence testing of *Listeria monocytogenes* strains in the immunocompromised mouse model (Siragusa et al., 1990). In this assay, induced listeriosis is monitored in groups of mice that are either untreated or have previously been injected with a polysaccharide to reduce their own peritoneal macrophage (white cells that ingest and destroy pathogens in host tissues) population. In untreated mice, the calculated LD<sub>50</sub> can be as high as 10,000 cells, vs the immunocompromised treated mice which manifest an infectious lethal dose of as few as 10 to 100 cells of *Listeria monocytogenes*. Although a stark example, this assay serves to illustrate the importance of the host immune response in fighting foodborne disease.

Since our population (potential hosts) are all food consumers and when viewed as a population are tending to be at a higher risk for foodborne disease (CAST, 1994; Buzby and Roberts, 1996), recognition that the host condition is a central part of the virulence equation is critical for formulating and implementing food safety policies and decisions. Although not discussed in this paper, the science of risk assessment will play more of a role in the coming years as demographic projections become realities.

### Virulence and the Disease Process

The disease process from bacterial pathogens is complex, yet can be described as a five step process: 1) entrance into the host, 2) penetration into the tissues, 3) resistance to host defenses, 4) ability to grow and multiply within the host, and 5) cause damage to the host (Smith, 1995). This paper will be limited to the alimentary route of entry. In general terms, pathogens are immediately confronted with a variety of antibacterial substances and defenses from the host including secretory antibodies, digestive enzymes, immune cells, low pH, intestinal bile salts, intestinal peristalsis, and a numerically overwhelming population of commensal intestinal bacteria.

From the microbiological viewpoint, bacterial pathogens must accomplish two goals in order to be successful in passing its genes to a new generation; they must survive and

**TABLE 1: Five Bacterial Requirements for Pathogenicity.**

- |    |                                  |
|----|----------------------------------|
| 1. | Entrance into the host.          |
| 2. | Penetration to the host tissues. |
| 3. | Resistance to host defenses.     |
| 4. | Ability to grow and multiply.    |
| 5. | Cause damage to the host.        |

**TABLE 2: The Enterovirulent or Diarrheogenic *Escherichia coli* (EEC).**

1.	Enteroinvasive	EIEC
2.	Enterohemorrhagic	EHEC
3.	Enteropathogenic	EPEC
4.	Enterotoxigenic	ETEC

multiply. The level of host injury does not necessarily correlate with evolutionary success (Mekalanos, 1992). A bacterial pathogen must be able to: 1) infect of the mucosal surface, 2) enter into the host through the mucosal surface, 3) grow *in-vivo*, 4) interfere with host defenses, and 5) inflict damage to the host from the survival or multiplication process.

Pathogenicity is the result of the organism's ability to accomplish goals of the disease process. To fulfill these steps, the pathogen must be equipped with the aforementioned requirements. Collectively these can be termed virulence determinants. These virulence determinants are genetically programmed and the expression of these genes are regulated by very sensitive mechanisms or sensors to be discussed later in this paper. The importance of a fully capable repertoire of virulence determinants is simply that in order to be pathogenic, an organism must manifest all five of these requirements. The loss of or lack of expression of any one factor will render that organism non-pathogenic.

**Enterovirulent *Escherichia coli*; the EEC**

Since the highly-publicized outbreak of hemorrhagic colitis and hemolytic uremic syndrome due to *E. coli* O157:H7 in undercooked ground beef (CDC, 1993), a veritable explosion of research has been initiated to address aspects of this foodborne disease. But despite these efforts, the causative agents can still be confusing; therefore for the food processing professional, a simplified overview of the diarrheogenic *E. coli* (EEC) is in order.

The enterovirulent diarrheogenic *E. coli* are classed into four recognized and distinguishable groups (Table 2). These divisions are based primarily on pathologic manifestations but at least four commonalities are shared by this group (Levine, 1988). Critical virulence factors are genetically programmed in genes found on plasmids (small pieces of DNA that are not part of the larger bacterial chromosome). Each group causes a distinctive interaction and infection of the intestinal mucosa. Enterotoxins can be produced that are either cell destructive, or non-destructive but invoke massive fluid accumulation in the gut. Within each category, strains tend to fall within certain 'O' serotypes (Table 3). These serotypes are based on the bacterium's reaction with specific antibodies. In the case of O157:H7, the strain reacts with 'O' serotype antibodies numbered 157 and 'H' serotype antibodies numbered 7.

ETEC (cause of traveler's diarrhea) and EIEC (bacillary

**TABLE 3: EEC Associated Serotypes.**

Enteropathogenic (EPEC)	
Class I:	O55, O86, O119, O125, O126, O127, O128ab, O142
Class II:	O18, O44, O112, O114
Enteroinvasive(EIEC) O28ac, O29, O124, O136, O143, O144, O152, O164, O167	
Enterotoxigenic (ETEC) O6, O8, O15, O20, O25, O27, O63, O78, O80, O85, O115, O128ac, O139, O148, O153, O159, O167	
Enterohemorrhagic (EHEC) O157, O26, O111	

dysentery) are unlikely to be foodborne disease agents within the United States, but are highly destructive pathogens in areas of the world with poorer hygiene standards and tend to affect mainly children of those geographies. Virulence of the EPEC strains is of particular interest to microbiologists because of its hypothetical relationship and role as the progenitor to the EHEC group of EEC. The proposed origin of the EHEC group will be discussed in a later section.

**Virulence of *E. coli* O157:H7**

This section will address the current understanding of the mechanisms of virulence of the EHEC and specifically *E. coli* O157:H7. The hallmarks of *E. coli* O157:H7 and the other EHEC is production of AE lesions and synthesis of copious amounts of shiga-like toxins (Acheson, 1996; Griffin, 1995; Robertson, 1988). The disease manifestations are hemorrhagic colitis and possibly subsequent diseases including hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). The initial illness results in abdominal cramping and watery diarrhea that quickly becomes grossly bloody. *E. coli* O157:H7 must meet the previously described five requirements for pathogenicity, and it does so by expressing or "switching genes on." These genes are collectively the pathogen's virulence factors which allow it to fulfill the five requirements outlined in Table 1. The following section will discuss some of these virulence factors.

Like all foodborne infectious pathogens, ingestion and transmission to the intestinal tract is quite a hostile environment to withstand and still retain viability. Immediately upon ingestion, the organism is subjected to higher temperatures, pH extremes, different and sparse nutrient density, high concentrations of membrane active bile salts, a myriad of digestive enzymes, other intestinal commensal bacteria (normal flora), and the constant peristalsis of the intestinal tract. Several acid resistance genes have been demonstrated to enhance the survival of *E. coli* O157:H7 in the pH extremes of the stomach (pH 1 to 3) and the intestinal tract (pH 4.5 to 7) with its content of antimicrobial fatty acids (Lin et al., 1996).

Once situated in the ileum or small intestine, this pathogen attaches to the brush border or microvilli of the epithelial cells of the villus. This attachment phenomenon is mediated by the expression of genes located on a large plasmid in

these strains known as the 60 MDa plasmid (in reference to its molecular weight). This genetic element codes for at least fourteen different virulence genes or determinants necessary for pathogenicity (Donnenberg, 1996). Bacterial attachment factors known as pili (not flagella) from a single organism can intertwine with other such pili forming bundles that allow initiation of an intimate attachment to the intestinal brush border cells. This process continues, resulting in making of another *E. coli* protein, known as intimin, that causes further destruction of the epithelial border resulting in a lesion termed attaching and effacing lesions (AE) since the brush border is essentially destroyed following attachment.

The second hallmark of the EHEC, including *E. coli* O157:H7, is the synthesis of large amounts of shiga-like toxins. The shiga-like toxins (currently termed Stx) are similar in nature to toxin synthesized by *Shigella dysenteriae* known as shiga-toxin (Calderwood, 1996; O'Brien and Holmes, 1987). EHEC produces two main types of Stx; Stx-1, Stx-2. A variety of acronyms have been used to describe EHEC shiga-like toxins including SLT (-1, -2 etc.) and Verotoxin or VT (-1, -2 etc.), these are synonyms for the Stx acronym recently adopted (EHEC strains acquire the genes for toxin synthesis from bacteriophage. EHEC strains have been known to produce all of one toxin type or both main types. Stx is a proteinaceous toxin composed of two subunits, A and B. The A subunit is responsible for disrupting intracellular protein synthesis in mammalian cells including human kidney glomerular cells. The B subunit (five B subunits are bound to one A subunit comprising one Stx molecule) is responsible for the attachment to the toxin receptor on human kidney cells. Stx-2 is more frequently associated with hemolytic uremic syndrome. In cell culture, for instance African green monkey kidney cells known as Vero Cells, toxin synthesis, along with AE lesion formation leads to cell degeneration and death. In young children and the elderly, toxin formation is the cause of one of the most frightening sequelae of EHEC infection, hemolytic uremic syndrome or HUS.

Stx bind to a receptor in humans known as Gb<sub>3</sub> (Lingwood, 1996). This receptor is located on kidney glomerular cells of infants. Toxin transmission to this site is thought to be mediated by toxigenic EHEC bound to the intestinal epithelium within AE lesions. The toxins are transported into the blood stream via capillaries which abound beneath the intestinal submucosa at sites of the AE lesions. Stx bind to Gb<sub>3</sub>, resulting in host cell death through inhibition of protein synthesis. Ultimately victims of HUS suffer either permanent kidney damage or even death.

The mechanism of HUS in the elderly is less well understood. Briefly, Stx can induce cytokine (cell messenger proteins that induce cell growth or metabolism) synthesis which results in the making of Gb<sub>3</sub> receptors on kidney glomeruli cells. It is thought that in the elderly, the cytokine response to an EHEC infection is somehow aberrant, resulting in Gb<sub>3</sub> receptor synthesis on glomeruli and subsequently HUS (Lingwood, 1996).

It is worth reiterating at this point that all of the afore-

mentioned mechanisms are the result of bacterial virulence factors which are encoded by the EHEC cell's DNA or its genome. The importance of this fact will be increasingly important in the next section.

### Where Did *E. coli* O157:H7 Come From?

It should be clear at this point that pathogen virulence is a highly complex and multifactorial occurrence which is the result of genes and their regulation (turning on and off). Expression of virulence genes or determinants are dependent on both the genome and the environment.

How does the bacterium's environment tell it what to turn on or switch off? This is a complex question but simply stated—there exists in bacteria, environmental signal sensors which respond to such things as temperature, low pH, nutrient density, osmolarity, anaerobiosis, and many other environmental signals. An example of one such sensor is a system known as the phoP/phoQ system studied in salmonellae (Miller et al., 1989). This system can detect low pH, such as that inside of a macrophage cell in which *Salmonella* are known to be able to survive (Leung and Finlay, 1991). In response to the low pH signal detected by *Salmonella*, the bacterium switches genes on that allow it to survive the low pH of its new environment.

What else do pathogens require to be virulent? In addition to environmental sensing capabilities, bacterial pathogens must be able to transport proteins into their environments. In the case of EEC which cause AE lesion formation (EHEC and EPEC), proteins which carry out these function are secreted by a system called a Type III secretion system (Mecasas and Strauss, 1996; Jarvis and Kaper, 1996). This system is a series of membrane proteins that are oriented within the bacterium's sacculus in order to secrete proteins that are synthesized within the bacterium out into the bacterium's environment and directly to the host cell.

Since sensors and secretion systems are essentially proteins entities, they are encoded by genes within the bacterium's DNA. Recently it has been discovered that important bacterial pathogen virulence genes are actually located within discrete segments of bacterial chromosomal DNA termed pathogenicity islands [PI] (Mecasas and Strauss, 1996). These entities are often the major distinguishing feature between a pathogen and its commensal cousin. PI DNA ranges in size from about 9 to 190 kilobase pairs. The DNA comprising PI's is considered "foreign" DNA in that it bears little resemblance to the rest of the bacterial genome in terms of guanine + cytosine content. PI's encode for virulence determinants including hemolysin, environmental sensors and, in the case of enterovirulent *E. coli*, the genes for AE (attaching and effacing) lesions. The PI identified within the EPEC and EHEC groups of *E. coli* has been termed the locus of enterocyte effacement or LEE.

Back to the central question of this section, the origin of *E. coli* O157:H7, one must remember that bacteria possess several means to cut and splice exogenous DNA into its genome. A proposed hypothesis states that a progenitor non-

pathogenic *E. coli* (such as a commensal *E. coli* cell thriving within an intestinal tract) acquired the pathogenicity island DNA encoding LEE. Next, this progenitor 'EPEC' containing the pathogenicity island LEE, acquires the genes for Stx (shiga-like toxin) synthesis from a bacteriophage resulting in an *E. coli* strain which now has a virulence arsenal that enables it to attach and invade the intestinal epithelium; causing hemorrhagic colitis as well as produce copious amounts of highly destructive toxins (Whittam et al., 1993). While this is a hypothetical scenario, it is based on measuring genetic distances by both genomic and multi-locus enzyme electrophoresis patterns which indicate the degree of allelic variation in strains of EEC.

### Summary and Some Internet Sources

In closing, it should be appreciated that for bacterial pathogens to be virulent, several complex requirements must be met. Although the example pathogen used in this primer (enterohemorrhagic *E. coli*) is readily destroyed by adequate cooking or heating, it is clearly in the best interest of the meat industry professional to have some understanding of these pathogenic mechanisms. The present climate of regulatory activity and producer/processor accountability dictate that more in-depth knowledge of food microbiology is a necessity.

Seldom does the food industry professional have time to perform detailed fact-finding literature searches and therefore could benefit by instant information. Two internet sources recommended for anyone needing such information are the U.S. Food and Drug Administration's "Bad Bug Book" at <http://vm.cfsan.fda.gov/~mow/intro.html> and "Dictionary of Cell Biology" at <http://www.mblab.gla.ac.uk/~julian/dict.html>.

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