Food Microbiology and Food Safety
Practical Approaches

Joshua B. Gurtler
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Foodborne Pathogens

Virulence Factors and Host Susceptibility

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Foodborne Pathogens

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Contents

Virulence Book Introduction

Food recalls in the United States are increasing, in part because of the increased sophistication of the country's foodborne disease surveillance system through the application of whole genome sequencing (WGS). Other countries are adopting this approach as well. Applying WGS to *Listeria* surveillance has increased the detection of outbreaks in the United States by 3.3-fold, and the median number of *Listeria* cases in an outbreak has decreased from 68 in the mid-1990s to 3 presently. There are about 625 times more estimated cases of salmonellosis (ca. 1 million) in the United States than listeriosis (1600). When WGS is fully implemented in salmonellosis outbreak detection, there is likely to be a considerable increase in the recognition and traceback of salmonellosis outbreaks annually. In 2017, WGS is anticipated to expand to other foodborne pathogens, which will likely lead to many new revelations about the sources of pathogens in our food supply and many food producers and processors will likely be adversely affected. The economic consequence is likely to be substantial.

With this revolutionary advancement in foodborne disease outbreak detection, it will be critical that all sectors of the food industry have world-class food safety systems in place and that robust methods are available for the detection and identification of pathogens in foods and food manufacturing facilities. This book addresses this latter point, in hopes that soon regulatory surveillance and policies regarding foodborne pathogens will be based upon the virulence of microbes rather than simply classifying entire groups of potential pathogens as harmful.

Further, all members of a particular bacterial genus (e.g., *Salmonella, Campylobacter*) or members of an individual bacterial species (e.g., *Listeria monocytogenes, Cronobacter sakazakii*) are often treated by public health and regulatory agencies as being equally pathogenic; however, this is not necessarily true and is an overly conservative approach to ensuring the safety of foods. Even within a species, virulence factors vary to the point that some isolates may be highly virulent, whereas others may rarely, if ever, cause disease in humans. Hence, many food safety scientists have concluded that a more appropriate characterization of bacterial isolates for public health purposes could be that of typing food-associated bacteria on the basis of their virulence factors. This concept can be addressed in a number of different

ways by examining the virulence factors of foodborne pathogens and their impact on the human host based on various intrinsic and extrinsic factors.

This book is divided into two sections. Part I, ***Foodborne Pathogens and Virulence Factors****,* focuses on specific virulence factors of foodborne pathogens and the role they play in regulatory requirements, recalls, and foodborne illness. The variability in virulence between strains of *Listeria monocytogenes, Salmonella, Campylobacter, Shigella,* and others will be addressed. This section will also examine known factors capable of enhancing virulence in foodborne pathogens. Part II, ***Foodborne Pathogens, Host Susceptibility, and Infectious Dose****,* is self-described and will address the ability of a pathogen to invade a human host, based on numerous exogenous factors relative to the pathogen and the environment. Some of these factors include host age, immune status, genetic composition, infectious dose, food composition, and probiotics. Readers of this book will develop a better understanding of foodborne bacterial pathogen virulence factors, pathogenicity, and host factors that influence the severity of disease in humans.

Part I
Foodborne Pathogens and Virulence Factors

Advantages of Virulotyping Pathogens Over Traditional Identification and Characterization Methods

Joshua B. Gurtler, Michael P. Doyle, Jeffrey L. Kornacki, Pina M. Fratamico, Andrew G. Gehring, and George C. Paoli

Abstract This chapter provides an overview regarding the advantages of virulotyping over historic serology-based, PCR, based on genes that identify an organism, or enzymatic and biochemical-based analyses of foodborne pathogens in clinical diagnostics and food industry microbiology testing. Traditional identification and characterization methods are designed to detect a given genus, species, serovar or genetic variant of bacteria via enzymatic, genetic or biochemical characterization. Virulotyping is a technique that identifies specific virulence genes within the pathogen's genome; thus having the discriminatory power to differentiate between a virulent and an avirulent (nonpathogenic) strain of bacterium within a species. As an example, the United States Department of Agriculture, Food Safety and Inspection Service (USDA-FSIS) methods for detection, isolation and identification of Shiga toxin producing *E. coli* (STEC) use the presence of the *eae* and *stx* genes as an initial screen after selective culture enrichment to determine if an adulterant STEC may be present in a raw beef verification sample and as a confirmatory screen of STEC isolates. While virulence testing for STEC is just one example of potential advantages of virulotyping, further benefits may also be realized from virulotyping other common foodborne pathogens (e.g., *Campylobacter* spp.*, Salmonella enterica,* and *Listeria monocytogenes*), should avirulent subgroups one day be identified. While PCR and microarray analyses are currently used to perform virulotyping, genome sequencing-based methods may be a more robust and discriminatory technique, when they become practicable for food safety testing and clinical diagnostics. A potential future virulotyping-based medical diagnostics and food testing system might be envisioned in the United States, which could prevent illnesses.

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Keywords Virulotyping • Enterohemorrhagic *E. coli* • *Salmonella enterica* • Virulence genes

Abbreviations

eae	The intimin outer membrane protein involved in formation of the attaching and effacing lesions
EHEC	Enterohemorrhagic *E. coli*
STEC	Shiga toxin-producing *E. coli*
Stx1	Shiga toxin 1
*stx*1	The Shiga toxin 1 gene
Stx2	Shiga toxin 2
*stx*2	The Shiga toxin 2 gene

Introduction

This chapter introduces the concept of virulotyping as a potentially superior method for medical infectious disease diagnostics or food safety testing over historic microbiology-, serology-, or PCR-based typing, which only detects a given genus, species, serovar or genetic variant of bacteria by its morphological and biochemical properties, structural components (O, H, or K antigens), or identifying genes, respectively. In many cases, the discriminating properties are not directly related to the organisms' virulence potential. Virulotyping, on the other hand, is a method that identifies specific virulence genes within the pathogen's genome, thus having the discriminatory power to differentiate between virulent and avirulent (nonpathogenic) bacteria that would be otherwise indistinguishable. While PCR and microarray analyses are commonly used in virulotyping, genome sequencing with bioinformatics analysis may be a more robust and accurate technique.

The first usage of the term virulotyping was by Timothy et al. (2008) and again by Döpfer et al. (2008). Since then, more than 150 scientific journal publications (Table 1) have either used the term virulotype, or virulotyping, to describe the characterization of bacteria with unique genetic virulence factors. While the term "pathotyping" may sometimes be synonymous with, and can always be inferred from virulotyping, this chapter will focus specifically on the use of the latter, which is the more-recently introduced term. Many of the recommendations cited in this chapter may one day be implemented, enhanced, superseded and supplanted by next-generation, whole genome sequencing (WGS) for medical diagnostics and food safety testing, which is the direction the field of bacterial identification and characterization may follow (Bertelli and Greub 2013; Fournier et al. 2014; Lecoit and Eloit 2015). However, until DNA sequencing becomes as facile, fast, reliable and cost-effective as is needed for infectious disease diagnostics and industrial

Table 1 Peer-reviewed scientific journal publications (2008–2016) discussing virulotype or virulotyping

Year	Authors	Title	Journal
2007	Piekarska and Jagielski	Prevalence of virulence-associated genes of *Enterococcus faecalis* clinical strains isolated from patients and volunteers.	*Medycyna Doświadczalna i Mikrobiologia*
2008	Timothy et al.	Molecular epidemiology of a reproductive tract-associated colibacillosis outbreak in a layer breeder flock associated with atypical avian pathogenic *Escherichia coli*.	*Avian Pathology*
2008	Döpfer et al.	Assessing genetic heterogeneity within bacterial species isolated from gastrointestinal and environmental samples: How many isolates does it take?	*Applied and Environmental Microbiology*
2009	Habib et al.	Correlation between genotypic diversity, lipooligosaccharide gene locus class variation, and caco-2 cell invasion potential of *Campylobacter jejuni* isolates from chicken meat and humans: Contribution to virulotyping.	*Applied Environmental Microbiology*
2009	Islam et al.	Comparative genotyping of *Campylobacter jejuni* strains from patients with Guillain-Barré syndrome.	*PLoS One.*
2009	Khoo et al.	Virulotyping of *Salmonella enterica subsp. Enterica* isolated from indigenous vegetables and poultry meat in Malaysia using multiplex-PCR.	*Antonie Van Leeuwenhoek, International Journal of General and Molecular Microbiology*
2009	Park et al.	Identification of *Salmonella enterica* subspecies I, *Salmonella enterica* serovars Typhimurium, Enteritidis and Typhi using multiplex PCR	*FEMS Microbiology Letters*
2010	Baré et al.	Influence of temperature, oxygen and bacterial strain identity on the association of *Campylobacter jejuni* with *Acanthamoeba castellanii.*	*FEMS Microbiology Ecology*
2010	Wassenaar	Virulotyping of foodborne pathogens. In Tracing pathogens in the food chain.	*In Tracing Pathogens in the Food Chain*
2010	De Haan et al.	Association of *Campylobacter jejuni* Cj0859c gene (fspA) variants with different *C. jejuni* multilocus sequence types.	*Applied and Environmental Microbiology*

(continued)

Table 1 (continued)

Year	Authors	Title	Journal
2010	D. Döpfer et al.	Pathogenic potential and horizontal gene transfer in ovine gastrointestinal *Escherichia coli.*	*Journal of Applied Microbiology*
2010	Habib et al.	Survival of poultry-derived *Campylobacter jejuni* of multilocus sequence type clonal complexes 21 and 45 under freeze, chill, oxidative, acid and heat stresses.	*Food Microbiology*
2010	Hotter et al.	Binary genomotyping using lipooligosaccharide biosynthesis genes distinguishes between *Campylobacter jejuni* isolates within poultry-associated multilocus sequence types.	*Epidemiology and Infection*
2010	Huehn et al.	Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe.	*Foodborne Pathogens and Disease*
2010	Litrup et al.	Association between phylogeny, virulence potential and serovars of *Salmonella enterica.*	*Infection, Genetics and Evolution*
2010	Mertes et al.	High-throughput universal probe *Salmonella* serotyping (UPSS) by nanoPCR.	*Journal of Microbiological Methods*
2010	Molla et al.	*Salmonella enterica* in commercial swine feed and subsequent isolation of phenotypically and genotypically related strains from fecal samples	*Applied and Environmental Microbiology*
2010	Moran	The role of endotoxin in infection*: Helicobacter pylori* and *Campylobacter jejuni.*	*Sub-Cellular Biochemistry*
2010	Nógrády et al.	Molecular and pathogenic characterization of *Salmonella enterica* serovar Bovismorbificans strains of animal, environmental, food, and human origin in Hungary	*Foodborne Pathogens and Disease*
2010	Tatavarthy and Cannons	Real-time PCR detection of *Salmonella* species using a novel target: The outer membrane porin F gene (ompF).	*Letters in Applied Microbiology*
2010	Thong et al.	Multiple-locus variable-number tandem repeat analysis of *Vibrio cholerae* in comparison with pulsed field gel electrophoresis and virulotyping.	*Journal of Biomedicine and Biotechnology*

(continued)

Table 1 (continued)

Year	Authors	Title	Journal
2010	Zautner et al.	*Campylobacter jejuni*-the search for virulence-associated factors.	*Archiv Fur Lebensmittelhygiene*
2011	Beutlich et al.	Antimicrobial resistance and virulence determinants in European *Salmonella* genomic island 1-positive *Salmonella enterica* isolates from different origins.	*Applied and Environmental Microbiology*
2011	Bhowmick et al.	Genotypic characterization of *Vibrio cholerae* isolates using several DNA fingerprint techniques.	*Future Microbiology*
2011a, b	Bugarel et al.	A multiplex real-time PCR assay targeting virulence and resistance genes in *Salmonella enterica* serotype Typhimurium.	*BMC Microbiology*
2011	Franklin et al.	Rapid genoserotyping tool for classification of *Salmonella* serovars.	*Journal of Clinical Microbiology*
2011	Gonzales et al.	A high-throughput open-array qPCR gene panel to identify, virulotype, and subtype O157 and non-O157 enterohemorrhagic *Escherichia coli*.	*Molecular and Cellular Probes*
2011	Hardy et al.	Prevalence of potentially neuropathic *Campylobacter jejuni* strains on commercial broiler chicken products.	*International Journal of Food Microbiology*
2011	Hauser et al.	Diversity of *Salmonella enterica* serovar Derby isolated from pig, pork and humans in Germany.	*International Journal of Food Microbiology*
2011	Hermans et al.	Colonization factors of *Campylobacter jejuni* in the chicken gut.	*Veterinary Research*
2011	Pui et al.	Multiplex PCR for the concurrent detection and differentiation of *Salmonella* spp., *Salmonella* Typhi and *Salmonella* Typhimurium.	*Tropical Medicine and Health*
2011	Salisbury et al.	*Salmonella virchow* isolates from human and avian origins in England - molecular characterization and infection of epithelial cells and poultry.	*Journal of Applied Microbiology*
2011	Schwaiger et al.	Comparative analysis of the bacterial flora of vegetables collected directly from farms and from supermarkets in Germany.	*International Journal of Environmental Health Research*

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Table 1 (continued)

Year	Authors	Title	Journal
2011	Senior et al.	*Galleria mellonella* as an infection model for *Campylobacter jejuni* virulence.	*Journal of Medical Microbiology*
2011	Smith et al.	Eavesdropping by bacteria: The role of SdiA in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium quorum sensing.	*Foodborne Pathogens and Disease*
2011	Teh et al.	Genetic variation analysis of *Vibrio cholerae* using multilocus sequencing typing and multi-virulence locus sequencing typing.	*Infection, Genetics and Evolution*
2011	Woodford et al.	Multiresistant Gram-negative bacteria: The role of high-risk clones in the dissemination of antibiotic resistance.	*FEMS Microbiology Reviews*
2011	Zautner et al.	Epidemiological association of different *Campylobacter jejuni* groups with metabolism-associated genetic markers.	*Applied and Environmental Microbiology*
2011	Zou et al.	Microarray analysis of virulence gene profiles in *Salmonella* serovars from food/food animal environment.	*Journal of Infection in Developing Countries*
2012	Asakura et al.	Molecular evidence for the thriving of *Campylobacter jejuni* ST-4526 in Japan	*PLoS ONE*
2012	Borriello et al.	Diversity of *Salmonella* spp. serovars isolated from the intestines of water buffalo calves with gastroenteritis.	*BMC Veterinary Research*
2012	Chen et al.	Comparison of the pulsed field gel electrophoresis patterns and virulence profiles of the multidrug resistant strains of *Salmonella enterica* serovar Schwarzengrund isolated from chicken meat and humans in Taiwan.	*Food Research International*
2012	Cheng et al.	Experimental techniques in network pharmacology.	*Chinese Journal of Pharmacology and Toxicology*
2012	de Haan et al.	Association of *Campylobacter jejuni* metabolic traits with multilocus sequence types	*Applied and Environmental Microbiology*
2012	de Toro et al.	High clonality and diversity of virulence determinants among blaPSE-positive *Salmonella* Typhimurim isolates recovered in three geographically distant Spanish hospitals.	*Diagnostic Microbiology and Infectious Disease*

(continued)

Table 1 (continued)

Year	Authors	Title	Journal
2012	Ennis et al.	The prevalence, distribution and characterization of Shiga toxin-producing *Escherichia coli* (STEC) serotypes and virulotypes from a cluster of bovine farms.	*Journal of Applied Microbiology*
2012	Gautam et al.	The strain-specific dynamics of *Escherichia coli* O157:H7 faecal shedding in cattle post inoculation.	*Journal of Biological Dynamics*
2012	Gironde and Manceau	Housekeeping gene sequencing and multilocus variable-number tandem-repeat analysis to identify subpopulations within *Pseudomonas syringae* pv. maculicola and *Pseudomonas syringae* pv. tomato that correlate with host specificity.	*Applied and Environmental Microbiology*
2012	Hauser et al.	Clonal dissemination of *Salmonella enterica* serovar Infantis in Germany.	*Foodborne Pathogens and Disease*
2012	Hermans et al.	A tolerogenic mucosal immune response leads to persistent *Campylobacter jejuni* colonization in the chicken gut.	*Critical Reviews in Microbiology*
2012	Kulow et al.	Differences in colonization and shedding patterns after oral challenge of cattle with three *Escherichia coli* O157:H7 strains.	*Applied and Environmental Microbiology*
2012	Lam et al.	Multi-locus variable number tandem repeat analysis of 7th pandemic *Vibrio cholerae*	*BMC Microbiology*
2012	Louwen et al.	*Campylobacter jejuni* translocation across intestinal epithelial cells is facilitated by ganglioside-like lipooligosaccharide structures.	*Infection and Immunity*
2012	Maistro et al.	Microbiological quality and safety of minimally processed vegetables marketed in Campinas, SP - Brazil, as assessed by traditional and alternative methods.	*Food Control*
2012	Mingle et al.	Enhanced identification and characterization of non-O157 Shiga toxin-producing *Escherichia coli*: A six-year study.	*Foodborne Pathogens and Disease*
2012	Monaghan et al.	Serotypes and virulotypes of non-O157 Shiga-toxin-producing *Escherichia coli* (STEC) on bovine hides and carcasses.	*Food Microbiology*

(continued)

Table 1 (continued)

Year	Authors	Title	Journal
2012	Osama et al.	Genome sequence and comparative genomics analysis of a *Vibrio cholerae* O1 strain isolated from a cholera patient in Malaysia.	*Journal of Bacteriology*
2012	Revez and Hänninen	Lipooligosaccharide locus classes are associated with certain *Campylobacter jejuni* multilocus sequence types.	*European Journal of Clinical Microbiology and Infectious Diseases*
2012	Sellek et al.	Phenotypic and genetic analyses of 111 clinical and environmental O1, O139, and non-O1/O139 *Vibrio cholerae* strains from different geographical areas.	*Epidemiology and Infection*
2012	Taly et al.	Detecting biomarkers with microdroplet technology.	*Trends in Molecular Medicine*
2012	Teh et al.	Outbreak-associated *Vibrio cholera* genotypes with identical pulsotypes, Malaysia, 2009.	*Emerging Infectious Diseases*
2012	Zautner et al.	Epidemiological association of *Campylobacter jejuni* groups with pathogenicity-associated genetic markers.	*BMC Microbiology*
2012	Zhang et al.	Establishment and comparison of pulsed-field gel electrophoresis, multiple-locus variable number tandem repeat analysis and automated ribotyping methods for subtyping of *Citrobacter* strains.	*Biomedical and Environmental Sciences*
2012	Zou et al.	Molecular characterization of *Salmonella enterica* serotype Enteritidis isolates from humans by antimicrobial resistance, virulence genes, and pulsed-field gel electrophoresis.	*Foodborne Pathogens and Disease*
2013	Akter et al.	Prevalence and distribution of different diarrhoeagenic *Escherichia coli* virulotypes in major water bodies in Bangladesh.	*Epidemiology and Infection*
2013	Boko et al.	Identification and typing of *Salmonella enterica* serotypes isolated from guinea fowl (*Numida meleagris*) farms in Benin during four laying seasons (2007 to 2010).	*Avian Pathology*
2013	Botti et al.	*Salmonella* spp. and antibiotic-resistant strains in wild mammals and birds in north-western Italy from 2002 to 2010.	*Veterinaria Italiana*

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Table 1 (continued)

Year	Authors	Title	Journal
2013	Capuano et al.	Characterization of drug resistance and virulotypes of *Salmonella* strains isolated from food and humans.	*Foodborne Pathogens and Disease*
2013	Croxen et al.	Recent advances in understanding enteric pathogenic *Escherichia coli*.	*Clinical Microbiology Reviews*
2013	Day and Tiralongo	Sialic acid recognition, removal and surface presentation: Role in microbial pathogenesis of human hosts.	*In Sialobiology: Structure, biosynthesis and function. Sialic acid glycoconjugates in health and disease*
2013	Elemfareji and Thong	Comparative virulotyping of *Salmonella* Typhi and *Salmonella* Enteritidis.	*Indian Journal of Microbiology*
2013	Ellström et al.	Characterization of clinical *Campylobacter jejuni* isolates with special emphasis on lipooligosaccharide locus class, putative virulence factors and host response.	*International Journal of Medical Microbiology*
2013	Fratamico and Gunther	Advances in genomics and proteomics-based methods for the study of foodborne bacterial pathogens.	*In Advances in Microbial Food Safety.*
2013	Fresno et al.	Identification of diverse *Salmonella* serotypes, virulotypes, and antimicrobial resistance phenotypes in waterfowl from Chile.	*Vector-Borne and Zoonotic Diseases*
2013	Frye and Jackson	Genetic mechanisms of antimicrobial resistance identified in *Salmonella enterica*, *Escherichia coli*, and *Enteroccocus* spp. isolated from U.S. food animals.	*Frontiers in Microbiology*
2013	Guyard-Nicodème et al.	Characterization of *Campylobacter* spp. transferred from naturally contaminated chicken legs to cooked chicken slices via a cutting board	*International Journal of Food Microbiology*
2013	Imre et al.	Gene expression analysis of *Salmonella enterica* SPI in macrophages indicates differences between serovars that induce systemic disease from those normally causing enteritis.	*Veterinary Microbiology*

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Table 1 (continued)

Year	Authors	Title	Journal
2013	Ioannidis et al.	Distribution of six effector protein virulence genes among *Salmonella enterica enterica* serovars isolated from children and their correlation with biofilm formation and antimicrobial resistance.	*Molecular Diagnosis and Therapy*
2013	Lefterova et al.	A sensitive multiplex, real-time PCR assay for prospective detection of Shiga toxin-producing *Escherichia coli* from stool samples reveals similar incidences but variable severities of non-O157 and O157 infections in northern California.	*Journal of Clinical Microbiology*
2013	Lian et al.	Lack of association between TYK2 and STAT3 genes and Crohn's disease in the Malaysian population.	*Genetics and Molecular Research*
2013	Louwen et al.	A novel link between *Campylobacter jejuni* bacteriophage defense, virulence and Guillain-Barré syndrome.	*European Journal of Clinical Microbiology and Infectious Diseases*
2013	Mand et al.	Growth and survival parameter estimates and relation to RpoS levels in serotype O157: H7 and non-O157 Shiga toxin-producing *Escherichia coli*.	*Journal of Applied Microbiology*
2013	Osman et al.	Antimicrobial resistance and virulence-associated genes of *Salmonella enterica* subsp. *enterica* serotypes Muenster, Florian, Omuna, and Noya strains isolated from clinically diarrheic humans in Egypt.	*Microbial Drug Resistance*
2013	Park et al.	Evolution of the stx2-encoding prophage in persistent bovine *Escherichia coli* O157: H7 strains.	*Applied and Environmental Microbiology*
2013	Parsons et al.	Infection of chickens with antimicrobial-resistant *Salmonella enterica* Typhimurium DT193 and monophasic *Salmonella* Typhimurium-like variants: An emerging risk to the poultry industry?	*Avian Pathology*
2013	Pires-dos-Santos et al.	Genetic diversity and virulence profiles of *Escherichia coli* causing salpingitis and peritonitis in broiler breeders.	*Veterinary Microbiology*

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Table 1 (continued)

Year	Authors	Title	Journal
2013	Ricke et al.	Application of microarray analysis of foodborne *Salmonella* in poultry production: A review.	*Poultry Science*
2013	Yue et al.	Emergence of the virulence plasmid in *Salmonella* Onarimon and *S.* Blegdam from yak.	*Veterinary Microbiology*
2013	Zacharczuk	Investigation of molecular virulence factors of *Yersinia enterocolitica* 1B/08 human clinical isolates collected in Poland in 2009	*Medycyna Doświadczalna I Mikrobiologia*
2013	Zhang et al.	Development of a LAMP assay for rapid detection of different intimin variants of attaching and effacing microbial pathogens.	*Journal of Medical Microbiology*
2013	Zottola et al.	Prevalence and antimicrobial susceptibility of *Salmonella* in European wild boar (*Sus scrofa*); latium region - Italy.	*Comparative Immunology, Microbiology and Infectious Diseases*
2014	Béla and Ama	Present and future significance of molecular methods in microbial food safety examinations.	*Magyar Allatorvosok Lapja*
2014	Benavides et al.	Limitations to estimating bacterial cross-species transmission using genetic and genomic markers: Inferences from simulation modeling.	*Evolutionary Applications*
2014	Cernela et al.	Antimicrobial resistance patterns and genotypes of *Salmonella enterica* serovar Hadar strains associated with human infections in Switzerland, 2005–2010.	*Epidemiology and Infection*
2014	Chiari et al.	Isolation and identification of *Salmonella* spp. from red foxes (*Vulpes vulpes*) and badgers (*Meles meles*) in northern Italy.	*Acta Veterinaria Scandinavica*
2014	De Toro et al.	Antibiotic resistance and virulence factors in clinical *Salmonella enterica* isolates.	*Enfermedades Infecciosas y Microbiologia Clinica*
2014	Ellström et al.	Lipooligosaccharide locus class of *Campylobacter jejuni*: Sialylation is not needed for invasive infection.	*Clinical Microbiology and Infection*
2014	Franz et al.	Exploiting the explosion of information associated with whole genome sequencing to tackle Shiga toxin-producing *Escherichia coli* (STEC) in global food production systems.	*International Journal of Food Microbiology*

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Table 1 (continued)

Year	Authors	Title	Journal
2014	Gomez et al.	Gene markers of generic *Escherichia coli* associated with colonization and persistence of *Escherichia coli* O157 in cattle.	*Preventive Veterinary Medicine*
2014	Gordillo et al.	Quantification of viable *Escherichia coli* O157:H7 in meat products by duplex real-time PCR assays.	*Meat Science*
2014	Grigorenko et al.	Multiplex screening for blood-borne viral, bacterial, and protozoan parasites using an open array platform.	*Journal of Molecular Diagnostics*
2014	Guedda et al.	Antimicrobial and molecular analysis of *Salmonella* serovar Livingstone strains isolated from humans in Tunisia and Belgium.	*Journal of Infection in Developing Countries*
2014	Hoffmann et al.	Comparative genomic analysis and virulence differences in closely related *Salmonella enterica* serotype Heidelberg isolates from humans, retail meats, and animals.	*Genome Biology and Evolution*
2014	Islam et al.	Comparative population structure analysis of *Campylobacter jejuni* from human and poultry origin in Bangladesh	*European Journal of Clinical Microbiology and Infectious Diseases*
2014	Jamali and Thong	Genotypic characterization and antimicrobial resistance of *Listeria monocytogenes* from ready-to-eat foods.	*Food Control*
2014	Law et al.	Rapid methods for the detection of foodborne bacterial pathogens: Principles, applications, advantages and limitations.	*Frontiers in Microbiology*
2014	Mahapatra et al.	Cholera outbreaks in South and Southeast Asia: Descriptive analysis, 2003–2012	*Japanese Journal of Infectious Diseases*
2014	Mancini et al.	First isolation of *Salmonella enterica* serovar Napoli from wild birds in Italy.	*Annali Dell'Istituto Superiore Di Sanita*
2014a	Osman et al.	The consequences of a sudden demographic change on the sero prevalence pattern, virulence genes, identification and characterisation of integron-mediated antibiotic resistance in the *Salmonella enterica* isolated from clinically diarrhoeic humans in Egypt.	*European Journal of Clinical Microbiology and Infectious Diseases*

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Table 1 (continued)

Year	Authors	Title	Journal
2014b	Osman et al.	*Salmonella enterica* in imported and domestic day-old turkey poults in Egypt: Repertoire of virulence genes and their antimicrobial resistance profiles.	*OIE Revue Scientifique Et Technique*
2014c	Osman et al.	*Salmonella enterica* serotypes isolated from squabs reveal multidrug resistance and a distinct pathogenicity gene repertoire.	*OIE Revue Scientifique Et Technique*
2014d	Osman et al.	Isolation and characterization of *Salmonella enterica* in day-old ducklings in Egypt.	*Pathogens and Global Health*
2014	Ranjbar et al.	The prevalence of virulence sodC1 and sopE1 genes among the clinical serotypes of *Salmonella enterica* in Tehran, Iran.	*Journal of Military Medicine*
2014	Robino et al.	Urinary tract infection in Uruguayan children: Aetiology, antimicrobial resistance and uropathogenic *Escherichia coli* virulotyping.	*Journal of Global Antimicrobial Resistance*
2014	Toboldt et al.	Molecular epidemiology of *Salmonella enterica* serovar Kottbus isolated in Germany from humans, food and animals.	*Veterinary Microbiology*
2015	Ben Hassena et al.	Real time PCR gene profiling and detection of *Salmonella* using a novel target: The siiA gene.	*Journal of Microbiological Methods*
2015	Bolton	*Campylobacter* virulence and survival factors.	*Food Microbiology*
2015	Figueiredo et al.	Virulence characterization of *Salmonella enterica* by a new microarray: Detection and evaluation of the cytolethal distending toxin gene activity in the unusual host *S.* Typhimurium.	*PLoS ONE*
2015	Gautam et al.	Transmission of *Escherichia coli* O157:H7 in cattle is influenced by the level of environmental contamination.	*Epidemiology and Infection*
2015	Gharieb et al.	Non-typhoidal *Salmonella* in poultry meat and diarrhoeic patients: Prevalence, antibiogram, virulotyping, molecular detection and sequencing of class I integrons in multidrug resistant strains.	*Gut Pathogens*

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Table 1 (continued)

Year	Authors	Title	Journal
2015	Graziani et al.	*Salmonella enterica* serovar Napoli infection in Italy from 2000 to 2013: Spatial and spatio-temporal analysis of cases distribution and the effect of human and animal density on the risk of infection.	*PLoS ONE*
2015	Guyard-Nicodème et al.	Prevalence and characterization of *Campylobacter jejuni* from chicken meat sold in French retail outlets.	*International Journal of Food Microbiology*
2015	Jamali et al.	Prevalence, antimicrobial susceptibility and virulotyping of *Listeria* species and *Listeria monocytogenes* isolated from open-air fish markets.	*BMC Microbiology*
2015	Kalia et al.	Genome wide analysis for rapid identification of *Vibrio* species.	*Indian Journal of Microbiology*
2015	Khoo et al.	Pathogenicity and phenotypic analysis of sopB, sopD and pipD virulence factors in *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Agona.	*Antonie Van Leeuwenhoek, International Journal of General and Molecular Microbiology*
2015	Kuang et al.	Antimicrobial susceptibility, virulence gene profiles and molecular subtypes of *Salmonella* Newport isolated from humans and other sources.	*Infection, Genetics and Evolution*
2015	Lajhar et al.	Comparison of epidemiologically linked *Campylobacter jejuni* isolated from human and poultry sources.	*Epidemiology and Infection*
2015	Law et al.	Rapid methods for the detection of foodborne bacterial pathogens: Principles, applications, advantages and limitations.	*Frontiers in Microbiology*
2015	Murgia et al.	Antibiotic resistance determinants and genetic analysis of *Salmonella enterica* isolated from food in Morocco.	*International Journal of Food Microbiology*
2015	Nasfi et al.	A European epidemiological survey of *Vibrio splendidus* clade shows unexplored diversity and massive exchange of virulence factors.	*World Journal of Microbiology and Biotechnology*
2015	Ngoi et al.	Overview of molecular typing tools for the characterization of *Salmonella enterica* in Malaysia.	*Biomedical and Environmental Sciences*

(continued)

Table 1 (continued)

Year	Authors	Title	Journal
2015	Nüesch-Inderbinen et al.	Characteristics of *Salmonella enterica* spp. *enterica* serotype Schwarzengrund associated with human infections in Switzerland: 2006–2010.	*Archiv Fur Lebensmittelhygiene*
2015	Oh et al.	High prevalence of hyper-aerotolerant *Campylobacter jejuni* in retail poultry with potential implication in human infection.	*Frontiers in Microbiology*
2015	Rahaman et al.	Molecular tools in understanding the evolution of *Vibrio cholerae*.	*Frontiers in Microbiology*
2015	Retamal et al.	Genetic and phenotypic evidence of the *Salmonella enterica* serotype Enteritidis human-animal interface in Chile.	*Frontiers in Microbiology*
2015	Ricke et al.	Application of molecular methods for traceability of foodborne pathogens in food safety systems.	*Food Safety: Emerging Issues, Technologies and Systems*
2015a	Yang et al.	Prevalence, antimicrobial resistance and genetic diversity of *Salmonella* isolated from retail ready-to-eat foods in China.	*Food Control*
2015b	Yang et al.	Prevalence, enumeration, and characterization of *Salmonella* isolated from aquatic food products from retail markets in China.	*Food Control*
2015c	Yang et al.	Prevalence and characterization of monophasic *Salmonella* serovar 1,4,[5],12:I:-of food origin in China.	*PLoS ONE*
2015	Ye et al.	Prevalence, antimicrobial resistance and genetic diversity of *Yersinia enterocolitica* isolated from retail frozen foods in China.	*FEMS Microbiology Letters*
2016	Bado et al.	CTX-M-15 in combination with aac(6′)-ib.-cr is the most prevalent mechanism of resistance both in *Escherichia coli* and *Klebsiella pneumoniae*, including *K. pneumoniae* ST258, in an ICU in Uruguay.	*Journal of Global Antimicrobial Resistance*
2016	Issa et al.	A 3-year long study of *Staphylococcus aureus* isolates from subclinical mastitis in three azawak zebu herds at the Sahelian experimental farm of Toukounous, Niger.	*Tropical Animal Health and Production*

(continued)

Table 1 (continued)

Year	Authors	Title	Journal
2016	Kot et al.	Virulence gene profiles in *Staphylococcus aureus* isolated from cows with subclinical mastitis in eastern Poland.	*Journal of Dairy*
2016	Pan et al.	Molecular characterization, antimicrobial resistance and caco-2 cell invasion potential of *Campylobacter jejuni/coli* from young children with diarrhea	*Pediatric Infectious Disease Journal*
2016	Sánchez del Rey et al.	Screening of virulence-associated genes as a molecular typing method for characterization of *Streptococcus suis* isolates recovered from wild boars and pigs.	*Veterinary Journal*
2016	Vignoli et al.	Extended-spectrum β-lactamases, transferable quinolone resistance, and virulotyping in extra-intestinal *E. coli* in Uruguay.	*Journal of Infection in Developing Countries*
2016	Volpe et al.	Development and evaluation of an ELIME assay to reveal the presence of *Salmonella* in irrigation water: Comparison with real-time PCR and the standard culture method.	*Talanta*

quality control testing, traditional methods will continue to be utilized. Nevertheless, in recent years, the USDA-FSIS has begun implementing the use of WGS for characterization of pathogen isolates, which is useful in determining virulence factors, assessing antimicrobial resistance, conducting foodborne-outbreak tracebacks, and studying the persistence and environmental harborage of pathogens in food processing facilities. A goal of the USDA-FSIS and other public health agencies is to eventually utilize WGS for characterization of all foodborne pathogen isolates (USDA-FSIS 2016a).

Virulotyping Enterohemorrhagic *E. coli*

All enterohemorrhagic *E. coli* (EHEC) represent pathogenic bacterial strains because they possess a cadre of well-characterized virulence factors. Some isolates that are assumed to be Shiga toxin-producing *E. coli* (STEC), based on their serogroup (e.g., O26, O103, O111, O121, O45, and O145) or serotype (O157:H7), occasionally lack the characteristic *stx* and/or *eae* genes, necessary to induce the pathological cascade in the colon and bloodstream that leads to hemorrhagic colitis and hemolytic uremic syndrome (HUS). It is to be noted, however, that isolates that

contain the *stx* and *eae* genes occasionally have deletions around the start codons that inhibit the bacteria from producing the toxins; hence, one could envision a day when these deletions could even be screened in addition to testing for the presence of *stx* and *eae*.

Bugarel et al. (2011a) described a method where virulotyping was used to distinguish "EHEC and EHEC-like *E. coli* O26 strains" which the authors state might have application to hospital service laboratories or public health laboratories to test strains isolated from stools of patients suffering from diarrhea. They reported that EHEC and EPEC O26 strains phenotypically resemble O26 EHEC-like and apathogenic *E. coli* O26 strains and are therefore indistinguishable by cultural methods. The authors identified several STEC-like O26 strains expressing all features of STEC except Stx production and carrying remnants of Stx phages that were probably derivatives of EHEC O26. They developed an assay for specific detection of EHEC and EHEC-like O26 strains, by high-throughput PCR for selection of discriminative genetic markers via the BioMark real-time PCR system (Fluidigm, San Francisco, CA). They detected the genes *ECs1822*, *nleH1-2*, *nleA*, *nleC*, *nleH1-1*, *nleG*, *nleG2*, *nleG6-1*, *nleG6-2*, *espJ*, *espM2*, *nleG8-2*, *espG*, *ent* (or *espL2*), *nleB*, *nleE*, *efa1*, and *espB* at different frequencies in O26 EHEC, EHEC-like, and EPEC strains, and concluded that O26 *wzx* (wzx_{O26}), *eae*-beta, *stx*, *espK*, and *arcA* genotyping is highly discriminative for clear identification of EHEC and EHEC-like *E. coli* O26 strains (Bugarel et al. 2011a, b).

Tseng et al. (2014) also utilized the Fluidigm system to examine the presence and absence of 69 virulence genes in STEC strains recovered from finishing swine. The authors found three main pathotypes with 16 combinations of virulence gene profiles, and serotypes with 9 virulence genes (i.e., stx_{2e}, *iha*, *ecs1763*, $lpfA_{O113}$, *estIa* (*STa*), *ehaA*, *paa*, *terE*, and *ureD*) characterizing the most common serotype O59:H21. They also discovered *eae*, *nleF*, and *nleH1-2* genes in serotype O49:H21. Other genes (149) were identified, including those encoding adhesins, such as *iha.* Their results demonstrated the differences in virulence genes among the members of the swine STEC population and enhanced understanding of the dynamics of transmission of STEC strains among pigs housed in the same barn (Tseng et al. 2014).

Another example of virulotyping was illustrated by Patel et al. (2016) in which the FDA-ECID (FDA *Escherichia coli* Identification) microarray, containing features that enable comprehensive molecular serotyping and virulence profiling along with genome-scale genotyping and SNP analysis was used for identification and virulence characterization of *E. coli.* The FDA-ECID is a molecular toolbox that stratifies strain identification and pathogenic potential in the contexts of epidemiology and phylogeny. The authors used the ECID to characterize strains from food, environmental, and clinical sources, resulting in significantly greater phylogenetic and strain-specific resolution than previously reported for available typing methods (Patel et al. 2016).

The USDA-FSIS regulates seven serogroups of STEC (aka, the "top seven"); namely, STEC serotype O157:H7 and the six most common non-O157 O-serogroups (O26, O103, O111, O121, O45, O145; aka, the "big six"). An isolate is considered a regulated

STEC by USDA-FSIS if it (1) Has one of the "top seven" serogroups associated with human illness and, (2) Contains the virulence genes *stx* and *eae*. Furthermore, USDA-FSIS classifies these "top seven" STEC as adulterants in selected beef products, as will be discussed later. The USDA-FSIS published a risk profile for non-O157 STEC that details much of the information used in the selection of the "big six" non-O157 STECs (i.e., *E. coli* O26, O103, O111, O121, O45, and O145) as adulterants, based on serogroup and the *stx* and *eae* virulence genes (USDA-FSIS 2012). This risk profile shows the considerations that USDA-FSIS took to distinguish strains with a high probability to cause illness from strains with a low probability to cause illness.

Although only isolates of the "top seven" STEC are currently USDA-FSIS regulated adulterants, 200 serotypes of *E. coli* have been reported to harbor the *stx* genes, and Nataro and Kaper (1998) reported that about 50 of these serotypes have been directly associated with hemorrhagic colitis in humans. Twelve years later, Mathusa et al. (2010) reported that there are more than 120 of STEC serotypes that can cause human illness. Still, others have reported that more than 470 serotypes of STEC have been isolated from humans, and over 435 STEC serotypes have been isolated from cattle (Gyles 2007), although not all of these are capable of eliciting illness in humans (Nataro and Kaper 1998). For another example, in one study Allen et al. (2013) analyzed clinical isolates of STEC and determined that O118, O165:NM, O8:H16;, O146:H21, and O73 all produced Shiga toxin. Kobayashi et al. (2013) screened 282 strains of *E. coli* of different serogroups for 17 major virulence genes and concluded that the following STEC serogroups may also be pathogenic for humans: O14, O16, O45, O63, O74, O119, O128, O165 and O untypeable.

Buvens et al. (2011) reported a 2007 outbreak of foodborne illness from EHEC serotype O145:H28 and O26:H11-contaminated ice cream, when five girls between 2 and 11 years of age developed HUS. The Belgian authors mentioned that few labs in their country searched for non-O157 STEC in outbreaks by use of *stx*1 and *stx*2 gene testing. Consequently, they surmised that the actual incidence of non-O157 STEC involved in outbreaks of foodborne illnesses is underestimated in their country. In their study, colony sweeps and individual colonies isolated from fecal samples were tested for the *stx* genes with the consensus primer pair MK1 and MK2 (Karch and Meyer 1989). The isolates were also tested for pathogenicity via screening for the presence of O-island 122 (OI-122). Virulence testing revealed that *E. coli* O145:H28 was positive for *stx*2, while O26:H11 was only positive for *stx*1. *E. coli* O145:H28 was confirmed to have a complete OI-122 (COI-122) while O26:H11 contained an incomplete OI-122. The authors concluded that O145:H28 was most likely the cause of the HUS, because it has been previously demonstrated that isolates with *stx*2 and COI-122 are more likely to cause severe disease and HUS, whereas isolates with only *stx*1 and an incomplete COI-122 are less likely to cause illness. Buvens et al. (2011) also mentioned that the CDC recommends testing for *stx* genes in stool while simultaneously detecting STEC by culture-based methods with selective-differential media (Gould et al. 2009).

Based on the information published by Mathusa et al. (2010), the authors concluded that, *"using the results of serotype screening alone could be misleading if it is assumed that all positive results represent pathogenic non-O157 STEC. If appropriate*

virulence factors are not targeted as part of food sample screenings, it will be difficult to know whether or not identified STEC strains are pathogenic." Scheutz (2007) substantiates this assertion, suggesting that classification of a virulent STEC should be based solely on its virulence profile (e.g., Stx production, *eae* presence, etc.) rather than inferring virulence based on serogroup classification, alone.

Traditional testing for putative EHEC involves serological analysis for the somatic (O) and flagellar (H) antigens of, for example, *E. coli* O157:H7. More recently; however, analyses include testing for the O-antigens of the "big six" non-O157, STEC (O26, O45, O103, O111, O121, and O145). These have been classified by the USDA-FSIS as adulterants in raw non-intact beef such as ground beef, mechanically tenderized beef, and raw intact beef intended for non-intact use such as beef manufacturing trim, unless further analyses confirms that the bacteria do not contain the *stx* and *eae* genes (USDA-FSIS 2013, 2016b). Hence, when further analyses are performed, isolates of these serogroups are only classified as adulterants if they contain both the *stx* and *eae* genes, as well as serogroup-specific genes, as defined in the latest version of the USDA-FSIS Microbiology Laboratory Guidebook (MLG). The MLG contains a: "*Flow Chart Specific for FSIS Laboratory Non-O157 Shiga Toxin-Producing Escherichia coli (STEC) Analysis*" (USDA 2014c) which outlines that on day 5 of testing, any "big six"-positive colonies that test negative for these two virulence genes by BAX (DuPont Qualicon, Wilmington, DE)-PCR are not considered adulterants (Fig. 1).

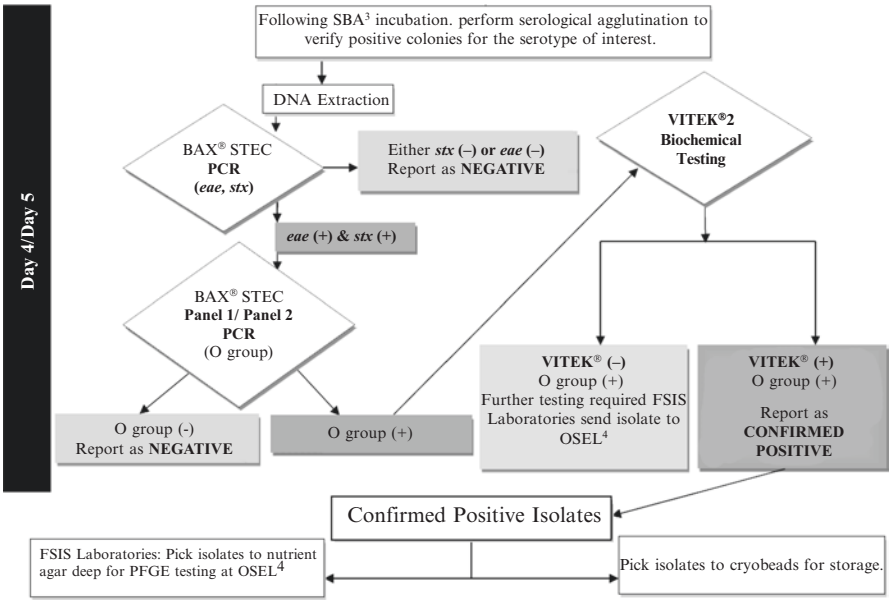

Fig. 1 USDA, FSIS Microbiology Laboratory Guidebook (*MLG*) *Flow Chart Specific for FSIS Laboratory non-O157 Shiga Toxin-Producing Escherichia coli (STEC) Analysis*, which outlines that on Day-5 testing, any putative "big six"-positive colonies that test negative for these two virulence genes by BAX-PCR are not considered adulterants

At present, approximately 50% of STEC infections in Europe are caused by non-O157 STEC (Anonymous 2011) and there is a higher number of non-O157 STEC infections in the United States each year, compared to O157:H7 infections (Bosilevac and Koohmaraie 2011). However, some isolates of the six most common serogroups are occasionally known to contain neither the *stx1* or *stx2* genes; thus, they are unable to secrete the verotoxins (cytotoxins), which induce bloody diarrhea, HUS and kidney failure. For this reason, the USDA-FSIS virulotypes putative adulterant STEC isolates for the *stx* and *eae* genes, to determine pathogenic potential, prior to confirming that an isolate is one of the seven regulated serogroups. Van Kessel et al. (2011) analyzed 536 bulk tank milk samples and 519 in-line milk filters, testing for the *E. coli stx*1 and *stx*2 genes, generic intimin (*eaeA* gene) and the γ-allele of the translocated intimin receptor (γ-*tir*), rather than testing for the specific *E. coli* serogroups. Fifteen percent of the *E. coli* isolates from the bulk tank milk samples were positive for an *stx* gene and 16% positive for the *eaeA* gene; however, 51% of isolates from in-line milk filters were positive for an *stx* gene and 65% positive for the *eaeA* gene (both of which are important for enterohemorrhagic illness). It should be noted, however, that some *E. coli*, which are positive for the *eaeA* gene but negative for an *stx* gene (enteropathogenic *E. coli*) have been associated with infantile diarrheal illness, but not hemorrhagic colitis or HUS. In Van Kessel's study, only 1.1% of bulk tank milk and 6.3% of milk filters were positive for *stx*2*, eaeA,* and γ-*tir,* a combination which is suggestive of the presence of *E. coli* O157:H7 (Van Kessel et al. 2011). The conclusion of the study was that much more useful information could be gleaned by baseline food testing for EHEC through virulence gene screening, rather than by targeting specified serogroups, which may or may not contain *stx* or attachment-associated virulence genes.

Molecular methods have been developed to enable the virulotyping of *E. coli* strains in food or clinical samples. For example, Oh et al. (2014) developed a multiplex PCR assay with nine primers to detect five diarrheagenic *E. coli* types. Detectable virulotypes include the genes *stx*1 and *stx*2 for EHEC, *lt*, *st*h and *st*p for ETEC (enterotoxigenic *E. coli*), *eaeA* and *bfpA* for EPEC (enteropathogenic *E. coli*), *aggR* for EAEC (enteroaggregative *E. coli*), and *ipaH* for EIEC (enteroinvasive *E. coli*). The authors validated their method on 150 *E. coli* isolates with no false-positive results (100% specificity). Hence, results from this PCR-based virulotyping assay would provide more useful information in terms of pathogenicity than testing only for specified STEC serogroups.

Further analysis of virulence gene profiles could provide even more refined and useful information, as the USDA-FSIS will soon be doing with WGS for all pathogen isolates. For example, Karmali et al. (2003) recommended subdividing the STEC into five seropathotypes, A – E, which the USDA-FSIS took into consideration when developing the regulatory definition of adulterant STEC. The final seropathotype (i.e., "E") would represent STEC serogroups not implicated in human illness. This categorization is based on the fact that Stx2 is known to be 1000 times more lethal to human kidney microvascular endothelial cells than is Stx1. Therefore, Stx1 is infrequently implicated in cases of HUS, and STEC strains carrying *stx*2 only are far more commonly implicated in HUS than are isolates bearing both *stx*1

or *stx*1 and *stx*2 (Gyles 2007). Nevertheless, not all Stx2-positive strains are of equal virulence. For example, the most common subtypes of the *stx*2 gene associated with hemorrhagic colitis and HUS are *stx*2a, *stx*2c, and *stx*2dact (now more commonly referred to as *stx*2d). Shen et al. (2015) determined that *stx*2 subtypes b, g, and e had lower cytotoxicity scores than subtypes a, c, and d, with subtypes b and g showing negligible cytoxicity scores, similar to that of nonpathogenic, biosafety level 1, *E. coli* K12. These findings are consistent with results that others have published (Prager et al. 2011; Zhang et al. 2005). Thus, a virulotyping method that would allow discrimination among *stx* subtypes will provide information on potential virulence of the isolates.

Perelle et al. (2006) obtained 88 *E. coli* isolates of the "top seven" serogroups from raw foods, and 16% (or 14 of the 88 isolates) of these presumptive STEC did not harbor an *stx* gene. Pradel et al. (2000) screened 220 presumptive STEC isolates from food and clinical sources and determined that 32% of them did not harbor *stx*1 or *stx*2. Further, only 5% of the isolates carried the *eae* gene. Citing Scheutz (2007), Mathusa et al. stated (2010) that *"Researchers have suggested that the pathogenicity of a non-O157 STEC strain may depend on the individual organism's virulence profile rather than simply on its serotype, co-classification of certain non-O157 STEC strains as adulterants based on serotype alone should be carefully considered."* For this reason, the USDA-FSIS tests for the virulence genes (*eae* and *stx*1 and *stx*2) in addition to serogroup. Nonetheless, *stx*1/*stx*2-containing STEC that are not one of the seven USDA-FSIS regulated serogroups are currently not considered adulterants by USDA-FSIS.

In one study, the U.S. CDC determined that the "big six" *E. coli* serogroups were responsible for more than 70% of all non-O157 STEC illnesses in the U.S. (Bosilevac and Koohmaraie 2011; Brooks et al. 2005). CDC FoodNet data, revealed that from 2000 to 2007, the "big six" STEC were responsible for 82% of all non-O157 STECs isolated from human illnesses (Gould 2009). Hence, 18% of all non-O157 STECs that were isolated from patients were not within the USDA-FSIS adulterant STEC definition. Currently, in their Microbiological Testing Program for *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *Escherichia coli* (STEC) (USDA-FSIS 2014a), the USDA-FSIS does not list the number of non-O157 and non-"big six" STEC isolates identified that carry the *stx*2 and *eae* genes. Nevertheless, if human illnesses are associated with a particular product that contains a specific pathogen (even an STEC that is not one of the seven considered adulterants) the agency can declare the product adulterated in that instance, by the virtue of having caused illnesses.

Monaghan et al. (2011) analyzed bovine fecal samples and crop soil samples and determined that 40% (480/1200) and 27% (162/600), respectively, contained non-O157 STEC (i.e., *stx*1 and/or *stx*2 positive by PCR). In this study, 107 non-O157 STEC isolates were collected from 27 STEC-positive samples (23 fecal and 4 soil samples). Only 15 of the 107 STEC (14%) were "big six" serogroups. Interestingly, only 19 of the 107 isolates (18%) were positive for *eae* by PCR, and 4 of these (21% of eae^+ STEC, [4/19]; or <4% of the STEC isolates [4/107]) were not "big six" serogroups. That is to say, 79% of stx^+/eae^+ isolates identified in this study would be

considered adulterants by USDA-FSIS if they were isolated from foods. The most common serotype (29%) isolated by Monaghan et al. (2011) was O113:H4. The gene *stx*1 was found in twenty-eight of the thirty-one (90%) O113:H4 strains (24 fecal and 4 soil isolates), whereas *stx*2d was found in all O113:H4 isolates (27 fecal and 4 soil isolates). The CDC, however, reported that in 2012, serogroup O113 only accounted for 0.1% of STEC illnesses in the U.S. (CDC 2014a). The dramatic difference in incidence of serogroup O113 isolates by Monaghan et al. (2011) and CDC may be attributable to the sources from which these isolates were obtained, bovine feces/soil and human clinical isolates, respectively. Interestingly, none of the STEC O113:H4 strains isolated by Monaghan et al. (2011) were positive for *eae*, which encodes the attaching and effacing protein, intimin, which is important for virulence.

The primary virulence factors responsible for producing serious illness in humans are Shiga toxins 2a, 2c, and 2d and the intimin (*eae*) outer membrane protein. Shiga toxin 2a (not Stx1) is the most important variant associated with strains causing HUS; in addition, there are two other *stx*2 types of relevance to human HUS (*stx*2c and *stx*2d), but *stx*1 is generally not associated with HUS. The USDA-FSIS testing involves use of the BAX system to screen for *stx* and *eae* genes while also testing for O-group-specific genes belonging to one of the "big six" STEC serogroups (USDA-FSIS 2014a, b, c).

Scallan et al. (2011) reported that there are an estimated 63,153 annual cases of human illness in the United States associated with STEC O157:H7; however, there is an estimated 79% higher number of non-O157 STEC cases each year (n = 112,752). The CDC reported that 5.9% of all laboratory-confirmed STEC illnesses reported to them in 2012 were neither O157 nor one of the "big six" STECs (CDC 2014a). If this is extrapolated to the total yearly estimate of 175,905 cases of STEC illnesses, it suggests that up to 10,378 cases of non-O157 or non-"big six" illnesses may occur per year in the United States (Scallan et al. 2011; CDC 2014a). These latter cases were associated with at least 110 serotypes of STEC other than O157 and the "big six" between the years 2002 and 2012(CDC 2014b). Further, the CDC reported that between 2002 and 2012, numerous non-O157, non-"big six" STEC serogroups caused illness, including STEC of O118 (154 illnesses), O69 (90 illnesses), O91 (75 illnesses), and O76 (48 illnesses) (CDC 2014b). Nevertheless, whereas 93.3% of all STEC illnesses in 2012 were associated with either serogroup O157 or one of the "big six," the next most prevalent serogroup in the CDC report (O118) only accounted for 0.8% of laboratory-confirmed illnesses (CDC 2014a).

Kappeli et al. (2011) reported that 13 non-O157:H7 STEC were associated with hemorrhagic colitis and HUS in patients in Switzerland, and that none of these was of the "big six" STEC. These *E. coli* included O91:H21/H–, O113:H21, O128:H2/H–, O20, O146, O148, O174, O117:H7, O20, O82, O148, O153, and O181. Further, Momtaz et al. (2013) isolated non-O157 STEC from patients in Iran that included O91, O113, and O128, similar to the findings of Kappeli et al. (2011). A study of STEC in beef burgers by Mohammed et al. (2014) revealed the presence of O55:H7, O126:H5, and O128:H2. Further, the World Health Organization (WHO) has warned that *E. coli* O55 and O128 are of particular concern, being capable of producing severe hemorrhagic colitis and HUS (Trabulsi et al. 2002).

Several single-state STEC outbreaks associated with *E. coli* strains not considered to be adulterants have also been reported. An outbreak of EHEC O84 infection occurred in a Colorado prison in 2007, sickening a confirmed 135 inmates (CDC 2007). An outbreak of EHEC O84:NM infection occurred in an Oklahoma jail in November 2010, with 21 confirmed illnesses (CDC 2010). Other outbreaks associated with STEC in the U.S. that were neither O157 nor members of the "big six" serogroups (and thus not considered adulterants) included the following (state, year and number of illnesses, respectively, given in parenthesis): O6:H16 (California, 2010–19), O6 (Illinois, 1998–916), O27 (Oregon, 2002–49) (CDC 2010). Luna-Gierke et al. (2014) reported on many U.S. outbreaks of EHEC infections, including those associated with serotypes O165:NM, O-undetermined, O69:H11, O84:H2, O141:H49, O104:H21, OUnd.:H8, and others.

Virulotyping *Salmonella* Serovars

Many serovars of *S. enterica* that are isolated from foods, and potentially result in recalls, may possibly be less pathogenic to humans than typical outbreak strains (Foley and Lynne 2008). Specifically, five *Salmonella* pathogenicity islands (SPI) have been identified in *S.* Typhimurium, whereas only two, SPI-1 and SPI-2, have been associated with causing human illness. These findings demonstrate that it is possible that some SPI-associated genes may elicit illness in humans (Gerlach and Hensel 2007; Rychlik et al. 2009). This suggests that there may be a difference in the degree of virulence of salmonellae, which may provide an explanation as to why there is a predilection for certain *Salmonella* serovars to predominate in foodborne illness outbreaks (viz., Enteritidis, Typhimurium, Heidelberg, Newport, Javiana and serovar I 4,[5],12:i:-), while other serovars are not commonly associated with widespread outbreaks (Graziani et al. 2015). Nevertheless, nonpathogenic salmonellae serovars have yet to be identified and classified, thus the USDA-FSIS considers all *Salmonella* serovars to be pathogenic to humans. It should be noted that while USDA-FSIS considers all *Salmonella* serovars to be potentially pathogenic, they are not considered adulterants unless they have been associated with human illness derived from eating a specific product (i.e., *Salmonella* associated with a specific outbreak can be considered adulterants in those cases).

Huehn et al. (2010) analyzed 523 *S. enterica* isolates of the five most commonly reported serovars in Europe, namely, Enteritidis, Typhimurium, Infantis, Virchow and Hadar. The bacteria were screened for ten common virulence genes using PCR (viz., *avrA, ssaZ, mgtC, siiD* (*spi4*_D), *sopB, gipA, sodC1, sopE1, spvC, and bcfC*). Despite the large number of strains tested, only 14 total virulotypes were detected. The greatest variation in virulence genes were in those present on either a prophage or a plasmid, such as the *spvC* and *sodC1* genes. A subset of 77 isolates were then subjected to microarray analysis, which revealed that *S.* Typhimurium had the greatest diversity in virulotypes, and *S.* Infantis had the least. The virulence gene *msgA* is essential to *Salmonella* virulence in mice and was detected in all *S.* Typhimurium

but not in other *Salmonella* serovars, which may be why Typhimurium is frequently implicated in foodborne illness. Some have proposed that virulotyping may be an alternate approach to distinguishing salmonellae of public health concern, although much more work is needed in this area (Elemfareji and Thong 2013; Galanis et al. 2006; Huehn et al. 2010; Khoo et al. 2009).

Elemfareji and Thong (2013) tested 94 *S.* Enteritidis isolates by multiplex PCR (followed by REP-PCR) for the presence of 22 virulence genes. Fifteen genes (*agfA, agfC, invA, lpfA, lpfC, sefD, prgH, spiC, sopB, sopE, iroN, sitC, misL, pipD, and orfL*) were detected in 100% of the isolates. Other virulence genes were detected in 98.9% (*sifA* and *spvC*), 97.8% (*pefA, spvB and mgtC*), and 90.4% (*sefC*) of the isolates. The virulence gene *cdtB* was not detected in any of the *S*. Enteritidis isolates. The authors concluded that the differences in virulence factors might contribute to host adaptation and diversity in *Salmonella* pathogenesis. The study also revealed that neither virulotyping by multiplex PCR nor REP-PCR differentiated *S.* Enteritidis strains either geographically or temporally, which may be a function of the highly clonal nature of the strains that lack genetic diversity. Nevertheless, in the future, use of a broader cross-section of virulence genes in typing schemes may provide a more accurate characterization of pathogenic strains of *S. enterica*. To date, this differentiation is not possible; hence, all salmonellae are considered pathogenic.

More Clinical Benefits to Virulotyping

Ethelberg et al. (2004), in addressing virulence factors associated with HUS in Denmark, concluded that in comparison to testing for O-antigens of *E. coli,* STEC isolates that tested positive for *stx*2 and *eae* (intimin) were much more predictive of the illness. STEC obtained from 343 patients belonged to 74 serotypes, including 49 different O-groups. They also determined that, based on multivariate analysis, the virulence factors *stx*1 and *ehxA* (hemolysin A) were not associated with an increase in the odds of contracting either HUS or bloody diarrhea. They reported that 2 of 23 HUS patients had co-infections with more than one strain of STEC, potentially indicating that the presence of multiple STEC isolates working synergistically to induce HUS. The investigators concluded that testing for the presence of virulence factors is more predictive of HUS than simply testing for specific STEC O-groups.

Another benefit of virulotyping over traditional bacterial characterization methodologies is to determine if virulence factors (specifically *stx*) are present or absent before administering antibiotics, which are generally contraindicated in cases of STEC/EHEC infections. Treatment with many (but not all) antibiotics is discouraged in cases of STEC, as they induce the so-called "SOS response" (Nassar et al. 2013) in EHEC, which induces the *stx* prophage to move into the lytic phase and produce exorbitant amounts of Stx, which may induce HUS. The antibiotics of the quinolones, sulfonamides, macrolides (e.g., azithromycin), aminoglycosides (e.g., gentamicin), and beta-lactam classes are often contraindicated for EHEC infections as they increase Stx production, which can illicit the above-described pathological

cascade and increase the likelihood of developing HUS (Nassar et al. 2013; Operario et al. 2014; McGannon et al. 2010; Panos et al. 2006; Rocha and Piazza 2007; Smith et al. 2012; Wong et al. 2000, 2012; Zangari et al. 2014). In unpublished ongoing studies, we have determined that both levofloxacin and metronidazole are capable of increasing the production of Stx in select outbreak strains of *E. coli* O157:H7. Although there are some conflicting reports regarding the amount of Stx produced in the presence of different antibiotics, the fact that several studies reveal that treatment with these antibiotics results in production of increased amounts of Stx is evidence that their administration in cases of EHEC is counter indicated. For example, in a 2013 clinical case, a woman with STEC/EHEC was misdiagnosed with a false-positive *Campylobacter jejuni* infection by a relatively inexpensive, common and cost-effective (though somewhat inaccurate) serological, lateral flow, immunoassay test. This prompted the physicians to administer a fluoroquinolone, which putatively induced the SOS cascade of Stx production, leading to the development of severe HUS and hospitalization for a month (Operario et al. 2014).

Conclusion

In many respects, virulotyping can be considered a superior method for disease diagnostics and food safety testing for particular bacterial pathogens, compared to traditional methods. Virulotyping is defined as a technique that identifies specific virulence genes within the pathogen's genome, thereby having the discriminatory power to differentiate between a virulent and an avirulent (nonpathogenic) strain of bacterium within an otherwise indistinguishable species. The advantages of virulotyping over traditional serology-based, PCR-based, or enzymatic and biochemical-based analyses of *Salmonella* and EHEC in clinical diagnostics and food industry microbiology testing have been addressed. Traditional identification and characterization methods are designed to detect a given genus, species, serovar or genetic variant of bacterium by its enzymatic and biochemical characteristics, its serology (O, H, or K serotyping), and/or its unique genes, but they do not necessarily address the virulence of a putative pathogen. While PCR and microarray analyses are commonly used in virulotyping, DNA sequencing, which is a more robust and discriminatory virulotyping technique, could be applied to food safety testing and clinical diagnostics, once more facile data acquisition and analysis methods are developed. Using bioinformatics tools, the presence of a wide array of relevant genes can be determined in the isolate. Applying a virulotyping-based testing system for analysis of potential pathogens from foods in the United States may prevent illnesses by targeting virulence genes in isolates that may otherwise be considered non-pathogenic. While there are an estimated 175,905 cases of STEC illnesses in the U.S. annually, the U.S. CDC determined that 5.9% of all laboratory-confirmed STEC illnesses reported to them were neither *E. coli* O157:H7, nor one of the "big six" STEC. Hence, an estimated 10,378 cases of non-O157:H7 or non-"big six" STEC illnesses may occur annually in the U.S. While this is just one example of potential advantages of applying virulotyping

to food industry testing programs and clinical-laboratory-based diagnostics, additional benefits may some day be gained by virulotyping other foodborne pathogens such as *Campylobacter* spp., *Salmonella enterica,* and *Listeria monocytogenes*, should avirulent subgroups of these bacteria one day be identified.

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Varying Pathogenicity of *Campylobacter jejuni* Isolates

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Abstract *Campylobacter jejuni* and *C. coli* are typical zoonotic pathogens that are leading causes of bacterial gastroenteritis in humans worldwide. The *C. jejuni* genome has a high plasticity and the molecular basis for the commensalism in some animals and the expression of virulence in humans is not well understood. *C. jejuni* colonizes the distal ileum and colon in humans, and ingestion of a few hundred live cells can infect humans. Adherence of *C. jejuni* to the target cells involves various adhesion factors, and the flagellar export machinery is a crucial secretory device to help *C. jejuni* entering the host cell. Although the intracellular survival of *C. jejuni* in non-phagocytic cells has been well documented, there are conflicting reports on the persistence capacity of *C. jejuni* in phagocytic immune cells. Several animal model systems have been used to study different aspects of this pathogen's invasion and pathogenicity phases. One of these models shows that the host-specific composition of microbiota is an important determinant in the occurrence of related disease, which complicates even more the study of the interaction of *C. jejuni* with mammalian host cells. The infant mice model shows promise to be a model system that closely mimics what happens in human cells after invasion by *C. jejuni*.

Keywords *Campylobacter Jejuni* • Adhesion • Animal model • Epidemiology • Invasion • Pathogenicity

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Introduction

The genus *Campylobacter* is in the Family *Campylobacteraceae*, which in turn belongs to the Order Campylobacterales and Class Epsilonproteobacteria. The genus *Campylobacter* comprises a group of bacteria with about 30 taxa with a mol% G + C content of the DNA of 27–31. These bacteria have a minimal growth temperature of 30 °C and require microaerobic environments, in which the oxygen concentration is between 5% and 10% (Lastovical et al. 2014). Four *Campylobacter* species, *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis,* can grow at 42 °C and are called "thermotolerant" species. The 42 °C temperature has been extensively used for isolation purposes, especially for the isolation of single *C. jejuni* and *C. coli* colonies from food and clinical samples.

The term "microaerobic" refers to oxygen concentration between 3% and 15%. The usual atmosphere for isolation contains around 5% oxygen, 10% carbon dioxide and 85% nitrogen, which is an inert gas. In general, all *Campylobacter* species need higher concentrations of carbon dioxide (ca. 10%) than the small amount in ambient air (0.04% or 400 parts per million). However, some *Campylobacter* species are "capnophilic" because they require 35% carbon dioxide to grow. Some species require hydrogen or formate with fumarate to grow under microaerobic conditions. If not available, anaerobiosis becomes an optimal growth condition for these species (On 2001; Vandamme et al. 2005).

Campylobacter species are chemoorganotrophs and they do not ferment or oxidize carbohydrates. These bacteria require nutrient-rich culture media under controlled laboratory conditions and acquire energy from amino acids or intermediates of the tricarboxylic acid cycle (Stingl et al. 2012). In contrast to *Salmonella enterica* and related pathogens, the metabolic potential of *C. jejuni* is highly restricted because various genes encoding common pathways for utilization of carbohydrates are either incomplete or entirely missing (Hofreuter 2014). Therefore, the lack of carbohydrate utilization is a limitation for identification purposes, and the test media used to identify other enteric bacteria based on carbohydrate fermentation are not reliable for the identification of *Campylobacter* species (Miller et al. 2010). *Campylobacters* are typically highly motile, with a characteristic corkscrew-like behavior by means of a single polar unsheathed flagellum at one or both ends of the cell. This particular type of motility is an important feature for detection using phase contrast microscopy during the isolation steps from clinical or food samples.

Disease outcome in humans varies substantially from mild, non-inflammatory, self-limiting diarrhea to severe, inflammatory, bloody diarrhea and abdominal pain lasting for several weeks. In some patients, *C. jejuni* is also associated with more severe sequelae such as the development of reactive arthritis and peripheral neuropathies, the Miller-Fisher and Guillain-Barrè syndromes (Nachamkin et al. 2008).

This chapter summarizes the key epidemiologic events related to human campylobacteriosis, and then focuses on the molecular mechanisms related to the virulence, primarily of *C. jejuni*, in mammalian cells. The emphasis is on *C. jejuni* because of its importance in public health and its significance as a foodborne pathogen.

Epidemiology and Transmission Routes of *C. jejuni*

The work of Elizabeth O. King in the 1950s, working at what is now known as the Centers for Disease Control and Prevention in Atlanta, GA, USA, brought attention to the importance of "related vibrios" as the etiological agents of foodborne diseases (King 1957). These "related vibrios" were different from *Vibrio fetus* (now *Campylobacter fetus* subspecies *fetus*), a group of bacteria well-known in veterinary medicine since 1918 (Smith 1918). These "related vibrios" were narrowed down to *Vibrio jejuni*, now called *Campylobacter jejuni*, a group of bacteria that were already implicated in dysentery in cattle (Jones et al. 1931) and suspected in the case of human campylobacteriosis 53 years ago (King 1962). But it was not until the early 1970s, when the isolation of *Campylobacter* spp. was made easier by the work of Jean-Paul Butzler, that the testing for *Campylobacter* spp. in stool specimens was incorporated in clinical laboratories.

During the last decade, *C. jejuni* has been recognized as a leading cause of bacterial gastroenteritis worldwide (Young et al. 2007; Nachamkin et al. 2008), with an annual incidence that varies substantially among different countries. For instance, in the USA, the latest data from the Centers for Disease Control and Prevention reveals an incident rate of 14 cases of campylobacteriosis per 100,000 population (Crim et al. 2015). In Canada, the incidence is approximately 30–35 cases per 100,000 population, whereas the incidence in Australia is approximately 110 cases per 100,000 population (Anonymous 2012) and in New Zealand is 160 cases every 100,000 population (Sears et al. 2011).

In developing countries, infections are endemic and this disease disproportionately affects young children. Non-*C. jejuni/coli* infections may be of similar or greater importance than *C. jejuni/C. coli* infections in several developing countries (Platts-Mills et al. 2014). In developed countries, infections have seasonal variations and more cases occur in late summer/early fall. The reason for this seasonal pattern is still not fully understood. Asymptomatic cases in adults and children are also common in both developing and developed countries. Most cases are sporadic and outbreaks only account for a small number of all annual cases.

Campylobacter infections in humans are primarily associated with handling raw poultry and meat, and the consumption of undercooked poultry, mainly chickens, or raw milk. *C. jejuni* can colonize the intestine of different host animals as a commensal resident. In general, *C. jejuni* inhabits the gut of a large variety of domestic and wild birds, as well as other food animals as a commensal. Therefore, handling and consumption of contaminated poultry and other meat products, raw milk and untreated drinking water have been recognized as common origins of *C. jejuni* infection of humans (reviewed by Oyarzabal and Backert 2011). The high prevalence of*Campylobacter* on chicken carcasses and the potential cross-contamination that can easily occur during food preparation make chicken meat an important vehicle of transmission. Recent studies have revealed that kitchen hygiene continues to be underestimated as the point of pathogen contamination, even in individuals that have suffered from campylobacteriosis (Millman et al. 2014).

The acquisition of *Campylobacter* enteritis, however, is complex and exposure is not the only factor in the development of disease. Workers in poultry processing facilities have higher titers of *Campylobacter*-specific IgG antibodies than the rest of the population and rarely become ill by *C. jejuni* infection (Cawthraw et al. 2000). However, a recent report evaluating the transmission of *Campylobacter* from slaughtered chickens to the workers in chicken processing plants revealed a complex picture. Asymptomatic *Campylobacter* infections occur even in individuals with only limited earlier exposure to *Campylobacter*, further complicating the study of disease development (Ellström et al. 2014).

In addition to poultry, red meats and raw milk, the consumption of raw vegetables, shellfish and contact with domestic animals are also known vehicles of transmission. An important source of *Campylobacter* spp. in the USA is the consumption of raw milk, which has increased in the last decade in some states. In the states where consumption of raw milk is allowed, there has been a higher incidence of bacterial foodborne diseases transmitted by milk, with campylobacteriosis being one of them (Langer et al. 2012).

Strain Variability

C. jejuni has a high plasticity in its genome, which is expressed in a large diversity of strains as verified with various DNA typing methods. However, it appears that, despite this diversity, the host environment and the associations that *C. jejuni* creates with different hosts are important driving forces in the evolution of this pathogen (Read et al. 2013). The ecological factors that affect this type of evolutionary pattern are not well understood, but distant host animals can harbor closely related strains (Sheppard et al. 2011).

Birds, especially domestic chickens, are an important reservoir of both *C. jejuni* and *C. coli*. The intestine of chickens is colonized by large numbers of *C. jejuni*/*C. coli*, with counts of 5-log colony forming units (CFU) per g of fecal material, or more, commonly occurring in chickens at the time of processing (Potturi-Venkata et al. 2007). The large variability in the potential for colonization by *C. jejuni* strains of chicken ceca has been recognized for many years, and several studies have tried to identify the genetic mechanisms for these differences to enable the development of vaccines or interventions to control the colonization of the chicken ceca by *C. jejuni*. Recently, studies with peptidoglycan (PG) hydrolase have revealed the importance of these enzymes in the modulation of cell shape and changes in the pathogenic attributes of *C. jejuni*. DL-carboxypeptidase PGP1 breaks down monomeric PG tripeptides to dipeptides, and *C. jejuni* mutants that are deficient in PGP1 exhibit rod-shaped cells, instead of the typical helical or spiral shape. These mutants also exhibit less motility and biofilm formation, a reduction in the colonization of chickens *in vivo* and induce an increased secretion of the pro-inflammatory chemokine IL-8 in epithelial cell infections (Frirdich et al. 2012; Frirdich and Gaynor 2013).

The genomic variability of*C. jejuni* also accounts for the wide range of phenotypic behavior exhibited by this organism. In a recent study (Thibodeau et al. 2015), scientists grouped strains of *Campylobacter* according to their colonization potential (agglutination, chemotaxis and adhesion and invasion of chicken cecal cells) and then molecularly typed these same strains using comparative genomic fingerprinting (Taboada et al. 2012). By comparing the gene content of the strains, some strains had different phenotypical scores and exhibited different outcompeting capacities in chicken colonization trials. Those strains that were able to outcompete others were unable to be predicted by any scoring system based on comparative genomic fingerprinting. These differences in genetic variability determined by molecular typing methods and the phenotypical behavior of *C. jejuni* illustrates the need for having enhanced methodological tools for the study of the complex mechanism by which this pathogen invades mammalian cells and produces disease.

This strain variability also can influence the efficacy of intervention strategies. Vaccination of commercial chickens has been studied as an intervention option with practical application to prevent or reduce the colonization of *C. jejuni*/*C. coli*. Studies with mice almost 30 years ago revealed that passive immunization with antibodies against *Campylobacter* flagella can diminish *C. jejuni* infection (Ueki et al. 1987), and a recent study suggests that single-domain antibodies specific against the flagellum could reduce bacterial colonization in chickens. In this latter study, single-domain antibodies, specific for *C. jejuni,* were isolated from a phage display library and then pentamerized to express multi-binding targets. The motility of *C. jejuni* was reduced in the presence of these flagellum-specific pentabodies, perhaps due to impairments in the motility of the flagella or antibody-mediated aggregation. There was a clear binding of the anti-flagella pentabodies to the flagellin subunit and reduced *C. jejuni* colonization of the ceca when the antibodies were given orally to *C. jejuni*-infected two-day old chicks (Riazi et al. 2013). But the high DNA variability among *C. jejuni* strains also resulted in large antigenic variations.

The requirement for various antibody combinations to target multiple antigenic variances on *C. jejuni* is an important limitation in the development of vaccines to control colonization in chickens or infection in humans (Alemka et al. 2013; Buckley et al. 2010; Connell et al. 2012; Hermans et al. 2011; Jagusztyn-Krynicka et al. 2009). In addition, the variability in the immune response by different animal species, with the limited understanding of the molecular mechanisms used by *C. jejuni* to colonize the lower intestine, primarily the ceca, of commercial chickens is an area that needs additional research to make progress on intervention strategies for food animals.

Molecular Virulence Mechanisms of *C. jejuni*

C. jejuni enters the host intestinal tract *via* the fecal-oral route and colonizes the distal ileum and colon of humans. The molecular basis for the commensalism in some animals and the expression of virulence in humans is not well understood.

One obvious aspect is that *C. jejuni* is amazingly successful in competing with the intestinal human microbiota (Masanta et al. 2013). An infectious dosage of a few hundred live *C. jejuni* cells is commonly enough to defeat the colonization resistance barrier of the human host, eventually resulting in campylobacteriosis (Masanta et al. 2013). Another aspect is the apparently increased number of *C. jejuni* cells invading epithelial cells in the human host as compared to the low invasion rates in chicken cells (Young et al. 2007). Interestingly, it appears that chicken mucin, which is the main component of intestinal mucus, inhibits *C. jejuni* internalization into chicken epithelial cells. However, human mucin does not have the same inhibitory effect (Alemka et al. 2010). This finding suggests that both *C. jejuni* adherence to epithelial cells and entrance into epithelial cells may be essential stages for human disease development. The molecular mechanisms underlying pathogenesis of *C. jejuni* infections are yet to be fully elucidated.

Adhesion to Epithelial Cells

After entering the human gastrointestinal tract, *C. jejuni* cells first interact with the mucus layer before they bind to the epithelial cells of the intestine. This attachment, as well as cellular invasion and transmigration, appear to be necessary requirements for successful colonization and pathogenesis by these bacteria (Fig. 1). Adherence of *C. jejuni* to the target cells involves various adhesion factors (called adhesins) and their corresponding host cell receptors. These adhesins are important for efficient

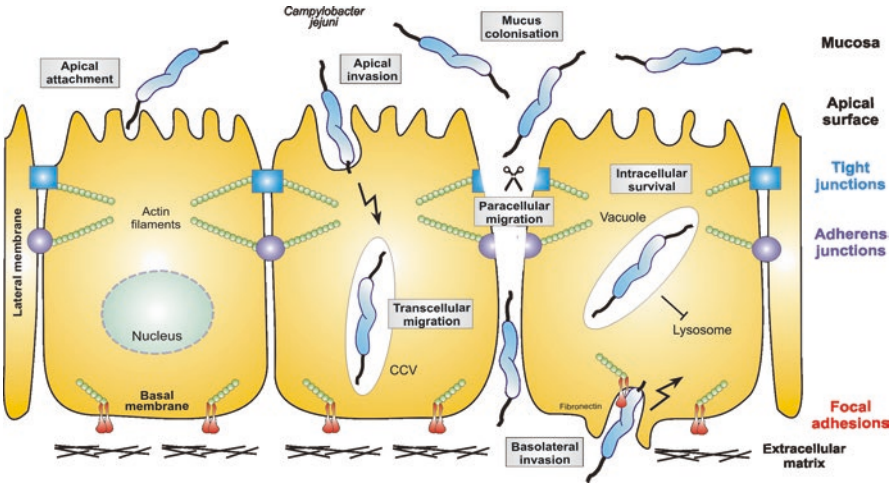

Fig. 1 Hypothetical model for important steps and *C. jejuni* mechanisms of infection in humans. The pathogen can bind to, invade into, transmigrate across and survive within polarized intestinal epithelial cells as shown (This figure has been modified with permission from Backert and Hofreuter (2013). See text for more details)

Table 1 Major adhesins described for *C. jejuni*

Name	Short name	Protein size	Host receptor
Campylobacter adhesion to fibronectin	CadF	37-kDa outer membrane protein	Fibronectin
Fibronectin like protein A	FlpA	46-kDa protein	Fibronectin
Periplasmic binding protein 1	PEB1	28-kDa lipoprotein	unknown
Lipoprotein A	JlpA	43-kDa protein	HSP90-α
Campylobacter autotransporter protein A	CapA	116-kDa lipoprotein	unknown
Major outer membrane protein	MOMP	59-kDa protein	unknown
Protein 95	p95	95-kDa protein	unknown

See ÓCróinín and Backert (2012) for a comprehensive review

interaction with host target cells; therefore, the presence of receptor(s) on target cells allows for direct participation of the cell in the invasion process. Table 1 provides a list of the major known adhesins that have been described for *C. jejuni.*

CadF is by far the best studied binding factor of *C. jejuni*. This protein allows *C. jejuni* cells to attach to the extracellular matrix protein fibronectin of target cells (Konkel et al. 1997, 1999, 2005). A Δ*cadF* mutant exhibited reduced levels of adherence to, and invasion into INT-407 intestinal epithelial cells (Monteville and Konkel 2002; Monteville et al. 2003; Krause-Gruszczynska et al. 2007) and was incapable of colonizing chickens (Ziprin et al. 1999). CadF appears to be essential for the uptake of *C. jejuni* at the basolateral host cell surface (Monteville and Konkel 2002). Many studies on the infection of INT-407 cells revealed that *C. jejuni* can attach to fibronectin by CadF and activate downstream signal transduction to small Rho GTPases (Krause-Gruszczynska et al. 2011; Boehm et al. 2011; Eucker and Konkel 2012).

FlpA also contributes to the attachment of *C. jejuni* to fibronectin and consequently epithelial cells. The interaction of FlpA with fibronectin is a dose-dependent process and an Δ*flpA* mutant bound to INT-407 cells significantly less compared to the corresponding wild-type *C. jejuni* (Konkel et al. 2010; Eucker and Konkel 2012). Studies have also revealed that CadF and FlpA act cooperatively in mediating *C. jejuni* attachment to host cells via fibronectin.

Studies with a *jlpA* mutant revealed that a non-functional JlpA protein reduces adherence of *C. jejuni* to HEp-2 cells by approximately 20% (Jin et al. 2001, 2003). Moreover, pre-treatment of HEp-2 cells with purified JlpA led to decreased binding by *C. jejuni* in a dose-dependent response (Jin et al. 2001). Similar studies have revealed that inactivating *peb1A*, which renders PEB1 proteins non-functional, decreased the adherence of *C. jejuni* to HeLa cells and colonization of mice (Pei et al. 1998).

CapA (Ashgar et al. 2007), p95 (Kelle et al. 1998) and MOMP (Moser et al. 1997) have also been reported as *C. jejuni* adhesins, but these proteins have not been examined in sufficient detail and some conflicting results have been reported in the literature.

Cellular Invasion

Early studies of intestinal biopsy samples from infected patients and cultured non-phagocytic cell lines using, among other techniques, electron microscopy revealed that *C. jejuni* can enter human intestinal epithelial cells (van Spreeuwel et al. 1985; Oelschlaeger et al. 1993). In general, epithelial cell invasion has been described as the primary mechanism leading to tissue damage and pathogenesis caused by *C. jejuni* in humans. Using specific inhibitors, studies revealed that *C. jejuni* can enter cultured cells by a microtubule-dependent (actin-filament-independent) and/or actin-filament-dependent (microtubule-independent) mechanism (De Melo et al. 1989; Konkel and Joens 1989; Oelschlaeger et al. 1993; Russell and Blake 1994; Hu and Kopecko 1999; Biswas et al. 2003; Monteville et al. 2003).

In contrast to highly invasive *Salmonella* or *Shigella* species, *C. jejuni* does not encode typical disease-related type-III or type-IV secretion systems (T3SS or T4SS) (Parkhill et al. 2000; Fouts et al. 2005; Hofreuter et al. 2006). Rather, *C. jejuni* relies on its flagellum and rapid motility to pass through the high viscosity of the intestinal mucosa to adhere and entry into host cells (Fig. 1) (Szymanski et al. 1995). The flagellum can also function as a T3SS for the export of proteins that control bacteria–host interactions (Young et al. 1999; Konkel et al. 1999; Christensen et al. 2009; Neal-McKinney et al. 2010). The substances secreted through this T3SS are called flagellar co-expressed determinants (FedA-D) and *Campylobacter* invasion antigens (CiaA-H) (Konkel et al. 1999; Eucker and Konkel 2012; Barrero-Tobon and Hendrixson 2012, 2014). However, the overall delivery system and the function of secreted proteins are still poorly understood for *C. jejuni*.

One of the best characterized Cia proteins is the ~70-kDa protein CiaB. CiaB is necessary for the secretion process itself, and is required for maximal invasion of *C. jejuni* into host cells (Konkel et al. 1999). Gentamicin protection assays revealed significantly lower invasion rates of a Δ*ciaB* deletion mutant in human cells as compared to wild-type *C. jejuni*. The Δ*ciaB* mutant also had reduced colonization of chickens (Ziprin et al. 2001). Another protein that is secreted from the flagellar export apparatus of *C. jejuni* is FlaC. Mutants of *flaC* are still motile and express a functional flagellum, but they are deficient in entering epithelial cells (Song et al. 2004).

Taken together, these observations suggest that the flagellar export machinery could be a crucial secretory device that plays a major role in the invasion process of host cells by *C. jejuni*. In line with these ideas, it was reported that Cia proteins, such as CiaC, can be delivered into the host cell cytoplasm using the adenylate cyclase domain of *Bordetella pertussis* CyaA as a marker (Neal-McKinney and Konkel 2012). However, another study raised doubt about the importance of another Cia protein, (i.e., CiaB, as a relevant virulence factor involved in host cell entry) (Novik et al. 2010). Hence, it is not clear if CiaB is indeed directly involved in exporting Cia proteins that trigger bacterial internalization, or if the observed invasion defects of flagellar deletion mutants are due to lack of flagella-based mobility and consequently reduced bacterial contact with the host cell.

Transmigration Across the Epithelial Barrier

Previous studies have revealed that *C. jejuni* can not only colonize the surface of intestinal epithelial cells, but also is able to invade underlying tissues such as the lamina propria and bloodstream followed by entering other organs such as the spleen, mesenteric lymph nodes or liver (reviewed by Backert et al. 2013). However, the molecular mechanism, as well as the major bacterial and host cell factors, involved in these activities are poorly understood.

There are various models to explain the paracellular and/or transcellular mechanisms by which *C. jejuni* may trigger its own transmigration across polarized intestinal epithelial cells *in vitro* (Fig. 1). *C. jejuni* strains that express ganglioside-like lipooligosaccharides can better attach to the apical surface of Caco-2 cells and have increased cell invasion and transmigration by the transcellular route than *C. jejuni* isolates lacking such structures (Louwen et al. 2012).

Other *in vitro* studies have revealed that a family of proteins, called high temperature requirement A (HtrA), are secreted virulence factors having important roles in adhesion, invasion and transmigration of *C. jejuni* (Brøndsted et al. 2005; Baek et al. 2011; Boehm et al. 2012; Hoy et al. 2012). The HtrA family of proteins is widely conserved in the bacterial kingdom and represents a class of heat-shock serine proteases with additional chaperone activity (Kim and Kim 2005; Clausen et al. 2011; Backert et al. 2013). HtrA protease members are typically composed of a signal peptide, a trypsin-like serine protease domain and 1–2 PDZ modules to perform protein-protein interactions (Skorko-Glonek et al. 2013). These proteases commonly operate intracellularly, in the periplasm, where they assemble proteolytically active HtrA oligomers that control protein quality (Ingmer and Brøndsted 2009). These proteases influence the survival and virulence capabilities of many bacterial pathogens (Ingmer and Brøndsted 2009; Backert et al. 2013) and in *C. jejuni* HtrA plays a pivotal role in inducing host cell apoptosis and immunopathology in infected mice (Heimesaat et al. 2014b, c). In addition, the growth of Δ*htrA* knockout *C. jejuni* mutants is significantly impaired at 44 °C and under oxygen stress conditions as compared to the corresponding wild-type strain (Brøndsted et al. 2005).

Until very recently, it was believed that the HtrA protein family was acting only inside the bacterial cells. However, a novel characteristic of HtrA during infection has been recently disclosed. In *C. jejuni* and its relative *Helicobacter pylori*, HtrA proteins are actively secreted in the extracellular environment, where they can hijack host cell proteins (Hoy et al. 2010, 2012; Backert et al. 2013). Infection studies using a transwell system with polarized cell monolayers revealed that secreted HtrA can proteolytically cleave the 125-kDa E-cadherin receptor in the adherens junctional complex. This cleavage leads to the opening of the cell-to-cell junctions between neighboring epithelial cells followed by paracellular transmigration of *C. jejuni* (Boehm et al. 2012, 2013, 2015). E-cadherin shedding and transmigration of *C. jejuni* was widely impaired when the *htrA* gene was deleted, or when a protease-inactive mutation was expressed using gene swapping (Boehm et al. 2012).

Furthermore, using Δ*htrA* deletion mutants, it was determined that HtrA is also necessary for efficient epithelial cell adherence by *C. jejuni* (Baek et al. 2011). Inactivating HtrA resulted in reduced bacterial adherence to host cells, with a reduction that was 5–10 times more effective than inactivating any other proposed *C. jejuni* adhesin. The specific activity of HtrA in this regard needs further elucidation.

Intracellular Survival

Early gentamicin protection assays and electron microscopy studies have revealed that *C. jejuni* can survive for extended periods of time in several intestinal epithelial cell lines and tissues both *in vitro* and *in vivo* (van Spreeuwel et al. 1985; De Melo et al. 1989; Konkel et al. 1992; Babakhani et al. 1993; Watson and Galan 2008). Upon invasion of mammalian cells, *C. jejuni* localizes in a specific membrane-surrounded compartment in the cytoplasm (Fig. 1). This compartment is called *C. jejuni*-containing vacuole (CCV), which appears to be distinct from the conventional cellular lysosomes. It has been proposed that *C. jejuni* is able to survive within the CCVs of different types of non-phagocytic epithelial cells for up to 24 h (Gaynor et al. 2005; Watson and Galan 2008) or even 72 h (Konkel et al. 1992; Naikare et al. 2006). Electron microscopy studies revealed that approximately 5 h after invasion, *C. jejuni* is present in a survival-permissive vacuolar compartment in the perinuclear region of the target cell (Hu and Kopecko 1999; Watson and Galan 2008). These CCVs were immunostained with GM130, a typical Golgi marker protein (Watson and Galan 2008), and it has been proposed that the transport of the CCVs from the cell periphery to the perinuclear space could be facilitated by the vesicle transport system, possibly involving microtubules and the dynein motor protein (Hu and Kopecko 1999; Watson and Galan 2008). Future studies should clarify the overall role of the CCVs and their perinuclear localization during the complete infection cycle.

The CCV originates from the canonical endocytic pathway immediately following epithelial cell entry, thus avoiding delivery of the bacteria into lysozomes (Watson and Galan 2008). It appears that the CCV can interrelate with early endosomal vesicles (Watson and Galan 2008; Louwen et al. 2012). *C. jejuni* co-localizes with the transiently expressed GFP-fusion proteins of the GTPases Rab4 and Rab5. Afterwards, the CCV also co-localizes with transiently expressed GFP-Rab7, a late endosomal marker (Watson and Galan 2008), but this co-localization is not part of the canonical endocytic trail.

The CCV is also associated with late-expressed endosomal markers, such as Lamp-1, which is unique and distinct from lysosomes (Watson and Galan 2008; Louwen et al. 2012). CCVs are not accessible to certain endocytic tracers and the involvement of Lamp-1 very early in the maturation of the CCVs suggests an unusual pathway. The subset of *C. jejuni* genes involved in the above processes is largely unknown, but a few putative enzymes are known.

Other genes that influence survival have also been identified over the years. A summary of these findings include:

- Mutagenesis of *aspA* and *aspB* has shown substantial deficiencies in bacterial survival, and lower number of recovered bacterial cells from both mutants is due to undetermined effects on bacterial physiology (Novik et al. 2010).
- Inactivation of the stringent response regulator SpoT resulted, among other defects, in diminished intracellular survival rates of *C. jejuni* (Gaynor et al. 2005).
- Genes encoding two other enzymes, the polyphosphate kinases Ppk-1 and Ppk-2, which are involved in polyphosphate metabolism, also enhance the fitness of *C. jejuni* within CCVs (Candon et al. 2007; Gangaiah et al. 2010).
- An active superoxide dismutase, SodB, is required for intracellular persistence (Novik et al. 2010; Pesci et al. 1994), and a mutant of the fumarate reductase A (frdA) gene, having a defect in fumarate respiration, also exhibited a lower survival capacity (Liu et al. 2012).
- The secreted Cia family member CiaI was determined to be involved in efficient intracellular survival (Buelow et al. 2011); however, this was not confirmed in another study (Barrero-Tobon and Hendrixson 2014).
- The absence of the *cprS* gene can positively regulate bacterial survival within the host cell. Mutation of CprS, the sensor kinase of a two-component regulatory module controlling planktonic bacterial growth, can enhance the intracellular fitness of*C. jejuni* (Svensson et al. 2009).

In a counterintuitive way, other genes have not been shown to enhance survival. For example, the catalase gene *katA*, which facilitates the detoxification of reactive oxygen metabolites, was determined not to be essential for the survival of *C. jejuni* in infected Hep-2 cells (Day et al. 2000). All these findings highlight the importance of using not only one, but a series of different assays to identify and describe factors with proposed function in adhesion and host cell entry by *C. jejuni*.

Interaction with Immune Cells

Even though the intracellular survival of *C. jejuni* in non-phagocytic epithelial cells has been well documented, several conflicting papers on the persistence of *C. jejuni* in phagocytic immune cells have been published. Multiple studies have revealed that *C. jejuni* can survive for several days after uptake within macrophages of the mouse macrophage-like cell line J774 from BALB/c mice (Kiehlbauch et al. 1985), in mouse peritoneal macrophages (Day et al. 2000), in the human monocytic cell line 28SC, and in freshly isolated human monocytes (Hickey et al. 2005). In contrast, other studies have revealed that *C. jejuni* can be killed following uptake by peripheral macrophages of chickens (Myszewski and Stern 1991), by macrophages originating from peripheral monocytes of humans (Wassenaar et al. 1997) and by bone marrow-derived macrophages from mice (Watson and Galan 2008). The

reasons for these different results are not clear, but one explanation may be variations in the experimental parameters. For example, the use of different types of phagocytic immune cells, diverse *C. jejuni* strains, inconsistent CFUs and variable infection times, as well as the application or lack of gentamicin to inhibit extracellular growth of *C. jejuni,* may have had a significant influence on experimental outcomes. Moreover, the induction of immune cell differentiation and activation varied among the experiments, which can also influence *C. jejuni* killing or survival in the studies. Hence, more research is needed to clarify the capacity of *C. jejuni* to survive, or be killed, within macrophages derived from different hosts, and to identify the bacterial factors that may be involved in survival.

Animal Infection Models

Efforts to elucidate *C. jejuni* infections have been limited by the absence of useful *in vivo* animal models. Various infection models have been tested in the past, including newborn piglets, weanling ferrets, chicken, primates, gnotobiotic canine pups and isolator-raised germ-free mice. But all these models have been only moderately successful in studying *C. jejuni*-mediated pathogenesis (reviewed by Jansen et al. 2008; Quin et al. 2011; Masanta et al. 2013).

Early mouse models were hampered by issues with sporadic colonization and/or absence of clinical disease manifestation (Kist and Bereswill 2001). However, the gnotobiotic mouse model, developed by treating conventional mice with a quintuple antibiotic cocktail for about 12 weeks to remove the mouse microbiota (Heimesaat et al. 2006), has helped reveal important findings. Gnotobiotic mice exhibit a fully developed immune system as opposed to isolator-raised germ-free animals (Bereswill et al. 2011). Using this strategy, the microbiota of the animals were substituted by peroral re-colonization to produce humanized flora-associated mice (hfa) and gnotobiotic mouse flora-associated mice (mfa) model systems (Bereswill et al. 2011). While mfa and conventional control mice can eliminate *C. jejuni* within a few days post infection, hfa mice are highly susceptible and harbor the pathogen at high density, revealing that colonization resistance against *C. jejuni* is at least partially due to host-specific composition of microbiota (Masanta et al. 2013). Following infection, gnotobiotic and hfa mice exhibit immunopathological properties similar to those observed in infected patients (i.e., pro-inflammatory responses and apoptosis of colonic epithelial cells), whereas severe clinical campylobacteriosis symptoms such as bloody diarrhea were absent.

Gnotobiotic mice lacking certain toll-like receptors (TLR-2, TLR-4 and/or TLR-9), adapter protein MyD88 or interleukin-10 (IL-10) have greatly contributed to our current understanding of the innate and adaptive immune reactions occurring during *C. jejuni* infection (Bereswill et al. 2011; Haag et al. 2012b). Surprisingly, within 6 days following *C. jejuni* infection, gnotobiotic IL-10-deficient (IL-$10^{-/-}$) mice exhibited substantial ulcerative enterocolitis, that was not self-limiting and thus is similar to severe human campylobacteriosis as known from immune-compromised

persons (e.g., AIDS patients) (Haag et al. 2012a). It was also observed that there was significantly less immunopathology when gnotobiotic IL-$10^{-/-}$ mice were also deficient in TLR-2 and/or TLR-4 revealing that TLR-mediated sensing of *C. jejuni* factors plays a crucial function in the immunopathology of campylobacteriosis (Bereswill et al. 2011; Haag et al. 2012b).

Another useful animal model that has contributed to our understanding of *Campylobacter*'s pathogenicity is the infant mouse model. Studies revealed that infant mice colonized by *C. jejuni* at 3 weeks of age (immediately after weaning) developed acute self-limiting ulcerative enterocolitis, similar to typical campylobacteriosis in humans (Haag et al. 2012a). Unexpectedly, following the acute stage of infection, infant mice were commonly asymptomatic carriers of *C. jejuni* for several months. But remarkably, and in spite of the lack of clinical sequelae, specific inflammatory parameters were observed to be upregulated within the gastrointestinal tract, as well as in extra-intestinal tissues such as the liver, lungs and kidneys (Haag et al. 2012a). In these cases, the infiltrating immune cells were predominantly $CD3^+$ T-lymphocytes, and to a less extent $B220^+$ B-lymphocytes. Therefore, the *C. jejuni* infection mouse infant model does not only mimic self-limiting human campylobacteriosis, but is also useful for the study of post-infectious sequelae (Heimesaat et al. 2014a, b).

Conclusions and Perspectives

Although *C. jejuni* has a relatively small genome, the plasticity of this genome provides this pathogen with the tools to successfully colonize animals, especially poultry, and infect humans. Campylobacters use unique pathways to interact with host cells, and hence, their mechanism of pathogenicity is dissimilar from other enteric bacterial pathogens such as *Salmonella*.

There are several adhesion factors that have been described for *C. jejuni*, although the actual contribution of each adhesion factor to the colonization process is still not fully understood. The flagellar export components have emerged as crucial secretory contributors to enable *C. jejuni* to enter host cells. The flagellum can function as a T3SS for the export of proteins that control adhesion to and invasion of host cells.

Conflicting reports on the persistence of *C. jejuni* in phagocytic cells needs to be addressed to better understand potential venues to activate the immune system against this pathogen. Several vaccines are under study to protect humans from infection and chickens from colonization by this pathogen. Although not addressed in this chapter, vaccines may provide an important alternative to prevent campylobacteriosis, especially in children in developing countries.

Several animal infection models have been useful to obtain information on different aspects of the invasion and pathogenicity phases of this pathogen. The host-specific composition of the microbiota is an important determinant in the occurrence of this disease, and this determinant could not have been fully addressed without

one of these animal models. However, the use of animals in human research is coming under closer scrutiny and will likely be limited in the future. Therefore, the search for cell lines or other alternative models is extremely important to continuing research needed to understand how to control this pathogen.

Although not addressed in this chapter, there are several limitations with the current methodologies available to study *Campylobacter* spp., some of which include the monitoring and isolation and identification of *Campylobacter* spp. from foods. For instance, in the USA, the only regulated testing for *Campylobacter* spp. is a performance standard for poultry carcasses after chilling. Yet, there are limited studies on the prevalence rates found after carcasses are portioned into parts and at the retail level.

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Strain-Specific Virulence Differences in *Listeria monocytogenes*: Current Perspectives in Addressing an Old and Vexing Issue

Sophia Kathariou, Peter Evans, and Vikrant Dutta

Abstract The query of whether all strains of *Listeria monocytogenes* should be considered to be human pathogens and of equal public health relevance has long attracted keen interest and generated debates among public health professionals, regulatory agencies, the food industry, and scientists investigating virulence and pathogenesis of *L. monocytogenes*. Animal models have often given contradictory and ambiguous results and have frequently lacked sufficient biological relevance, further aggravating the controversy. Different regulatory bodies have instituted regulations varying in threshold (e.g. "zero" tolerance in ready-to-eat foods vs. a certain number of CFUs) permissible in a sample of a certain size, depending on the nature of the sample and the extent to which it is permissive of *L. monocytogenes* growth. The issue has remained unsettled. However, recent breakthroughs in *L. monocytogenes* genotypic assessments and integration of whole genome sequence data with human susceptibility and disease outcome metrics are beginning to provide substantial evidence for differences in virulence among *L. monocytogenes* strains. Increasing evidence supports the disproportionately high contribution of a relatively small number of "hypervirulent clones" to human listeriosis.

Keywords *Listeria monocytogenes* • Virulence • Pathogenicity • Strain variation • Dose response • Hypervirulent clones

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The query of whether all strains of *Listeria monocytogenes* should be considered to be human pathogens and of equal public health relevance has long attracted keen interest and generated debates among public health professionals, regulatory agencies, the food industry, and scientists investigating virulence and pathogenesis of *L. monocytogenes* (Hof and Rocourt 1992). Animal models have often given contradictory and ambiguous results and have frequently lacked sufficient biological relevance, further aggravating the controversy. Different regulatory bodies have instituted regulations varying in threshold (e.g. "zero" tolerancein ready-to-eat foods vs. a certain number of CFUs) permissible in a sample of a certain size, depending on the nature of the sample and the extent to which it is permissive of *L. monocytogenes* growth. The issue has remained unsettled.

Nonetheless, the *Listeria* field is at a rather different stage today than it was two or three decades ago. Major advances have been made in strain subtyping and analysis of the population structure of *L. monocytogenes*, the identification and characterization of virulence determinants, and the elucidation of pathogenesis mechanisms and host-pathogen interactions. These advances have recently reached a new phase with the application of whole genome sequencing (WGS) in surveillance and outbreak investigations of *L. monocytogenes*, complementing and expanding data from targeted screening of strains for the presence of specific virulence genes or virulence-impacting mutations. Thus, we are currently at the cusp of a novel level of understanding of the range and type of diversity in virulence within the species *L. monocytogenes*. The remainder of this chapter will address the current understanding of this issue, and also utilize a synthesis of available data towards hypothesis building for future work to further elucidate virulence diversity within *L. monocytogenes*.

Omics Tools in the Determination and Characterization of Differences in Virulence Among Different Strains of *Listeria monocytogenes*

"Omics" tools are used to characterize and quantify biological molecules in a highly parallel manner, so that the structure, function and dynamics of an organism or a community of organisms can be understood comprehensively, minimizing target-specific biases. Virulence-related applications of omics tools for *L. monocytogenes* such as genomics, transcriptomics, methylomics and proteomics assess entire systems without the need to target or experimentally isolate individual genes, RNAs or proteins. This provides the capability of gauging the importance of single or multiple genes, RNAs, or protein targets of interest within a comprehensive, complex system. Omics tools have been applied in numerous studies with *L. monocytogenes* with a key stated or implicit objective to elucidate virulence-related attributes and adaptive responses. We are now witnessing unprecedented increases in the frequency of whole genome sequencing, as well as increasingly accessible analyses of other attributes at the global level (e.g., transcriptome, proteome, methylome, and metabolome analyses). Such advances are poised to revolutionize our understanding of virulence of bacterial pathogens, including *L. monocytogenes*. A major focus of our discussion will be directed to genomics applications.

The *L. monocytogenes* genome includes the circular DNA chromosome, which has a similar size in different strains (approx. 2900 kb), and plasmids. Genome sequences encode proteins, structural RNAs (ribosomal and transfer RNAs) and non-structural (enzymatic, regulatory etc.) RNAs. Other genome sequences are dedicated to either promoting or repressing transcription or translation and as transcription terminators.

Many sequence-based tools targeting selected sites across the entire genome have been applied to *L. monocytogenes* to aid in the characterization of population structure, facilitate outbreak investigation and other applications. The intent is to use these tools to supplement and eventually replace the genomic DNA restriction fragment-based pulsed-field gel electrophoresis (PFGE) molecular subtyping system, which since 1996 has been the gold standard for *L. monocytogenes* molecular epidemiology and the pivotal tool for PulseNet (Halpin et al. 2010). Such sequence-based tools targeting multiple sites across the genome have included Multilocus sequence typing (MLST) and a variant thereof that includes virulence loci, Multi-virulence locus typing (MLVST); Multilocus genotyping (MLGT) based on detection of a large array of single-nucleotide polymorphisms (SNPs) on the chromosome; Multilocus variable-number tandem-repeat analysis (MLVA); microarray-based DNA-DNA hybridizations with oligonucleotides or PCR fragments representing the *L. monocytogenes* "pan-genome", i.e. the core genome of all conserved genes as well as other sequences, encountered in some but not all strains (Chenal-Francisque et al. 2013 and references therein; Doumith et al. 2006; Ducey et al. 2007; Salcedo et al. 2003; Ward et al. 2008).

Generally, these tools do allow unambiguous placement of strains in phylogenomic groups designated as "lineages" ("Genomic Divisions" in the earlier *L. monocytogenes* literature) and largely congruent with serotype-based clusters. Of the two major lineages which account for most strains implicated in human listeriosis, lineage I includes strains of serotype 1/2b, 3b and most serotype 4b strains whereas lineage II includes serotype 1/2a, 1/2c, 3a and 3c strains (Piffaretti et al. 1989; Kathariou 2002; Orsi et al. 2011). Strains in the less common lineage III have a propensity for animal infections and include serotypes 4a, 4c and certain serotype 4b strains while lineage IV (formerly designated as lineage IIIB) is even less common (Orsi et al. 2011).

There is generally high conservation of *L. monocytogenes* genes encoding known virulence determinants among different strains, serotypes and lineages (Maury et al. 2016). Sequence polymorphisms, while of subtyping potential, have generally not been correlated with differences in virulence. However, there are certain notable exceptions, which include (1) virulence-related sequences harbored by some, but not all strains and (2) virulence-associated polymorphisms in conserved genes.

Virulence-Related Genomic Sequences with Non-homogenous Distribution in *L. monocytogenes*

Even though genes for most virulence determinants are conserved within *L. monocytogenes*, certain strains do harbor virulence-related genes that are not uniformly present within the species. Such differences in gene content can now be readily detected via whole genome sequence comparisons. For example:

1. The Listeriolysin S *Listeria* Pathogenicity Island (*Listeria* Pathogenicity Island 3, LIPI-3). In *L. monocytogenes*, LIPI-3 is present in the chromosome of certain lineage I strains (serotypes 1/2b and 4b) but not other lineage I strains; furthermore, LIPI-3 has not yet been detected in other lineages. In strains harboring it, LIPI-3 has been experimentally determined to affect virulence in murine infections (Cotter et al. 2008).
2. The Clonal Complex 4 (CC4)-specific *Listeria* Pathogenicity Island (Listeria Pathogenicity Island 4, LIPI-4). Analysis of a large panel of *L. monocytogenes* isolates from France suggested that CC4 was one of the human-"hyperivurent" clones that will be discussed in detail later in this chapter, with higher relative representation of human clinical isolates (71.3%) than any other clones (Maury et al. 2016). A 6-gene cluster, LIPI-4, appears to be unique to CC4 and in murine models LIPI-4 was found important for virulence, especially the ability to invade the brain, thus providing the first evidence for neuro-invasiveness-specific determinants in *L. monocytogenes* (Maury et al. 2016).
3. The large family of internalins. Genes encoding certain members of the large internalin protein family have strain-specific distribution, though the virulence impacts of such variations in distribution have not been rigorously determined (Bierne et al. 2007). Internalins are chromosomal determinants, but at least one strain with plasmid-borne internalin genes has been described (Den Bakker et al. 2012). Diversity in internalins was exemplified by the in-depth comparison of three serotype 1/2a strains which revealed that several internalins were harbored by two of the three strains and two were only found in one strain (Bécavin et al. 2014).
4. Integrative chromosomal elements (ICEs). Certain genes of the Tn*916*-like integrative chromosomal element (ICE) in strain EGD-e, including genes associated with the cadmium-resistance *cadAC* cassette, are induced in the liver of mice infected with this strain and are required for normal virulence levels (Camejo et al. 2009). However, these genes and the associated ICE are uncommon in *L. monocytogenes* genomes (Kuenne et al. 2013; Lee et al. 2014). The fact that in at least certain lineage I strains the integration site of the ICE is occupied by LIPI-3, mentioned above (Cheng et al. 2010), may partially contribute to the relative scarcity of the Tn*916*-like ICE in *L. monocytogenes.*
5. Prophages. Prophage sequence content and prophage-chromosome junction sequences are variable among different strains (Verghese et al. 2011), with potentially important impacts in virulence based on data suggesting that prophage excision is induced intracellularly, leading to enhanced escape from the vacuole and virulence (Rabinovich et al. 2012).
6. CRISPR elements. Studies with clustered, regularly interspersed short palindromic repeats (CRISPRs) revealed an "orphan" CRISPR, *rliB*, devoid of a CRISPR-associated protein (*cas* gene) (Sesto et al. 2014). In the presence of type I A CRISPR Cas, *rliB* conferred protection against invading phage DNA. However, type I A CRISPR Cas was only harbored by some (12/40) of the examined strains of *L. monocytogenes* (Sesto et al. 2014). Earlier studies using EGD-e, which lacks type I A CRISPR-Cas, revealed that *rliB* was involved in modulation of virulence in the murine model, as the *rliB*-deficient mutant exhibited more efficient liver colonization of mice than its parental counterpart (Camejo et al.

2009). These results suggest virulence involvement of *rliB* in strains such as EGD-e which lack type I A CRISPR-Cas, whereas in strains harboring type I A CRISPR-Cas the function of *rliB* may instead focus on phage defense (Sesto et al. 2014). Further deployment of *in silico* and experimental tools for characterization of the *L. monocytogenes* "CRISPRome" and other genes encoding small, noncoding RNAs (Mandin et al. 2007; Toledo-Arana et al. 2009; Schultze et al. 2014; Sesto et al. 2014) may identify additional virulence-related small, noncoding RNAs that are not uniformly harbored by all *L. monocytogenes* strains. Some small, non-coding RNAs were not shared even among strains of the same serotype (1/2a) (Bécavin et al. 2014). Furthermore, the extent to which these small RNAs may in fact encode small polypeptides remains to be determined.

7. Serotype-associated "decorations" on teichoic acids of the cell wall. In *L. monocytogenes*, cell wall teichoic acid (WTA) is variable in composition among different serotypes. In serogroup 1/2, phospho-ribitol phosphate in the WTA backbone is glycosylated ("decorated") with rhamnose and N-acetylglucosamine, whereas in the WTA of serotype 4b, 4d and 4e the N-acetylglucosamine is part of the backbone and is itself decorated with glucose and galactose moieties (Fiedler 1988; Fiedler et al. 1984). Insertional inactivation of *gtcA* of serotype 4b abolished galactose in the WTA and recognition with serotype 4b-specific monoclonal antibodies (Promadej et al. 1999), and was found to result in reduced virulence in a murine gastrointestinal model (Faith et al. 2009). Such *gtcA* mutants also are resistant to serotype 4b-specific phage and spontaneous mutants with a similar phenotype have been frequently detected (Clark et al. 2000; Cheng et al. 2007, 2008).

As mentioned above, in serogroup 1/2 the WTA of serogroup 1/2-associated decoration consists of N-acetylglucosamine and rhamnose. Evidence for virulence attenuation in mutants with impaired rhamnosylation of WTA has been provided through deletion in strain EGD-e of a gene cluster associated with rhamnose biosynthesis (*rmlABCD*) and glycosylation (*rmlT*) (Carvalho et al. 2015). The resulting mutants lacked rhamnose on the WTA and had enhanced susceptibility to antimicrobial peptides (AMPs); further characterization revealed that the rhamnose component of the WTA in serogroup 1/2 *L. monocytogenes* delayed access of the AMPs to the cell membrane. Interestingly, the mutants lacking rhamnose in the WTA were also less virulent in mice, with reduced numbers in the liver and spleen following oral gavage and especially following intravenous inoculation, thus providing further evidence for involvement of the serogroup 1/2a-specific rhamnnose component of the WTA in pathogenesis (Carvalho et al. 2015). Evidence for the role of WTA decoration in AMP resistance has also been obtained with other Gram-positive pathogens, with the notable difference that in other pathogens amino acids such as alanine are added onto the WTA (Peschel et al. 1999; Kovács et al. 2006), whereas in *L. monocytogenes* WTA is decorated with sugars (Fiedler et al. 1984; Fiedler 1988).

Genome sequence analysis of spontaneous phage-resistant mutants of the serotype 1/2a strain 10403S revealed mutations in teichoic acid biosynthesis genes (Denes et al. 2015). Interestingly, substantial portions of *L. monocytogenes*

serogroup 1/2 isolates from the environment of turkey processing plants were found to be phage-resistant (Kim et al. 2008). The underlying reasons are unknown, but if they include loss of WTA-glycosylation with rhamnose it is conceivable that such isolates may also have enhanced susceptibility to AMPs and be attenuated in virulence. With further elucidation of the genetic basis for WTA decoration in strains of different genotypes, screening for relevant mutations can be significantly optimized. Risk assessments of food contaminated with such mutants will benefit substantially from (1) more accurate determinations of the frequency of such mutations and (2) further elucidation of the impact of such mutations on virulence. As with other mutations, WGS data will contribute to more accurate determinations of the frequency and distribution of mutations in these and other virulence-related genes in *L. monocytogenes* from various sources.

Virulence-related sequence polymorphisms in conserved virulence determinants Targeted analysis of virulence determinants has led to discovery of several naturally encountered strains harboring mutations in genes important (and on several occasions essential) for virulence in animal models. Such mutations can have major implications for risk assessment of *L. monocytogenes* contaminating the food supply if they are encountered frequently in foods and if they result in attenuation or loss of virulence. Of the types of mutations that will be discussed here, those harboring premature stop codons in *inlA* result in the most vexing issues regarding assessment of the risk that strains harboring such mutations pose for the food supply.

1. Strains with premature stop codons in *inlA*, encoding the enterocyte invasion determinant Internalin A. In 1998, the extensively studied strain LO28 (human fecal carriage isolate, serotype 1/2c) was shown to harbor a premature stop codon (PMSC) in *inlA*, a chromosomal gene encoding Internalin A and required for enterocyte invasion (Jonquières et al. 1998). This finding was followed with numerous reports of the frequent presence of *inlA* SNPs that resulted in premature stop codon (PMSCs) (Jacquet et al. 2004; Van Stelten et al. 2010; Ward et al. 2010). Internalin A encoded by such alleles is truncated and thus no longer associated with the bacterial surface, as it lacks the terminal LPXTG motif required for InlA anchoring into the cell wall. The absence of surface-associated InlA in these PMSC-harboring strains prevents binding of *L. monocytogenes* to the InlA receptor E-cadherin (Lecuit et al. 1999). Importantly, *inlA* alleles with PMSCs were found to be frequent (often >30%) among serotype 1/2a, 1/2b and 1/2c (i.e. serogroup 1/2) isolates from foods and food processing environments, whereas most clinical isolates of the same serotypes expressed full-length Internalin. Intriguingly, PMSCs have been extremely uncommon among serotype 4b isolates, regardless of source (Jacquet et al. 2004; Van Stelten et al. 2010; Ward et al. 2010). Besides detection via targeted sequencing of *inlA*, known *inlA* PMSCs have been incorporated in multi-locus genotyping (MLGT) schemes, allowing simultaneous subtyping and *inlA* PMSC detection (Ward et al. 2010).

   The fact that the *inlA* PMSCs are rare in clinical isolates suggests that they have impaired virulence; in fact, dose response analysis in intragastrically inoculated guinea pigs revealed that a significantly higher dosage was required for

infection by a strain harboring a PMSC in *inlA*, in comparison to a strain with full-length InlA (Van Stelten et al. 2011). Such findings may prompt the proposal that food-derived strains with PMSCs in *inlA* should not be subjected to the same restrictions by government regulatory agencies as other *L. monocytogenes* strains, since they appear to be virulence-attenuated. However, isolates with truncated Internalin A due to *inlA* PMSCs may still be isolated, albeit rarely, from clinical cases of listeriosis (Jacquet et al. 2004). Even though we cannot exclude the possibility that the PMSCs accrued during isolation from a clinical sample or subsequent passage in the laboratory, it is also conceivable that such strains can still cause listeriosis in certain individuals with unique predisposing conditions or in response to other aspects in the host-pathogen-food interface. This need for caution in considering the potential risk associated with such strains is supported by animal model data which revealed that strains with *inlA* PMSCs could still cause fetal infections in orally inoculated mice and guinea pigs (Holch et al. 2013). The *inlA* PMSCs represent a rather unique and challenging case of naturally-encountered alleles with virulence-impacting sequence polymorphisms, because such alleles occur frequently in *L. monocytogenes* serogroup 1/2 strains which tend to be common in foods and food processing plants.

2. Naturally encountered mutations in *hly* and other LIPI-1 virulence determinants: The major pathogenicity island (LIPI-1) is conserved among *L. monocytogenes* strains and harbors several extensively characterized, key virulence determinants including, among others, *hly,* encoding the hemolysin Listeriolysin O (LLO); *actA,* mediating actin polymerization and intracellular motility; *plcA*, encoding phosphatidyl-inositol phospholipase C; and *prfA*, the positive regulatory factor for these and numerous other virulence genes (Vazquez-Boland et al. 2001). Strains of various serotypes harboring inactivating mutations in LIPI-1 genes have been occasionally identified, especially from foods and food processing plants (Témoin et al. 2008; Roche et al. 2005, 2012). Certain mutations (base substitutions or deletions) in *prfA*, for instance, can result in occasional strains that are non-hemolytic, impaired in LLO and other PrfA-regulated determinants. In contrast, certain other PrfA mutations (especially Gly145Ser) render the bacteria hyper-hemolytic and constitutively over-producing not only LLO but also lecithinase and other PrfA-controlled determinants (Ripio et al. 1997; Wong and Freitag 2004; Vasanthakrishnan et al. 2015). Non-hemolytic mutants such as those from mutations in *hly* or *prfA* are expected to be severely attenuated or avirulent for humans, and are not typically implicated in invasive listeriosis, while the human virulence impact of PrfA mutations leading to the hyper-hemolytic phenotype remains unknown. However, hyper-hemolytic strains are generally not encountered among human clinical isolates, possibly because of impaired fitness in foods or food processing environments or because of attenuated virulence. The prevalence of mutations in these and other virulence genes can be determined from analysis of WGS data, as well as via targeted sequencing or by incorporation of relevant sequence polymorphisms in MLGT and other high-throughput subtyping schemes.

3. Mutations impacting *iap*, encoding a 60 kDa extracellular protein (P60). In *L. monocytogenes*, *iap* (invasion-associated protein) is a conserved gene essential for cell division, and mutations result in rough (filamentous) variants that are considered attenuated in virulence (Pilgrim et al. 2003). However, some rough mutants were virulent in intraperitoneal murine models and also exhibited some degree of virulence perorally (Lammerding et al. 1992). Similarly to the *hly* and *prfA* mutants discussed above, rough mutants are infrequently detected in foods or food processing plants, and are not typically recovered from invasive human listeriosis.
4. Mutations in *inlB*. Analysis of the genome of F2365 (Jalisco cheese, 1985 California outbreak strain) (Nelson et al. 2004) revealed a premature stop codon mutation in *inlB*, a cell wall-associated host cell invasion protein of *L. monocytogenes* (Braun et al. 1997; Disson et al. 2008). The *inlB* mutations was confirmed by re-sequencing of the F2365 genome and found to be absent from other strains of the same clone (CC1, or ECI) (Nightingale et al. 2007). However, inclusion of F2365 in other virulence studies has indicated that this strain exhibits normal virulence (Faith et al. 2012). The prevalence and virulence impacts of such spontaneously-arising *inlB* mutations in *L. monocytogenes* need to be further characterized. The growing number of strains with WGS data will undoubtedly contribute to elucidating the prevalence of these mutations.

Modified threshold criteria for *inlA* PMSC mutants or for uncommon isolates with mutations in other virulence genes (e.g. *hly*, *prfA*, *iap*, *inlB*) are currentlynot well justified, either because of the above-discussed ambiguity about the actual potential of the strains to cause human disease or due to low incidence. In addition, detection of rare variants in foods or food processing plants may well indicate the simultaneous presence of other, undetected strains with potentially typical phenotypes and virulence, an argument that also holds in regard to PMSC mutations in *inlA*.

Whole Genome Sequencing (WGS)-Based Insights on Virulence Differences Among *L. monocytogenes* Strains

Since 2013 there has been an unprecedented increase in the numbers of sequenced *L. monocytogenes* genomes with thousands of strains already having WGS determinations. In conjunction with other federal agencies in the US, the Centers for Disease Control and Prevention (CDC) has been validating the use of WGS in routine *L. monocytogenes* surveillance in illness, foods and food processing plants as well as in outbreak detection and investigation, and an entire chapter in this book is in fact dedicated to the molecular epidemiology applications of WGS and other next generation sequencing approaches.

I. **Early WGS investigations: focus on major "epidemic clones" and outbreak-associated strains**. The first WGS efforts with *L. monocytogenes* were directed towards the extensively studied strain EGD-e (Glaser et al. 2001) followed by

strains from different outbreaks (Nelson et al. 2004), as well as strains of the same epidemic clone obtained at different times from the same processing facility and from food-patient pairs (Orsi et al. 2008). The findings consistently reaffirmed the conservation of known virulence genes in *L. monocytogenes* of different serotypes (1/2a, 4b) and clonal complexes ("clones"). These earlier WGS investigations with focus on outbreak-related strains also revealed the presence of unique genes and strain-specific gene clusters, i.e. components of the accessory genome portion. Examples were prophage sequences, plasmids and unique chromosomal genes or gene cassettes of variable size (from a few kb to almost 50 kb), such as the large (ca.80 kb) plasmid pLM80 of strain H7858 (CC6) and the large (ca. 50 kb) *Listeria* genome island, LGI1, of the Canadian outbreak strain (CC8) (Nelson et al. 2004; Cheng et al. 2010; Gilmour et al. 2010; den Bakker et al. 2013; Kuenne et al. 2013). Only a few of these members of the accessory genome have yielded clues regarding potential impacts on functional attributes or adaptations, including those related to virulence. These include:

1. Tn*916*-like ICEs. *L. monocytogenes* EGD-e harbors a Tn*916*-like ICE which, as discussed above, includes a *cadAC* cadmium resistance cassette. Expression of *cadC was* markedly induced in the liver of infected mice and determined to be required for normal virulence levels in the murine model, with virulence assessed by extent of growth of the bacteria in the liver following intravenous inoculation (Camejo et al. 2009).
2. Gene islands mediating resistance to chemical stressors or phage. WGS of strain H7858 (CC6, also designated ECII) revealed that it harbored not only pML80 which encodes a composite transposon with genes for resistance to cadmium (*cadAC*), quaternary ammonium disinfectant (e.g. benzalkonium chloride) (*bcrABC*) and triphenylmethane dye (e.g. crystal violet, malachite green) (*tmpA*) (Nelson et al. 2004; Elhanafi et al. 2010, Dutta et al. 2014) but also a number of unique chromosomal islands (Nelson et al. 2004; Cheng et al. 2010). One of the latter includes a gene cassette with a novel, thermoregulated restriction-modification system. The restriction enzyme gene is expressed primarily at low temperature (≤30 °C) thus rendering the host bacteria uniformly and completely resistant to phage provided that they were grown at a low temperature (Kim et al. 2008, 2012). Such functions may or may not have direct virulence involvements but do have direct food safety implications. In addition to *bcrABC*, at least two other, unrelated genomic islands are associated with quaternary ammonium disinfectant resistance in *L. monocytogenes* (Müller et al. 2014; Kovacevic et al. 2015). Disinfectant-resistant strains may have an advantage in processing plants, and exposure to disinfectants has been found to induce virulence genes (Kastbjerg et al. 2010). Strains with resistance to disinfectants are more likely to remain viable and undergo such gene expression alterations. Phage resistance at low temperature confers greater fitness to these strains in food processing plants and foods, where low temperatures generally prevail, or in applications of phage as biocontrol.

Another pertinent example is a chromosomal island (designated *Listeria* genomic island 2, or LGI2) in strain Scott A (CC2, also known as ECIa or ECIV) which harbors multiple genes with putative heavy metal (arsenic and cadmium) resistance functions along with a gene encoding a putative cell wall-associated protein with the LPXTG motif and ABC transporters (Briers et al. 2011; Lee et al. 2013; C. Parsons and S. Kathariou, unpublished). Many ECIa strains besides Scott A harbor these heavy metal resistance genomic islands (Lee et al. 2013; Lee and Kathariou, unpublished). Additional studies are needed to elucidate the potential role of the LGI2-mediated adaptations on virulence or other food safety-related attributes.

Several studies have revealed that different *L. monocytogenes* strains harbor islands with different sequence content at the same chromosomal locus, which appears to constitute a "hotspot" for acquisition of new genes (den Bakker et al. 2013; Kuenne et al. 2013). Different virulence-associated genes were detected on some of these hotspot-associated islands. For example, the Listeriolysin S pathogenicity island (LIPI-3) described above is harbored by certain strains of lineage I (serotypes 1/2b, 3b and 4b) while EGD-e harbors the Tn*916*-like ICE, also described above, at this same genomic location (Cheng et al. 2010).

II. **Real-time, large-scale WGS and implications for virulence gene screening and analysis**. There is currently a major paradigm shift in *Listeria* surveillance and outbreak investigations in the US, with all *L. monocytogenes* isolates from patients, foods and other sources undergoing WGS in real time. The query arises, what are some anticipated contributions of WGS of this type and scale to our understanding of potential virulence differences among *L. monocytogenes* strains? We are currently at the cusp of this unprecedentedly game-changing and massive wave of information, and many outcomes remain to be discerned. Nonetheless, there are also several documented as well as foreseeable applications and insights, including the following:

1. Genotyping and surveillance though WGS-driven platforms: detection of zproblematic strains. Described as "the first distributed network of laboratories to utilize whole genome sequencing for pathogen identification", the FDA's Genome Trakr Network is now involved in routine surveillance, source tracking and outbreak investigation for foodborne pathogens (http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS/ucm363134.htm). Genome Trakr is currently deployed in the US in conjunction with PFGE and other genotyping tools (e.g. MLVA) but has the capacity to eventually be implemented on its own for molecular epidemiology applications at the local, national and international level. Strain genotyping and cluster detection via Genome Trakr will provide real-time data with tremendous potential for revealing virulence-related information. Strain genotypes determined by a variety of analytical tools such as high-resolution applications of multi-locus sequence typing (core genome MLST, whole genome MLST etc.) and single nucleotide polymorphism (SNP)-based genotypic schemes can rapidly determine whether isolates from food and environmental sources match those previously implicated

in human disease. Detection of isolates representing "hypervirulent" clones implicated in multiple outbreaks or frequent human disease (Kathariou 2003; Ragon et al. 2008; Cantinelli et al. 2013; Chenal-Francisque et al. 2011; Maury et al. 2016) would signal clear potential for high-risk contamination, since such strains are "repeat offenders" with undisputed capacity to cause human disease. Thus, WGS-based data can be used to inform estimates of the likelihood for implication of certain genetic elements in disease.

The same reasons, i.e. the high genotypic resolution and real-time analysis afforded by WGS-based tools will also guide attention to any other food or environmental strain with a genotype closely related to one previously detected in human listeriosis, even if such a strain type has been rarely encountered before. After all, several strain genotypes were uncommon prior to their first recognition in an outbreak. Poignant examples include clonal complex 6 (CC6, also known as ECII), which were not known to be associated with outbreaks until the 1998–1999 hot dog outbreak (Kathariou 2002; Graves et al. 2005) and new clones that were first recognized following their involvement in outbreaks in 2011 (cantaloupe) and 2014 (stone fruit, ice cream etc.) in the US (Lomonaco et al. 2013; Burall et al. 2016). Another example involves lineage III strains (serotype 4a, 4c and certain serotype 4b strains), which are often considered animal-adapted and virulence-attenuated for human listeriosis (Orsi et al. 2011). Human listeriosis outbreaks involving lineage III strains have not been reported, and the likelihood of such an outbreak is considered remote based on available epidemiologic data. However, some lineage III strains were identified among isolates from human sporadic cases of listeriosis in the US (Lee et al. 2014). It was intriguing that several of these isolates had the same (potentially human-adapted) haplotype based on the multi-locus genotyping scheme (MLGT) (Lee et al. 2014). Thus, in spite of their generally low representation in human listeriosis, certain lineage III strains would justify attention if detected in food.

2. Genotyping and surveillance though WGS-driven platforms: detection of potentially virulence-attenuated strains. The premature stop codon mutations (PMSCs) in *inlA* (Internalin) remain the most extensively characterized source of commonly encountered natural variants with potential attenuation in virulence. As described above, most *inlA* PMSCs are at specific sites in the coding sequence, and identification of these known *inlA* PMSCs resulting in truncated Internalin has already been successfully incorporated in sequence-based genotypic tools such as MLGT (Ward et al. 2010). Mining of WGS data can readily identify these and other, novel PMSCs as well as other genetic changes that might interfere with Internalin production or anchoring on the surface. It is anticipated that such a systematic and targeted analysis of WGS data will address many existing gaps regarding prevalence, serotype, source, and regional distribution of these strains. Such data will provide statistical strength for more accurate estimates of the likelihood of strains with specific *inlA* PMSCs being involved in human disease. If the incidence of a strain with a certain *inlA* PMSC mutation is either extremely low or below detection in human disease and yet genotypically similar isolates with the same muta-

tion are commonly encountered in foods, it may be justified to confer tentative low risk designations to these isolates, while recognizing their potential to serve as indicators for the presence of other strains that may be more likely to cause disease.

As discussed above, occasional human clinical isolates with *inlA* premature stop codons have been encountered (Jacquet et al. 2004). At some point in the future, culture-independent analysis of WGS or of specific *L. monocytogenes* genes (e.g. *inlA*) directly from clinical samples (e.g. blood, cerebrospinal fluid, placenta) may more accurately address the prevalence of such mutations in clinical isolates, to exclude the likelihood that mutations accrue in culture.

3. Genotyping and surveillance though WGS-driven platforms: detection of profile signature variants in *L. monocytogenes'* "virulome". The virulence gene repertoire of *L. monocytogenes* is impressively rich and is still being archived: Dozens of chromosomal determinants have been identified that play roles in virulence based on altered performance of mutants in various virulence models (Camejo et al. 2009; Cabanes et al. 2008). Their impact on virulence can be subtle or profound. The emerging paradigm with *L. monocytogenes* is one with a huge multiplicity of virulence-related sequence elements, both coding and non-coding, both in the core and the accessory genome, which can be termed the organism's "virulome" and which contribute to this pathogen's interactions with animal hosts and to the outcome of infection. It is also apparent that the virulence findings in the various model systems may not always or accurately reflect a determinant's involvement in human illness.

   The *L. monocytogenes* virulome is a genomic resource that, if analyzed carefully, appropriately and collectively, has huge potential for insights in regard to virulence assessments of specific strains. The same tools that have revolutionized our understanding of the virulence determinants of the pathogen are also deployable (and have already been deployed on several occasions) to elucidate host cell responses to *L. monocytogenes* infection, revealing processes never imagined before, ranging from host cell epigenetics ("patho-epigenetics") to mitochondrial fragmentation and modulated interference with mitochondrial functions (Cossart and Lebreton 2014). We are actually now at the infancy of a novel era in characterization of virulence of *L. monocytogenes* (and other pathogens), where WGS analysis of the genome and in depth assessments of expression profiles and proteomic signatures, among other approaches, together with similar, parallel host-centered approaches offer the promise of finally providing glimpses of the pathogen's capability of causing disease at levels of true biological relevance. In this just-now-emerging era, tools that can assess relative virulence may involve complex virulome signatures of the pathogen as well as host-response signatures at transcriptomic, patho-epigenetic, metabolomic or other levels. The sensitivity and overall capacity of such tools will have to be validated with epidemiologic data and virulence models. Such future validation efforts, however, will have major advantages over those of the past, largely due to the much higher molecular resolution and real-time capacity afforded by WGS platforms and other omics-based molecular biologic analytics not only for the pathogen but at the host level as well.

Strain-Specific Differences in Virulence:Insights from Epidemiological Investigations

Epidemiologic investigations frequently collect and present a rich assortment of data, and, as such, can serve as powerful "teachable moments" to place pathogens, hosts and the environment in meaningful contexts (Garner and Kathariou 2016). In the case of *Listeria* and listeriosis, studies on certain outbreaks can serve important hypothesis-building functions regarding virulence attributes of the implicated strains. Below are selected cases in point:

1. Chocolate milk febrile gastroenteritis outbreak, 1994: A hypovirulent clone, which at high levels causes different outcomes in healthy adults vs. children? Febrile gastroenteritis outbreaks typically involve previously healthy adults and foods with unusually high levels of *L. monocytogenes* (Schlech 1997). One such outbreak in the United States in 1994 involved chocolate milk consumed at a summer picnic and contaminated with a *L. monocytogenes* strain of serotype 1/2b, CC3 (Proctor et al. 1995; Dalton et al. 1997; Cantinelli et al. 2013). The levels of *L. monocytogenes* in the milk were quite high (approx. 10^9CFU/ml), apparently due to temperature abuse of the product which was contaminated at the dairy, and the median dose of *L. monocytogenes*/person could have been as high as 2.91×10^{11}cells (Dalton et al. 1997).

   Outside of the summer picnic event, only three invasive listeriosis cases involving this strain were epidemiologically traced to the contaminated chocolate milk (Dalton et al. 1997). Considering the wide market availability of the contaminated product, such relatively low incidence of invasive cases suggests that the implicated strain was of relatively low virulence. At the same time, it is intriguing that one of three invasive cases was a 2-year-old girl without known predisposing conditions, and the outbreak strain was also recovered during the outbreak period from another previously healthy child (5-year-old) with invasive listeriosis (Dalton et al. 1997; Proctor et al. 1995). *L. monocytogenes* can grow prolifically in chocolate milk, and it can be difficult to exclude temperature abuse of the product under certain conditions, even in situations beyond the summer picnic. Hence, it is conceivable that the children became ill with invasive listeriosis even in the absence of predisposing factors, following consumption of highly contaminated product, which, in adults, primarily caused febrile gastroenteritis.

   Further evidence supporting reduced virulence of the chocolate milk outbreak strain was obtained from a murine model of gastrointestinal listeriosis. In comparison to strain Scott A, intragastric inoculation of the chocolate milk outbreak strain yielded lower numbers of bacteria in the liver and spleen and less severe histopathological damage to these organs (Czuprynski et al. 2002). Furthermore, the chocolate milk strain appeared impaired in its capacity to grow in synthetic gastric fluid *in vitro* (Czuprynski et al. 2002).
2. Turkey deli meats outbreak, 2002 and Karoun cheese outbreak, 2014: A hypervirulent clone with subsequent diversification towards virulence attenuation? In 2002, a multistate outbreak of invasive listeriosis was traced to turkey deli meat

products contaminated with *L. monocytogenes* of serotype 4b, clonal complex 6 (CC6, also known as ECII) (Gottlieb et al. 2006; Cantinelli et al. 2013; Kathariou et al. 2006). One intriguing feature of the 2002 outbreak was that an estimated 13% (7 of the 54) cases lacked known predisposing conditions. Overall, case patients in this outbreak were significantly more likely than other listeriosis patients to be pregnant or nonelderly (<64 years of age) and with no known predisposing conditions (Gottlieb et al. 2006). As will be discussed below, this clone was among four that were recently designated as "hypervirulent" for invasive human invasive listeriosis based on epidemiological data from a large collection of strains in France, as well as animal model data (Maury et al. 2016). It would be of interest to determine the extent to which case patients without known comorbidities were also represented in the 1998–1999 hot dog-associated multistate outbreak in the US which also involved this clone and was traced to contaminated hot dogs (CDC 1999; Graves et al. 2005; Kathariou et al. 2006). CC6 (ECII) is a relatively recent clone, being first identified in the 1998–1999 hot dog-associated outbreak in the US, but has since been implicated in a significant portion of human listeriosis cases from the United States (Lee et al. 2014; den Bakker et al. 2008, 2010) as well as in human listeriosis cases in France and elsewhere (Ragon et al. 2008; Chenal-Francisque et al. 2011; Cantinelli et al. 2013; Maury et al. 2016).

The findings from the 2002 turkey deli meat-associated outbreak contrast with those from a more recent multistate outbreak in the US in which CC6 was implicated via contaminated soft cheese (CDC 2015b). Even though this latter outbreak was first detected in 2015, the WGS-aided outbreak investigation retrospectively identified several earlier cases over a 5-year (2010–2015) period, with 30 cases in total. These findings suggest a low incidence of illness in spite of long-term release of contaminated product. Even though other factors (e.g., levels of the bacteria in the product, consumption patterns) can also be involved, the findings raise the possibility that the implicated CC6 strains may represent a diversified population with lower virulence than observed with CC6–associated outbreaks before. In-depth comparative WGS analysis and experimental assessment of these strains in virulence models would be informative in this regard.

3. Caramel apple outbreak, 2014: an opportunity to assess differential virulence of the implicated strains? A multistate outbreak of listeriosis in 2014 involved caramel apples, a vehicle not previously identified in listeriosis (or any other foodborne disease). Two genetically distinct strains of serotype 4b were involved; one was a member of CC1 (also known as epidemic clone I, or ECI) whereas the other was a previously rare genotype, ST-382 (CDC 2015c; Garner and Kathariou 2016). Of the 35 case patients, 3 patients with meningitis were children (5–15 years old) without known predisposing conditions whereas approximately one third (11 of the 35 illnesses) were pregnancy-related (CDC 2015c).

   Of the two different genotypes involved in this outbreak, CC1 (ECI) is well known for its frequent involvement in outbreaks and sporadic listeriosis (Kathariou 2002; Ragon et al. 2008; Cantinelli et al. 2013; Lee et al. 2014; Maury et al. 2016). Similar to CC6 discussed above, and as will be further discussed below, CC1 is among the four clones recently identified as "hypervirulent" (Maury et al. 2016). It will be important to know whether in the caramel

apple outbreak CC1 (ECI) was more frequent among pregnancy cases or non-pregnant cases without known predisposing factors, thereby supporting the high virulence potential of this clone. Such analysis of the caramel outbreak cases may be complicated by the possibility of co-infection with the two strains, which was documented in one of the patients (CDC 2015c).

4. Cantaloupe outbreak, 2011: evidence for hypovirulent clones with unusually low proclivity for pregnant women? The largest listeriosis outbreak investigated to date (147 cases, 33 deaths) involved contaminated whole cantaloupe and occurred in the US in 2011 (McCollum et al. 2013). Besides the vehicle, which was novel for listeriosis, unusual attributes included the involvement of multiple strains of serotypes 1/2a and 1/2b, including members of two new clonal groups (Lomonaco et al. 2013; McCollum et al. 2013; Garner and Kathariou 2016). In comparison to previous outbreaks in which 12–66% of the cases are pregnancy-related (Swaminathan and Gerner-Smidt 2007), only 5% of the cantaloupe-associated outbreak cases involved pregnancy (McCollum et al. 2013). The attack rate among exposed pregnant women was estimated as approximately 1 per 10,000 (Imanishi et al. 2015). Additional studies are needed to compare this low attack rate to that in other outbreaks, e.g., the caramel apples-associated outbreak, in which 11 of the 35 cases involved pregnancy (CDC 2015c).

   In addition to the apparently low proclivity for pregnancy of the cantaloupe outbreak-associated strains, patients >60 years of age constituted an unusually high portion (86%) of cases in this outbreak, with the median age for men and nonpregnant women being 78 years (81 years among those who died). Most (85%) of the positive cultures were from blood, whereas only 5% were from cerebrospinal fluid (McCollum et al. 2013). No cases <60 years of age and not pregnant or immunocompromised were reported (McCollum et al. 2013). Currently available data suggest that this outbreak may have involved strains with limited potential to cause invasive illness via breaching the placental barrier or via central nervous system infection. Two of the clones implicated in this outbreak, CC5 (ECVI, of serotype 1/2b) and CC7 (ECVII, of serotype 1/2a), did not rank highly among those recently identified as hypervirulent and in fact CC7 was quite infrequent among either food (0.9%) or clinical isolates (2.9%) in a study of a large panel of *L. monocytogenes* isolates from France (Maury et al. 2016). Additional analysis of these strains in subsequent epidemiological investigations and experimental models will be needed to more definitively assess their relative virulence.

5. North Carolina outbreak, 2000: evidence for an unusual strain hypervirulent for pregnancy? This outbreak in Winston-Salem, NC in 2000 involved contaminated soft cheese and caused mostly pregnancy-related listeriosis (MacDonald et al. 2005). The serotype 4b strain that was involved had an unusual genotype, which has not been implicated in other outbreaks, and which was uncommon among human listeriosis isolates from the US (2005–2008) or France (2008–2013) (Lee et al. 2014; Maury et al. 2016). However, the fact that most of the cases were pregnant women suggested high virulence in this population. This was supported by experimental data, which revealed an unusually high virulence of the outbreak strain in an animal pregnancy model of listeriosis (Poulsen et al. 2011). The collective data suggest that this strain is hypervirulent for pregnant women,

but is uncommon in the human population for unknown reasons, possibly involving its fitness in foods or the environment.

6. Stone fruit outbreak, 2014; ice cream outbreak, 2014; and frozen vegetables outbreak, 2015: evidence for targeting highly specific risk groups? Detection and investigation of these outbreaks was greatly aided by WGS which identified cases retrospectively and also permitted the delineation of outbreak cases from cases infected with similar strains but not linked to the outbreak (Jackson et al. 2015; CDC 2015a, 2016; Burall et al. 2016). In all three outbreaks, the levels of *L. monocytogenes* were likely quite low due to the nature of the implicated products; the products were widely distributed; and the number of cases was small, suggesting an unusually low attack rate. For instance, only two patients were conclusively linked to the contaminated stone fruit, in spite of the wide distribution of the product (Jackson et al. 2015). The frozen vegetables-associated outbreak involved eight case patients over 2.5 years (CDC 2016). In the ice cream-associated outbreak, the product was distributed in hospitals, i.e. in populations with multiple co-morbidities, but the number of cases was still low (CDC 2015a). The collective findings suggest that these strain/product combinations may target a highly specific risk group, the attributes of which remain to be identified.

   It remains to be determined whether the continued use of WGS in surveillance will lead to more frequent detection of small outbreaks or single cases traced to widely distributed products with low levels of contamination, which may still lead to disease in highly specific risk group(s). In previous years, such incidents would have been considered sporadic cases without capacity for attribution to specific product(s).

Recent Applications of Genotype Surveillance, Whole Genome Sequencing and Targeted Assessments to Identify *L. monocytogenes* Populations (Clones) Varying in Virulence Potential and Host System Tropism

Pulsed-field gel electrophoresis (PFGE) has served as the gold standard for PulseNet foodborne disease outbreak surveillance since 1996 and relies on variable distribution of DNA sequences serving as recognition sites for enzymes (restriction endonucleases) which cut the DNA at the corresponding restriction site. For *L. monocytogenes* PulseNet applications, PFGE protocols utilize the enzymes AscI and ApaI (Halpin et al. 2010). Subsequent DNA sequence-based subtyping tools such as multilocus sequence typing (MLST) based on sequence analysis of fragments of seven conserved genes, multiple locus variable number tandem repeat analysis (MLVA), and multilocus genotyping (MLGT) have complemented the resolution of PFGE and provided additional advantages, especially in reduced subjectivity in the output and interpretation of the subtyping data and ability to provide identifiers unique to each subtype (Chenal-Francisque et al. 2013 and references therein; Ducey et al. 2007; Salcedo et al. 2003; Ward et al. 2008).

MLST has emerged as the system of choice for large-scale surveillance. In addition to the portability of the output in MLST, which, unlike banding patterns and images as in the case of PFGE, consists of unambiguous nucleotide sequence data, multiple laboratories can readily implement this typing scheme. A key attribute is the potential to obtain sequence type (ST) designations upon depositing the sequences electronically to the *Listeria* MLST database, maintained and curated at the Pasteur Institute (http://www.pasteur.fr/recherche/genopole/PF8/mlst/index.html). This can facilitate the assessment of worldwide distribution of specific sequence types (STs) as well as clonally-related groups of strains. Strains exhibiting identical sequences in 6 of the 7 gene fragments interrogated in the conventional MLST scheme are assigned the same "clonal complex" (CC) designation. Below are recently emerged and emerging trends and findings of major relevance to the theme of this chapter.

1. Applications of MLST and other sequence-based tools in detection of clones with potential differences in human virulence. The wide implementation and portability of MLST and other sequence-related tools for surveillance of *L. monocytogenes* strains from diverse sources, including humans, other animals, food and environment, has revolutionized our understanding of the worldwide distribution and diversity of *L. monocytogenes*, including association of certain genotypes with different sources and outcomes. A major investigation employing MLST analysis of a large panel (n = 6633) of clinical (n = 2584) and food (n = 4049) isolates of *L. monocytogenes* from France over a 9-year period (2005–2013), revealed 63 different clonal complexes ("clones"). Certain of these MLST-based clones were disproportionately represented among clinical vs. food isolates (Maury et al. 2016). For instance, the serotype 4b (lineage I) clonal complexes 1–4 (CC1, CC2, CC4 and CC6) were significantly more common among human clinical isolates than among those from food, whereas the opposite was observed with the most numerous clonal complex, CC121, of serotype 1/2a (lineage II), which was significantly over-represented among food isolates. When examined individually, certain clones were significantly more likely to consist of clinical isolates than isolates from foods. At the extreme ends of the spectrum were CC4, in which 71.3% of the isolates were of clinical origin, and CC121, which consisted primarily (93%) of food isolates (Maury et al. 2016). Such findings may suggest that clones such as CC1, CC2, CC4 and CC6 have higher virulence for humans i.e. they may constitute "hypervirulent" clones, whereas the opposite may be the case for clones such as CC121, CC8/CC16 and CC9, which may constitute "hypovirulent" clones (Maury et al. 2016). CC1 was also recently determined to be a major clone among 520 *L. monocytogenes* isolates of human and food origin from Australia, 1995–2015 (Kwong et al. 2016), suggesting worldwide predominance of this potentially hypervirulent clone and supporting previous reports (Ragon et al. 2008; Cantinelli et al. 2013; Haase et al. 2014).

   The recent massive increases in whole genome sequencing data for *L. monocytogenes* has conferred the capacity to infer ST designations based on the seven-locus MLST scheme directly from WGS data. When implemented by laboratories with sufficient automation, *in-silico* extraction of ST and CC designations from WGS data is actually less expensive and less labor-intensive than

conventional PCR-and-sequencing-based approaches (Kwong et al. 2016). An additional major advantage of the availability of WGS data is the capacity to massively expand the number of gene fragments employed for MLST, thus greatly expanding the resolution of MLST typing. For instance, a core genome MLST (cgMLST) scheme has been developed based on approximately 1700 coding sequences conserved (core genome) among all *L. monocytogenes* strains (Schmid et al. 2014; Ruppitsch et al. 2015), whereas whole genome MLST (wgMLST) utilizes the analysis of a pangenome of 4797 coding sequences identified based on all published *L. monocytogenes* genomes (Jackson et al. 2016). Routine implementation of cgMLST or wgMLST in surveillance is certain to yield more accurate data (than conventional MLST) on the incidence of specific genotypes in human listeriosis and their relative frequency in clinical versus food sources. Such differences might reflect differences in virulence, as postulated based on analyses using conventional MLST data (Maury et al. 2016).

2. Host system tropisms and co-morbidity profiles as potential indicators of differences in human virulence. One application of special relevance in regard to detection of potential differences in human virulence is the high-resolution identification of genotypes associated with different outcomes of invasive listeriosis and potential host system tropisms. Strains derived from blood cultures and implicated in septicemia are clearly invasive and virulent, having breached the intestinal barrier. Even so, they may be less virulent than strains isolated from cerebrospinal fluid and thus capable of also breaching the blood-brain barrier and become involved in central nervous system infections (CNS cases), or those that also breach the blood-placenta barrier and become implicated in pregnancy-related listeriosis and mother-neonatal infections (MN cases).

   Analysis of clonal distribution among different disease outcomes has indeed revealed that presumably hypervirulent clones such as CC1, CC2, CC4 and CC6, with higher representation among clinical than food sources, tend to be more often implicated in high-invasiveness outcomes such as CNS and MN cases and are less likely to be isolated from listeriosis that only involved septicemia (Maury et al. 2016). Interestingly, host tropism differences were observed among the hypervirulent clones, with CC1 being significantly associated with both CNS and MN cases whereas CC2 and CC4 had significant proclivity for MN infections. In contrast, food-associated clones such as CC121, CC8/CC16 and CC9 were significantly associated with septicemia without accompanying CNS or MN infections, supporting their presumed hypovirulence (Maury et al. 2016).

   Host co-morbidity profile analysis has provided further support for the presence of hypervirulent and hypovirulent clones among strains implicated in human listeriosis. An inverse relationship was observed between the number of predisposing conditions and the prevalence of certain clones. Specifically, the hypervirulent clones CC1, CC2, CC4 and CC6 were commonly found to cause disease in patients who seemed otherwise healthy or had few known co-morbidities. In contrast, patients infected with CC121 and CC9 (hypovirulent clones) tended to be highly immunocompromised, having multiple predisposing conditions (Maury et al. 2016). Such findings suggest that there are differences in viru-

lence potential among pathogenic *L. monocytogenes* strains. Even though listeriosis is generally associated with immunocopromised individuals, the degree of immunosuppression may make a difference in terms of the clone that is implicated. At one end of the spectrum are certain limited-virulence clones, which are only able to cause illness in highly susceptible individuals, in whom the illness appears to be primarily septicemia, without further CNS or MN involvement. At the other end of the spectrum are hypervirulent clones which can cause illness in individuals with few or no known predisposing conditions, with illness frequently becoming invasive, as in CNS and MN cases. This latter population of individuals appears to resist infection by hypovirulent clones.

Challenges and Opportunities Regarding Determination of Hyper- and Hypovirulent Clones

Elucidation and further interpretation of the findings described above regarding hyper-and hypovirulent clones is certain to become enhanced in the near future with the increased application of high-resolution strain genotyping and bacteriological assessment tools. One attribute that may confound the findings is the presence of pre-existing virulence-attenuating mutations in certain clones. A case in point involves premature stop codons (PMSCs) in *inlA*, which as discussed above are widespread among serotype 1/2a, 1/2b and 1/2c isolates from food, but are rare among food-derived serotype 4b isolates. Hypervirulent clones (CC1, CC2, CC4, CC6) are all of serotype 4b and unlikely to harbor *inlA* mutations when growing in food, thus constituting a relatively homogeneous population of potentially virulent cells. This may make up for their relatively infrequent presence in foods, which tend to be more commonly contaminated with isolates of serogroup 1/2. In contrast, populations of serogroup 1/2 clones may be partitioned into pathogenic (full length InlA) and attenuated-virulence populations with truncated InlA, with the latter unable or unlikely to cause human disease. Hence, even though serogroup 1/2 strains are more likely to contaminate food, a substantial fraction may be unable to cause illness. In attributing likelihood to be implicated in disease, it is important to consider not only the MLST-based clone designation but also the extent to which a specific clone is likely to be populated by isolates harboring virulence-attenuating mutations. In this context, the accuracy of identification of hypervirulent clones might be enhanced by assessments within each serogroup, e.g. comparing clinical versus food prevalence of clones within the serotype 4b population. It will be of interest to determine whether such an analysis will still identify CC1, CC2, CC4 and CC6 as potentially hypervirulent based on a greater likelihood to be encountered in clinical cases vs. foods than other serotype 4b clones.

Another challenge currently involves better elucidating the ingested doses of *L. monocytogenes* in foods implicated in illness by different clones as well as illness with different outcomes and in patients with different co-morbidity profiles. The

challenge concerns the lack of data on clone-specific growth potential of different clones in foods implicated in listeriosis. Clones that commonly contaminate food may not necessarily be able to grow to high levels, whereas the opposite may occur with other clones, which are infrequently associated with food but may be able to grow well there. Enrichment-based recovery protocols for *L. monocytogenes* in food typically do not enable the determination of actual *L. monocytogenes* populations in the food. Furthermore, the levels of listeriae in routinely surveyed foods may well be different from those in foods associated with specific illness incidents, and foods are frequently contaminated with multiple strains and clones of *L. monocytogenes*. Laboratory assessments of the potential of different clones to grow in listeriosis-implicated foods may partially address this data gap. Accurate assessments are difficult because it may be challenging (and frequently impossible) to adequately simulate food matrix, other microbiota (including additional listeriae or other *L. monocytogenes* clones), a contamination scenario and conditions pertinent to a food that was associated with disease. Metagenomic analysis of foods associated with illness and direct methods for determining quantitative levels (e.g. a qPCR assay specific for an illness-associated clone) would be useful in addressing these gaps. The more rapid detection of outbreaks now made possible with the use of WGS may be helpful in this regard, as the implicated foods will have a greater likelihood of being identified while still available at retail or at the processing plant for metagenomic and other analyses. The long incubation time for listeriosis will continue to create challenges in this regard. On the other hand, since 2013 all *L. monocytogenes* isolates obtained by the USDA-FSIS, FDA and the CDC are being sequenced on a real-time basis in the United States, greatly enhancing the system's ability to detect relevant foods and determine population levels of the clones contaminating the food (CDC 2015d).

Metagenomic and qPCR analyses of foods with similar parallel analysis of clinical samples may prove to be especially valuable in ascertaining relative *L. monocytogenes* levels in food vs. clinical samples for mixed-strain outbreak scenarios. Multiple strains were implicated in at least two recent common-source listeriosis outbreaks, including the 2011 cantaloupe-associated outbreak, with three distinct clones of two different serotypes (1/2a and 1/2b), and the 2014 caramel apple-associated outbreak, with two distinct clones of the same serotype (4b) (Garner and Kathariou 2016). In the latter outbreak, evidence for coinfection was obtained, albeit inadvertently (CDC 2015c). Determining relative ratios of the different clones in food vs. disease may prove highly informative in revealing potential virulence differences among these pathogenic clones. For instance, as mentioned the caramel apple outbreak involved two distinct serotype 4b clones: CC1, which is a hypervirulent clone (Maury et al. 2016) and ST382, which has been uncommonly implicated in human listeriosis (Garner and Kathariou 2016). It would have been of interest to determine (1) the extent to which the two clones grew in the product, and (2) whether CC1 made a greater contribution to illness than might be anticipated based on its prevalence in the product, in accordance with its hypervirulent status. It would also be of great interest to determine the relative incidence of the two strains in outbreak patients with different predisposing conditions. As described

previously, three patients in this outbreak were otherwise healthy children (3–15 years of age), hence it is tempting to speculate that the hypervirulent clone CC1 was implicated in their illness.

Experimental Evidence for Hypervirulent and Hypovirulent Clones with Different Invasiveness Potential, and Some Caveats on the Choice of Prototype Strains

Many different cell culture and animal models have been utilized to assess potential differences in virulence among different *L. monocytogenes* strains (e.g. Lecuit 2007; Cabanes et al. 2008; Hoelzer et al. 2012; Disson and Lecuit 2013; D'Orazio 2014; Bou Ghanem et al. 2013). Earlier studies systematically assessed strain panels of diverse serotypes and sources to identify potential differences in outcomes in various animal or cell culture models (e.g. von Koenig et al. 1983; Stelma et al. 1987; Hof and Hefner 1988; Pine et al. 1990, 1991; Hof and Rocourt 1992; Lammerding et al. 1992; Barbour et al. 1996, 2001; Czuprynski et al. 2002; Takeuchi et al. 2003, 2006; Faith et al. 2006). These investigations consistently determined that strains differed in virulence in the various models that were employed. However, the earlier studies were largely done prior to the advent of strain genotyping tools that allowed strain genotype designations to be readily transferrable among different laboratories, limiting assessment of whether certain strain genotypes and clonal groups differed in virulence in the respective model.

One of the early investigations revealed that serotype 4b strains tended to be more virulent in murine models infected via different routes, even though infection by any *L. monocytogenes* strains could confer protection to subsequent challenge (von Koenig et al. 1983). These are of special interest in light of the finding, more than three decades later, that all four hypervirulent clones identified and assessed with different murine models and route of inoculation also are of serotype 4b (Maury et al. 2016).

As the focus on elucidation of fundamental host-pathogen interactions and listerial pathogenesis mechanisms intensified, investigations increasingly utilized single strains for genetic constructs and other analyses of virulence and host-pathogen interactions. A few strains became especially prominent as models in the analysis of *L. monocytogenes* virulence; these largely include two closely related serotype 1/2a strains, EGD (originally isolated from guinea pigs) and 10403S (isolated from human skin lesions), and the serotype 1/2a strain EGDe, which was the first *L. monocytogenes* strain to have its genome completely sequenced (Glaser et al. 2001) but which is of unknown origin and has been found to be unrelated to strain EGD (Bécavin et al. 2014). The serotype 1/2c strain LO28 (isolated from human feces) has also been extensively utilized in virulence studies.

These strains have been extremely valuable in fueling much of our current understanding of basic listerial pathogenesis mechanisms and virulence determinants. However, their nonhuman (in the case of EGD) or noninvasive (in the case of

10403S and LO28) origin and their serotypes compromise extrapolation of findings to strains implicated in invasive human listeriosis, which frequently are of serotype 4b (together with 1/2a and 1/2b) and most of which are of other genotypes/clones. Indeed, a recent analysis of a large group of clinical and food isolates from France suggested that these strains represent clones that are not major contributors to human listeriosis: EGDe and LO28 belong to the food-associated clone CC9, which has been encountered in 10.3% of clinical isolates, whereas EGD and 10403S are members of CC7, which has been infrequently associated with isolates of either food (0.9%) or clinical sources (2.9%) (Maury et al. 2016). In addition, early studies using the intragastric inoculation in the murine model revealed that EGD was less virulent than serotype 4b strains based on the numbers of listeriae detected in the liver and spleen of the animals (Czuprynski et al. 2002; Faith et al. 2006).

Such findings were more recently confirmed and extended when strains EGDe and 10403S were compared to others using oral inoculation in a "humanized" murine model that allowed efficient binding between InlA on the bacterial surface and its receptor, E-cadherin, on the murine enterocytes (Disson et al. 2008; Maury et al. 2016). The hypervirulent clones CC1, CC4 and CC6 produced significantly greater weight loss than EGDe or 10403S; in addition, strains representing these clones were significantly less invasive in the liver or the brain than some of the hypervirulent clones (Maury et al. 2016). The brain invasion data are especially interesting: they suggest that even though these strains have been extensively utilized as prototypes in identification and characterization of virulence determinants and analysis of listerial pathogenesis overall, their neurotropic potential may not reflect that of clones frequently implicated in human listeriosis.

Using oral inoculations of *L. monocytogenes*in the humanized model described above has yielded agreement between infection outcomes and the virulence ranking of clones based on their relative prevalence among clinical vs. food isolates. Weight loss was not observed following inoculation of the animals with multiple isolates of CC9 and CC121, which are clones that tend to be significantly more common among food than among clinical isolates, whereas significant weight loss accompanied infection by hypervirulent clones such as CC1 or CC6 (Maury et al. 2016). In addition, in comparison to mice inoculated with CC9 and CC121 isolates, animals inoculated with CC1 and CC6 yielded greater numbers of listeriae in the liver as well as brain, whereas greater numbers exclusively in the brain were observed following inoculation with CC4 isolates (Maury et al. 2016).

In these assessments, isolates of each clone were from food as well as from representative types of clinical categories (septicemia, CNS or MN cases) (Maury et al. 2016), suggesting that the observed virulence metrics were clone-specific, and not detectably impacted by the source of the isolate. However, WGS analysis of isolates from different sources will greatly enhance our understanding of intra-clone diversity, especially as this might reflect source or virulence differences in the humanized mouse or other models.

Are There Virulence Determinants Unique to Hypervirulent Clones?

The focus on specific prototype strains for analysis of listerial pathogenesis delayed the identification of determinants and attributes that may uniquely contribute to the apparent hypervirulence of clones such as CC1, CC2, CC4 and CC6. Genome sequencing and subsequent studies with strains of CC1 and CC6 (earlier referred to as ECI and ECII, respectively) revealed several genes unique to these clones (Nelson et al. 2004; Yildirim et al. 2004, 2010; Chen and Knabel 2007; Cheng et al. 2010; Kim et al. 2012). However, the use of isogenic constructs has failed to yield direct experimental evidence for involvement of such clone-specific genes in virulence. Inclusion of isogenic constructs in alternative models such as the humanized mouse model and assessment of internal organ and brain invasion may further elucidate the potential involvement of clone-specific genes in virulence.

CC4 has recently received special attention in terms of possibly possessing unique virulence determinants. This serotype 4b clone has not been recognized in outbreaks in the US but was detected in a 1999 outbreak of listeriosis in France associated with pork rillettes (de Valk et al. 2001; Cantinelli et al. 2013) as well as a 2013–2014 outbreak in Switzerland associated with salads (Stephan et al. 2015; Tasara et al. 2015). As mentioned earlier, clinical isolates constituted a major portion (71.3%) of CC4, more so than was observed with any other clone (Maury et al. 2016). It can be recalled from earlier discussion in this chapter that, similarly to other hypervirulent clones, CC4 was more frequently implicated in CNS and MN cases than cases with septicemia, and infected individuals with relatively few or no detected predisposing conditions (Maury et al. 2016). Also, following oral inoculation in the humanized murine model, the levels of CC4 isolates in the brain were greater than those observed with any of the other clones tested, whereas the levels in the liver were not unusually high (and in fact were lower than observed with CC1 or CC6), suggesting potentially high neurotropism of CC4 (Maury et al. 2016).

WGS-based analysis revealed only a few (n = 19) CC4-specific genes (Maury et al. 2016). Of special interest was the six-gene cluster (cellobiose-family phosphotransferase system) designated as *Listeria* Pathogenicity Island 4 (LIPI-4), which, when deleted, specifically reduced brain invasion of the mutant without impacting bacterial levels in other organs. In a murine pregnancy model, the mutant was additionally impaired in fetal and placental infection (Maury et al. 2016). These findings support the hypothesis that LIPI-4 specifically contributes to the ability of this clone to cause human CNS and MN infections, and to the apparent hypervirulence of this clone. It remains to be determined whether other clone-specific genes of CC4 uniquely contribute to the neurotropism and MN infection potential of this clone.

Determinants and attributes that contribute to CNS and MN infection potential in other hypervirulent clones, specifically CC1, CC2 and CC6 remain to be elucidated. Similarly, attributes associated with the hypovirulence of clones such as CC9 and CC121 remain to be identified. It would be of interest to determine whether such hypovirulent clones can become endowed with a greater ability for causing CNS or

MN infections upon acquisition of genes or gene clusters (e.g. LIPI-4) associated with hypervirulent clones. LIPI-4 is an example of a genomic pathogenicity island that is highly restricted in its distribution among different *L. monocytogenes* strains, being only detected in CC4 but not other strains of the same serotype or lineage; in contrast, the earlier-mentioned LIPI-3 (which includes the gene encoding Listeriolysin S) was detected in several clones within lineage I (serotypes 4b and 1/2b) (Cotter et al. 2008) whereas LIPI-1 (which includes *hly*, encoding Listeriolysin O) is conserved among *L. monocytogenes* of diverse serotypes (Vazquez-Boland et al. 2001); LIPI-2 is the LIPI-1 counterpart in *L. ivanovii* (Dominguez-Bernal et al. 2006). If clone-specific genes and gene clusters (presumably acquired by horizontal gene transfer, or representing residues of ancestral systems) indeed uniquely mediate specific virulence responses in *L. monocytogenes*, it will be critical to elucidate ecological and evolutionary mechanisms for their acquisition, maintenance and potential dissemination among different *L. monocytogenes* strains.

Conclusions

It is evident that we are at an exciting time in regard to the issue of virulence differences among *L. monocytogenes* strains. The progress in elucidating virulence differences among clones and strains of *L. monocytogenes* is the fruit of the labor and commitment of numerous laboratories throughout the world. The past few decades have witnessed intense development, refinement and implementation of strain subtyping tools, including the current application of whole genomic sequencing and other omics technologies. These have been accompanied by parallel pathogenesis studies of ever-increasing depth and sophistication. Collectively, such efforts have brought us to a point where human listeriosis and its underpinnings in food and the environment can be investigated in real time, obtaining findings with direct biomedical relevance. We are thus now at a point where not only can we better address the earlier-articulated query, "Is any strain of *Listeria monocytogenes* detected in food a health risk?" (Hof and Rocourt 1992), but are also better prepared to address the query, "Is any strain of *Listeria monocytogenes* from human disease equally threatening to human health?" The next few years promise to lead to discoveries that will further increase our ability to address these and related queries and to approach *L. monocytogenes* in the food supply, the environment and disease utilizing more informed and proactive responses.

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The *Listeria monocytogenes* Triad for Success: Food Matrix, Stress Response and Virulence

Maria Leonor Faleiro

Abstract As with most foodborne pathogens, *Listeria monocytogenes,* during its life cycle, encounters many environmental stresses, beginning with its residence in soil, through the food production chain until it encounters host challenges to accomplish a successful infection process. Exposure to a single stress or a combination of stresses can compromise the integrity of the bacterial cell, and to circumvent those injuries the pathogen is equipped with mechanisms that can sense sublethal stress and trigger its gene expressing arsenal to reprogram its mode of survival by expressing stress tolerance factors. Such tolerance factors were initially identified using laboratory media. However, these approaches did not reliably translate those tolerance responses in real food matrices that the pathogen requires, and the molecular elements associated with those responses. Using different omics approaches, recent studies are revealing the tolerance responses associated with different food matrices and their possible impact in enabling the pathogen to better survive *in vivo* stress challenges. This knowledge is useful for developing new and more efficient control strategies and improving food safety, especially for minimally processed foods.

Keywords *Listeria monocytogenes* • Listeriosis • Food matrix • Stress response • Virulence

Introduction

Listeriosis, the disease caused by the foodborne pathogen *Listeria monocytogenes,* causes high mortality in at-risk populations, with an approximate 20% death rate (Silk et al. 2012). Despite low incidence of disease, an increased number of reported cases of listeriosis in the European Union (EU) in 2014 was reported (0.52 cases per 100,000 persons, corresponding to an 30% increase over the previous year (EFSA and ECDC 2015a, b). Also the number of deaths had increased; in 2014 210 deaths occurred (EFSA and ECDC c 2015b). In 2014, fishery products either at processing

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plant or at retail (mostly smoked fish) achieved the highest level of non-compliance with EU *L. monocytogenes* criteria followed by soft and semi-soft cheeses, RTE meat products and hard cheeses (EFSA and ECDC 2015b).

In contrast to the EU, in the USA, in 2013 and 2014, reported listeriosis cases were much less (0.26 cases per 100,000 persons in 2013 and 0.24 in 2014, respectively) (CDC 2014, 2015). In the USA, two recent outbreaks were reported, one associated with the consumption of caramel apples and the other with ice cream products (http://www.cdc.gov/listeria/outbreaks/index.html). Hence, there is a broad range of food products that pose a risk of disseminating *L. monocytogenes*. It is now well recognized that when *L. monocytogenes* encounters environmental stresses (either in a food processing environment, food products or during its host passage) it is capable of developing tolerance responses that not only enable the survival of the pathogen but also enhance its virulence. Besides this extraordinary ability of *L. monocytogenes* to respond to conditions that endanger its survival, several other factors contribute to the occurrence of this foodborne disease, such as the increasing number of individuals with a compromised immune system, an increase in the elderly population, and changing eating habits involving a greater demand for raw, fresh and minimally processed foods (Callejón et al. 2015; Newell et al. 2010).

This chapter provides an overview of the adaptation mechanisms of *L. monocytogenes* and how the food matrix can influence these responses and the pathogen's virulence.

Food Production and Induction of Stress Responses

The range of food products that are at risk of *L. monocytogenes* contamination is broad, but human listeriosis cases are most strongly associated with ready-to-eat (RTE) food that can become contaminated after post-processing with high levels of the pathogen. A zero-tolerance policy (no detected *L. monocytogenes* in a specified amount of food) for the presence of *L. monocytogenes* in RTE foods has been mandated by the U. S. Department of Agriculture (USDA) and the U.S. Food and Drug Administration (Todd 2007). In the European Union, food safety criteria for the occurrence of *L. monocytogenes* in food is regulated (EC N° 2073/2005). The criteria established for RTE foods is for those that can support the growth of the pathogen – a limit of 100 CFU/g is allowed during their shelf life or absent in 25 g immediately before the product is delivered for distribution. A stricter criterion is applied to RTE to be consumed by infants or is for particular medical purposes for which there is no *L. monocytogenes* tolerance in 25 g. To the contrary, a less stringent criterion is applied to RTE foods that do not support the growth of *L. monocytogenes,* for which the limit is 100 CFU/g during their shelf life (EC N° 2073/2005). Also, the Codex Alimentarius guidelines provide analogous criteria on the application of general principles of food hygiene to the control of *L. monocytogenes* in ready-to-eat foods-CAC/GL 61-2007 (CAC 2007). All these criteria and guidelines are key to drive food processors to examine the presence of this pathogen both at the

manufacture and storage stages. The extensive and frequent occurrence of *L. monocytogenes* in the environment of food production locations and processing facilities makes food contamination very difficult to control.

L. monocytogenes, as with many other foodborne pathogens, must overcome a series of stresses during their life cycle, starting during their presence in soil where they are thought to thrive on decomposed plant material (MacGowan et al. 1994) and then when the bacterial cells are transported through the food production chain until their passage into the host. These stress conditions range from both physical and chemical treatments to biological challenges. In nature, this pathogen struggles with the depletion of nutrients thereby experiencing starvation, and must compete for these few nutrients with the autochthonous microbial population, and also experience a gradient of pH and temperature and the presence of antibacterial compounds (naturally occurring antibiotics and bacteriocins). In the food processing environment, *L. monocytogenes* must overcome many treatments/conditions, including heat and cold treatments (either at processing or during storage and distribution), increasing osmolarity through the addition of carbohydrates or NaCl, drying (e.g., vacuum and freeze-drying), radiation exposure (e.g., gamma and UV), low pH (fermented food [vegetables and dairy], fruit juices and addition of acids), oxidative stress (use of oxidative sanitizers [e.g., chlorine, H_2O_2], exposure to air), and use of preservatives (e.g., nitrate, nisin and other bacteriocins). In many foods, in particular fermented foods, *L. monocytogenes* is confronted with the presence of the indigenous microbes (e.g., lactic acid bacteria, yeasts and molds) that, through their fermentative activity, maintain a low pH and may produce antimicrobials (such as bacteriocins and H_2O_2) (Bowman et al. 2008, 2010; Carpentier and Cerf 2011; Melo et al. 2015; Nilsson et al. 2012; Wesche et al. 2009).

Exposure to one or a combination of these stresses will cause cell injury, thereby compromising not only listerial growth but also the pathogen's survival (Adrião et al. 2008; Ilhak et al. 2011; Ribeiro et al. 2006; van der Veen et al. 2008; Werbrouck et al. 2009) by affecting the integrity of the cell wall, cellular membrane, DNA, RNA and proteins (Alonso-Hernando et al. 2010; He et al. 2015; Melo et al. 2013b; Tamburro et al. 2015; van der Veen et al. 2007; Wesche et al. 2009; Zhang et al. 2015). *Listeria* cells, when sensing sublethal stress, trigger their gene-expressing arsenal to mitigate cell injury by producing repair enzymes to preserve cellular homeostasis and initiate mechanisms to eliminate the vehicle of stress (Cheng et al. 2015; Cotter and Hill 2003; Ghandi and Chikindas 2007).

Another important cellular event is cross adaptation occurring from prior exposure to a single stress (e.g., salt or low pH), which confers protection against different types of stress (e.g., heat or cold) (Adrião et al. 2008; Bergholz et al. 2012; Shen et al. 2015). The occurrence of cross adaptation in a psychrotrophic bacterium, such as *L. monocytogenes,* is a special concern for refrigerated, minimally processed ready-to-eat foods, one of the leading food vehicles of listeriosis outbreaks (Allerberger and Wagner 2010; Swaminathan and Gerner-Smidt 2007). In those types of food, storage under abusive temperatures favourable to *L. monocytogenes* growth, allow the pathogen to reach levels greater than 100 CFU/g at the point of consumption, which is considered a public health hazard to the general population (Bergholz et al. 2012; Lungu et al. 2009).

Cross adaptation also enables the pathogen to more effectively pass through the host as a result of prior exposure to stress either in the food processing plant environment or in the food product which induces cross protection against stressful conditions occurring *in vivo* (Abdullah and Calicioglu 2013; Begley et al. 2002; Ilhak et al. 2011; Melo et al. 2013c).

Furthermore, the isolation of antibiotic resistant *L. monocytogenes* strains from food, the food processing plant environment and ill humans is increasing (Antunes et al. 2002; Barbosa et al. 2013; Bertsch et al. 2014; Camargo et al. 2015; Gómez et al. 2014; Harakeh et al. 2009; Khen et al. 2015; Morvan et al. 2010; Obaidat et al. 2015; Walsh et al. 2001; Wang et al. 2015). Therefore, concerns have been raised regarding the acquisition of antibiotic resistance after preexposure of *L. monocytogenes* to food processing treatments (Al-Nabulsi et al. 2015; Allen et al. 2015; Alonso-Hernando et al. 2009; Kastbjerg and Gram 2012; Laursen et al. 2015).

Altogether, these tolerance responses increase the likelihood of transferring pathogens with higher resistance profiles to humans via food, thereby making control of listeriae an even more difficult task.

Tolerance Responses that Influence *L. monocytogenes* Survival

As described previously, *L. monocytogenes* can encounter a variety of stress conditions during its passage through the food chain. The ability of the pathogen to adapt to and overcome these stresses is essential to its survival in foods and food processing facilities, and enable the pathogen to survive *in vivo* stress conditions. The main mechanisms that *L. monocytogenes* uses to overcome these hurdles which are of particular importance to the food industry and can have an impact on the ability of the pathogen to overcome the challenges of the infection process are described below.

Heat Tolerance Response

Thermal processing is one of the most efficient food processing techniques to mitigate microbial food spoilage and contamination by foodborne pathogens, especially in dairy and meat products. Several factors govern *L. monocytogenes* heat resistance, including characteristics of foods, such as water activity and acidity (Bergholz et al. 2012; Skandamis et al. 2008). When bacterial cells are exposed to high temperatures, the production of heat shock proteins (HSP) is promptly induced (at a minimum of 45 °C) (van der Veen et al. 2007, 2009). HSPs comprise highly conserved chaperones (e.g., GroEL and DnaK) or proteases (e.g., ClpB and ClpP) involved in the refolding of heat-injured proteins or degrading incorrectly assembled or denatured proteins.

Cold Tolerance Response

Refrigeration is a primary preservation technique that extends the shelf life of foods by inhibiting microbial growth. The psychrotrophic nature of *L. monocytogenes* provides a challenge to control the pathogen's growth in refrigerated ready-to-eat food (Arguedas-Villa et al. 2010; Chan and Wiedmann 2009). *L. monocytogenes* endures cold temperatures by synthesizing cold shock proteins (CSPs) (Chan and Wiedmann 2009; Tasara and Stephan 2006; Wemekamp-Kamphuis et al. 2002). CSPs accomplish diverse functions, mainly as chaperones involved in the course of DNA recombination, transcription, translation and proper protein folding (Schmid et al. 2009). Several studies have revealed the contribution of CSPs to other stress tolerance responses, including osmotolerance (Schmid et al. 2009), oxidative stress (Loepfe et al. 2010) and virulence (Loepfe et al. 2010; Schärer et al. 2013), illustrating the complexity of the responses of *L. monocytogenes* when challenged with stress conditions.

The uptake and accumulation of compatible solutes (e.g., glycine, betaine and carnitine) through the BetL, Gbu and OpuC transporter systems, which are involved in osmotolerance response, are also mediated by exposure to a low temperature (Sleator et al. 2003). The cryoprotective activity of these compounds was further revealed using mutants with deleted osmolyte transporter genes (Wemekamp-Kamphuis et al. 2002).

The exposure of *L. monocytogenes* to cold temperature also has an influence on the pathogen's ability to overcome a lethal acid challenge (Ivy et al. 2012). Using a full-genome microarray approach to evaluate gene transcription after challenging several *L. monocytogenes* strains (lineage I and II) with a simulated gastric juice (representing an acid shock treatment at pH 3.5), the investigators determined that bacterial cells grown at 30 °C better tolerated the rapid pH change than did the cells grown at 7 °C. Cells grown at 37 °C had an acid tolerance response (ATR), with the induction of a group of regulators, namely σ^B, σ^H, CtsR, HrcA, which participate in different adaptation responses. An interesting observation after an acid shock was the induction of prophage genes in cultures grown at 7 °C (Ivy et al. 2012). This effect seems to be linked with the induction of the SOS response that is also involved in the acid shock response (Waldor and Friedman 2005; van der Veen et al. 2010).

Surprisingly in the study of Cacace et al. (2010) that investigated the proteome response of *L. monocytogenes* ATCC 15313 grown in BHI medium at low temperature, no over-production of any cold proteins was observed. Instead the proteins that assisted the response to low temperature were those involved in the key steps of glycolysis and other metabolic pathways, such as the Pta-AckA pathway. These results revealed that at low temperatures *L. monocytogenes* requires significant levels of high-energy components in order to ensure its growth.

Listeria monocytogenes encounters cold temperatures during both food production and storage, which is also accompanied by other stress conditions, therefore it is important to examine the adaptation responses to a combination of stress challenges.

Osmotolerance Response

Salting, jointly with thermal processing and refrigeration, are practices used for preserving some foods. The addition of salt to food reduces its water activity as well as alters electrochemical potential of the membrane of microbial cells thus increasing the shelf life of food products. However, many foodborne pathogens, including *L. monocytogenes,* can cope with highly osmotic environments. The maximum NaCl concentration, that permits *L. monocytogenes* growth, ranges from 7% to 10% (Lado and Yousef 2007; Panfill-Kuncewicz et al. 2009). This osmotolerance is achieved by cells driving the synthesis or transport of compatible solutes, such as glycine betaine, proline and carnitine, to maintain the osmolarity of both the cells extracellular and intracellular surroundings (Angelidis and Smith 2003; Fraser et al. 2003; Gardan et al. 2003a; Okada et al. 2008; Sleator et al. 2003).

Several proteomic studies have examined the proteins involved in the osmotolerance response (OTR) of *L. monocytogenes*, namely the Ctc, DnaK, HtrA, GbuA, AppA, and OpuC induced proteins (Abram et al. 2008; Duché et al. 2002a, b). In a recent study (Melo et al. 2013a) also using a proteomic approach to elucidate the adaptation response of two dairy isolates (A9 and T8), results revealed that either in a mono sublethal condition (pH 5.5 or 3.5%, w/v NaCl) following a lethal stress (pH 3.5 [adjusted with lactic acid] or 20%, w/v NaCl) or in a combination of sublethal stresses (pH 5.5 and 3.5% NaCl) the tolerance response was identical. Specifically, *L. monocytogenes* A9 exhibited a classical ATR profile but no OTR was observed, whereas strain T8 was intrinsically resistant to low pH, but exhibited an OTR. The proteomic analysis revealed that the OTR-positive strain (T8) overproduced a group of proteins that were different from the group of proteins overproduced by the ATR-positive strain (A9). The most prevalent proteins associated with OTR were the Ctc (general stress protein), glycolysis-related proteins and detoxification proteins (Sod and iron-sulfur cluster proteins), whereas in the ATR-positive strain the most prevalent proteins were associated with protein synthesis, protein folding (GroEL, DnaK and TF), accomplishment of reducing power, cell wall and cell division, and biosynthesis of fatty acids and nucleic acids. Overproduction of the Ctc protein by strain T8 was anticipated because over-production of this protein is critical for protecting *L. monocytogenes* in the absence of compatible solutes (Gardan et al. 2003b). Studies by Melo et al. (2013a) revealed that the different adaptation responses between the two dairy isolates may mirror their past exposure to environmental stress factors (such as low pH or salt) and their adaptive responses to the cheese-processing environment. Such approaches not only elucidate the mechanisms of adaptation used by this pathogen but also highlight how difficult it is to achieve *Listeria* control using traditional measures (such as salt).

It is widely recognized that the role of the compatible solute uptake mechanism in *L. monocytogenes* is for osmotolerance. However, efforts have been made to identify other elements involved in the *L. monocytogenes* salt tolerance phenotype (Sleator and Hill 2005; Burall et al. 2012, 2015). The two-component regulatory system LisRK and the *htrA* gene that is under the transcriptional control of LisRK

were identified by Sleator and Hill (2005). Burall and collaborators (2012) have associated the Iap protein to the salt and cold tolerance of strain LS411 that was implicated in the Jalisco cheese listeriosis outbreak (Linnan et al. 1988). Iap protein is important for *L. monocytogenes* cell viability and virulence (Bubert et al. 1992; Pilgrim et al. 2003). Recently Burall et al. 2015 identified an acetyltransferase (LstC) that is associated with response to high salt resistance. However, its role in salt tolerance is still not clear.

The impact of salt stress on the expression of four known sigma factor B (σ^B) dependent genes (*opuCA*, lmo2230, lmo2085 and the σ^B gene itself) was studied by Utratna et al. (2011). The σ^B belongs to the group of stress-responsive sigma factors, which are subunits of bacterial RNA polymerase that recognizes specific DNA sequences in promotor sites allowing the start of RNA synthesis (see section "Tolerance Response to Low pH"). The gene expression was monitored during exponential growth in BHI supplemented with NaCl at concentrations ranging from 0 to 0.9 M NaCl. The expression of *opuCA* was dependent on the salt concentration, with a high correlation between the extent of salt stress and the induction of gene expression. Furthermore, the expression of other genes was also rapidly induced by the exposure to osmotic stress. Studies by Utratna et al. (2011) revealed that salt activates σ^B and responds proportionally to its concentration, and emphasized that osmotic stress tightly modulates the activity of this regulator. Besides σ^B, other sigma factors have been associated with salt response, namely the sigma factor RpoN (σ^{54}) (Okada et al. 2006), and also the CstR regulator that not only participates in salt response but also in other stresses, such as ATR and heat shock response (Nair et al. 2000a).

The capacity to accumulate compatible solutes in addition to assisting in osmotic stress response, also has been associated with the ability to tolerate low temperature and freezing conditions (Sleator et al. 2003).

Listeria monocytogenes experiences an osmotic challenge under different conditions, including in the food chain and the gastrointestinal milieu, which occur at different temperatures. Therefore, adaptation to osmotic stress can occur in a temperature-dependent manner (Berghloz et al. 2010, 2012). Bergholz et al. (2010) determined there were lineage-specific (lineages I, II and III) differences in the ability of *L. monocytogenes* to survive simultaneous osmotic and cold temperature stresses and that the preceding growth temperature had an influence on the ability of these lineages to overcome osmotic stress. After growth at 7 °C, all three lineages had similar salt tolerances. However, when grown at 37 °C, only lineages I and III were able to overcome salt stress, indicating that once in the host listeriae, those two lineages can better tolerate osmotic stress.

Bergholz et al. (2012) used a transcriptome approach to determine how *L. monocytogenes* strain H7858 (serotype 4b), which was implicated in a frankfurter outbreak in 1998 (Nelson et al. 2004), responds to short-term and long-term exposure to salt stress at 7 and 37 °C. The investigators observed over-expression of the σ^B and σ^B-regulated genes and an encoding sodium/proteon antiporter (*mrpABCDEFG*) at both temperatures during the short-term response to salt stress. For the long-term response at 7 °C, the transcription of genes encoding

chaperones and proteases (*dnaK*, *groES*, *groEL*, *grpE*, *htrA* and *clpP*) was increased. In contrast, for the long-term response at 37 °C, the transcription of *ctc*, *opuCA*, *lmo1580*, *gadT2D2*, *gadD3* and virulence genes (*prfA, mpl*, *actA*, *plcB*, *plcA* and *inlA*) was increased. At both temperatures, over-expression of the *kat* gene was observed. This gene encodes a catalase involved in the catalysis of H_2O_2, indicating a cross-protection to oxidative stress from exposure to salt. This study provided insights into how temperature modulates the molecular events of the response of *L. monocytogenes* to salt stress, and the implications of this exposure in developing protection to other shock conditions, such as exposure to hydrogen peroxide.

An overview of the systems involved in the osmotolerance response of *L. monocytogenes* is provided in Fig. 1.

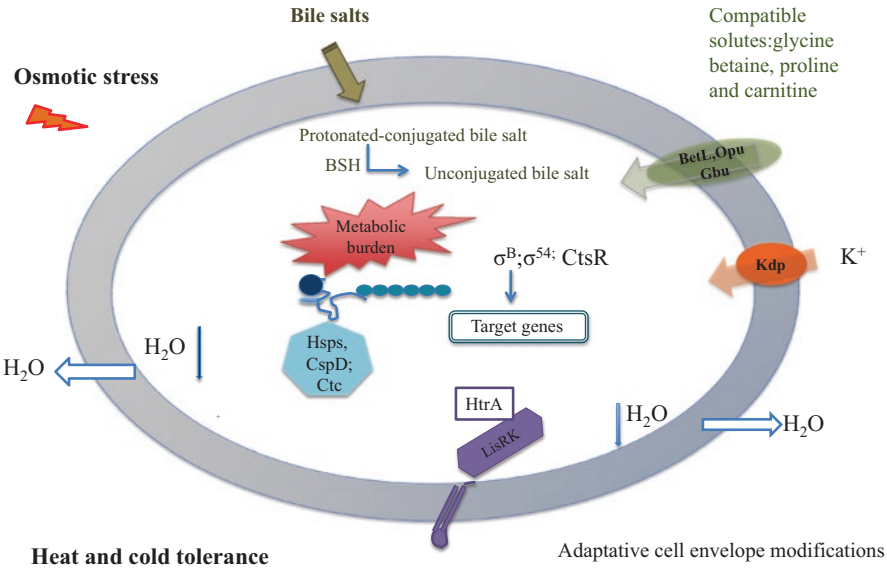

Fig. 1 Overview of the systems identified in response of *L. monocytogenes* to osmotic, heat and cold, and bile stress. Under high osmolarity, the uptake of K^+ initially occurs followed by the uptake of compatible solutes, i.e., glycine betaine, proline and carnitine. Cold stress conditions also cause the accumulation of these solutes. Cold shock proteins (*Csps*) have a role in both the cold and osmotolerance response. The regulator CstR participates in the osmotolerance and heat shock response. If cells lack protective osmolytes, Ctc becomes involved in the response to osmotic stress. The two-component regulatory system LisRK, which modulates the transcription of the *htrA* gene (a serine protease involved in the osmotolerance and heat response), possesses both osmosensing and osmoregulatory functions. In the presence of bile stress, BSH breaks down the protonated-conjugated bile salt to unconjugated bile salt (Adapted from Melo et al. (2015))

Tolerance Response to Low pH

While in the food chain *L. monocytogenes* can be exposed to both sublethal and lethal pH values (see section "Food Production and Induction of Stress Responses"). The pathogen also is exposed to different acidity levels during its passage through the gastrointestinal tract of the host. As with other stresses, the exposure of *L. monocytogenes* to sublethal low pH triggers an adaptive response, which in this condition permits the pathogen to cope with exposure to a lethal low pH. This phenomenon is designated as an acid tolerance response (ATR) (Davis et al. 1996; Booth et al. 2002). ATR can be induced either by organic or inorganic acids, and as observed in other bacteria, acid resistance is growth phase-dependent; stationary phase *L. monocytogenes* cells are naturally tolerant to acid-stress conditions (Davis et al. 1996; Lin et al. 1996; O'Driscoll et al. 1996; Phan-Thanh et al. 2000). ATR, as with many other tolerance responses, also induces cross-protection to other stresses, such as NaCl, ethanol, heat and hydrogen peroxide (Faleiro et al. 2003; Lou and Yousef 1997; Koutsoumanis et al. 2003).

L. monocytogenes uses different systems and mechanisms to cope with low pH, including the F_0-F_1 ATPase, the glutamate decarboxylase (GAD) system, the arginine deiminase system, and several regulators, including σ^B (Dowd et al. 2012; Faleiro 2012). These systems are illustrated in Fig. 2 and described in the following section "Systems that Sustain *L. monocytogenes* Acid Resistance".

Systems that Sustain *L. monocytogenes* Acid Resistance

Several acid resistance systems have been identified *L. monocytogenes*, including the F_1F_0-ATPase complex, decarboxylation reactions (by consumption of H^+) that include the participation of glutamate and lysine decarboxylases, production of ammonium ions by the action of amino acid deiminases (arginine deiminase), acetoin production and several transcriptional regulators. The functions of these systems are described below.

F_1F_0 ATPase Complex

The F_1F_0-ATPase complex provides a channel for proton translocation through the cell membrane using energy from ATP hydrolysis. Studies by Cotter et al. (2000), using N, N'-dicyclohexylcarbodiimide (DCCD), an inhibitor of ATPase activity, revealed the participation of the F_1F_0-ATPase complex in the acid tolerance response of *L. monocytogenes*. The exposure of *L. monocytogenes* LO28 cells to the (DCCD) inhibitor both before and during acid stress increased the acid susceptibility of acid-adapted cells. Acid-adapted *L. monocytogenes* LO28 cells (pH 5.5 adjusted with

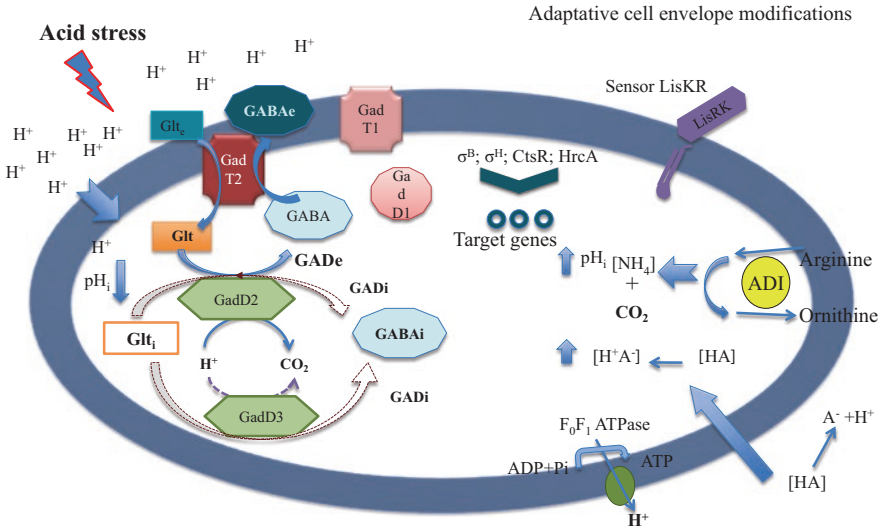

Fig. 2 Overview of the acid resistance systems and mechanisms identified in *L. monocytogenes* response to acid stress. Acid stress can be sensed by the two-component system LisRK and the sources of this stress are weak acids that diffuse into the cell and protons from inorganic acids. pH homeostasis is disturbed by the increased levels of acid anions entering the cell, which injures the cell membrane and disrupts essential metabolic pathways. The bacterial cell is protected from such effects by the action of the F_0-F_1-ATPase, GAD and ADI systems, and the involvement of sigma factors and regulators. At inhibitory pH (<4.5), the antiporter GadT2 uptakes extracellular glutamate (*Glt*) that is decarboxylated by GadD2 to ϒ-aminobutyrate (*GABA*) with the consumption of H^+. Following GadT2 ensures both the import of Glt and the export of GABA. This is carried out by the extracellular GAD system (GAD_e), which is represented by full arrows. Due to the decarboxylation of the intracellular GlT by GadD2 and GadD3, an intracellular accumulation of GABA ($GABA_i$) occurs. This process is promoted by the second GAD system, the GAD_i, which is depicted by *dotted lines*. At more aggressive acid stress, no support from GadD1 and GadT1 either on GAD_e or GAD_i occurs (Adapted from Melo et al. (2015))

3 M lactic acid) that were not exposed to DCCD survived better at low pH (pH 3.5) than DCCD-treated cells with 3 logs more survivors after a 2-h acid challenge.

The proteomics analyses (determined by two-dimensional gel electrophoresis) of the acid-adapted cells of *L. monocytogenes* L28 and EGD also revealed the involvement of F_1F_0-ATPase in response to acid exposure (Phan-Thanh et al. 2000).

The Glutamate Decarboxylase Acid Resistance (GAD) System

L. monocytogenes is capable of surviving acid stress by using the glutamate decarboxylase (GAD) system. The GAD system in *L. monocytogenes* is comprised of five genes: *gadD1*, *gadD2*, and *gadD3, gadT1* and *gadT2*. The first three codify for decarboxylases and the last two for antiporters. These genes are grouped in pairs, namely *gadD1T1* and *gadD2/T2,* and *gadD3* is independent (Cotter et al. 2001a, b Cotter et al. 2005). The proposed functional mechanism of the GAD system initiates

with glutamate being taken up by the cell via a specific transporter and is converted to ϒ-aminobutyrate (GABA) following cytoplasmic decarboxylation and consumption of an intracellular proton. After GABA is exported from the cell through the action of an antiporter positioned in the cell membrane, the pH of the cytoplasm increased by virtue of proton loss and the release of GABA (more alkaline) into the environment, which slightly increases the external pH (Booth et al. 2002). Karatzas et al. (2012) proposed a new operating model for the GAD system, since independent of GABA intracellular accumulation, the (Glt)/GABA antiport takes place. The investigators also observed that the system is medium-dependent and also strain-dependent.

The new proposed model of functioning divides the GAD system into two semi-independent systems; the extracellular (GAD_e) and the intracellular (GAD_i). The GAD_e uses Glt_e through GadD2, whereas GAD_i depends on Glt_i. Briefly, GAD_i cooperates to confer acid resistance and consists of GadD2 (transforms Glt in GABA by decarboxylation with H^+ consumption) and GadD3 (a Glt decarboxylase), with GAD_e (comprised of the GadD2/T2) assisting with the exportation of GABA by the action of GadT2 and at the same time the import of Glt by decarboxylation with H^+ consumption (see Fig. 2).

Arginine Deiminase (ADI) System and Thiamine

The acid tolerance response of *L. monocytogenes* can also be influenced by the arginine deiminase (ADI) system, which is comprised of three enzymes, the ADI (EC 3.5.3.6), catabolic ornithine carbamoyltransferase (cOTC) (EC 2.1.3.3) and carbamate kinase (CK) (EC 2.7.2.2). These enzymes are encoded by *arcA, arcB* and *arcC*, respectively, and are responsible for catalyzing the transformation of arginine into ornithine, NH_3, CO_2, and ATP. Through the transporter encoded by *arcD,* ornithine is moved outside of the cell in exchange for arginine (Ryan et al. 2009). Deletion of the *arcA* gene (*lmo0043*, an orthologue of *arcA*) of *L. monocytogenes* strain 10403S resulted in reduced growth at a sublethal acid-adaptation induction pH in BHI (pH 5.5) compared with the wild-type strain. Moreover, the exposure of a Δ*arcA* mutant to artificial gastric fluid reduced its survival, suggesting that ArcA plays a role in *L. monocytogenes* acid resistance (Cheng et al. 2013).

Both the arginine and agmatine systems (*lmo0036-lmo0043* locus) contribute to the ATR of *L. monocytogenes* influencing the balance of the cytoplasmic pH (Chen et al. 2011; Cheng et al. 2013; Ryan et al. 2009). The input of these systems to ATR is based on the combination of the catabolic products of arginine and agmatine with intracellular protons, i.e., discharging ammonium ions (NH_4^+), which reduce the acidity of the cell thereby preserving the pH homeostasis.

The observation that thiamine-depleted cultures were significantly more sensitive to acid than thiamine-sufficient cultures lead Madeo et al. (2012) to investigate the role of thiamine on the acquisition of acid tolerance by *L. monocytogenes*. Thiamine uptake is crucial to cell viability as thiamine is an essential co-factor for several enzymes involved in central metabolism. ThiT, a membrane protein, enables

thiamine transport. The study performed with transposon mutants prompted the investigators to anticipate that thiamine deficient cells would not produce acetoin, a proton-dissipation compound obtained from pyruvate, which is essential for pH homeostasis (Madeo et al. 2012).

Besides these elucidating studies on the contribution of the ADI system to *L. monocytogenes* acid tolerance, the involvement of the ADI system in vacuum-packaged sliced/smoked salmon, was recently reported by Tang et al. (2015). However, additional studies are needed to further elucidate the contribution of the ADI system to *L. monocytogenes* survival in acidic foods.

Sensors and Regulators

For *L. monocytogenes* to survive, the many different stresses to which it's exposed the pathogen must be capable of sensing environmental changes. The two-component system LisRK has been associated with the regulation of *Listeria's* acid tolerance response by sensing the environmental drivers of acid stress (Cotter et al. 1999).

The involvement of stress-responsive sigma factors has been identified in both Gram-negative and Gram-positive bacteria (Castanie-Cornet et al. 1999; Chan et al. 1998; Brigulla et al. 2003; Wemekamp-Kamphuis et al. 2002). It is known that *L. monocytogenes* possesses five sigma factors, four of which are alternative sigma factors (σ^B, σ^C, σ^H and σ^L) involved in the control of gene expression under conditions of environmental stress. The sigma factor that controls housekeeping gene expression is σ^D (Glaser et al. 2001). Sigma factors are subunits of RNA polymerase that ensure the proper recognition of target gene promoters. Thus, when bacterial cells are exposed to environmental stress conditions, the expression of sigma factor and its respective activity provides an efficient system to promptly switch the direction of transcription thereby changing the cell's gene expression in order to cope the challenges of environmental stress. In *L. monocytogenes*, the most studied sigma factor is σ^B that modulates nearly 150 genes (Hain et al. 2008; Raengpradub et al. 2008). The sigma factor σ^B has been associated with several stress responses, including acid, salt, heat, low temperatures and oxidative stresses and nisin (Abram et al. 2008; Ferreira et al. 2003a, b; Heavin et al. 2009; Kazmierczak et al. 2003; Sue et al. 2004). The acid resistance presented by stationary-phase bacterial cells also involves σ^B activity (Ferreira et al. 2001). The influence of σ^B on gene expression affecting the infection process has been reported (Camejo et al. 2009; Ferreira et al. 2003a; Kazmierczak et al. 2003), hence contributing to *L. monocytogenes* virulence.

The role of σ^B on the response of *L. monocytogenes* to stress factors in foods has been studied, and strain variability in the σ^B response has been observed (Alessandria et al. 2013; Oliver et al. 2013; Rantsiou et al. 2012). Rantsiou et al. (2012) studied the expression of σ^B together with three virulence genes (*hly*, *iap* and *plcA*) of different isolates (an isolate from meat, another from cheese, and a collection strain [NCTC 10527]) in fermented sausage, minced meat, soft cheese and ultrahigh temperature-treated milk. There was considerable diversity in the expression of virulence genes,

but the expression of σ^B was similar among the strains, except for the cheese isolate in which there was an over-expression of σ^B in UHT milk. Alessandria et al. (2013) evaluated the expression of σ^B in a soft cheese (Crescenza with pH 5.5 and 1% [w/w] NaCl) at 4 and 12 °C using 11 *L. monocytogenes* strains (both food and human isolates). For two strains, *L. monocytogenes* EGD and a soft cheese isolate (strain 70), the transference from BHI to cheese at both temperatures caused an over-expression of σ^B. The gene expression analysis did not reveal any association with the gene expression profile and the source of the strains (Alessandria et al. 2013).

Oliver et al. (2013) determined the limited role of σ^B in response to acid and oxidative stress, as well as the participation of this sigma factor in infection both tested *in vitro* (ability to invade Caco-2 cells) and *in vivo* (guinea pig model) of *L. monocytogenes* strain F2365 that was implicated in the listeriosis outbreak associated with contaminated Mexican-style soft cheese in California in 1985. From these studies, it is evident that an unusual σ^B-independent response to acid and other stresses is disseminated among *L. monocytogenes* strains.

Evidence from several studies has emerged revealing that other sigma factors (such as sigma H (σ^H), Mar R, sigma L (σ^L)) have a role in the response of *L. monocytogenes* to low pH, and also the establishment of transcriptional networks between σ^B and the negative regulators HrcA and CtsR (Hu et al. 2007a, b; Matilla et al. 2012; Olesen et al. 2009). Therefore, *L. monocytogenes* response to diverse environmental stresses includes the activation of a variety of elements of the stress-related machinery, and in a coordinated manner.

Oxidative Stress

The ability of *L. monocytogenes* to overcome environmental stress conditions and its ability to cause disease includes its competence to respond to oxidative stress. *L. monocytogenes* must cope with oxidative stress both in the food processing environment, food products, and also during its passage in the host (Lungu et al. 2009). The use of sanitizers and disinfectants, and modified atmosphere packing (MAP) in the food industry introduces oxidative stress to *L. monocyogenes* (Gandhi and Chikindas 2007; Hill et al. 2002; Tompkin 2002). Reactive oxygen species (ROS), such as superoxide anions ($O_2^{\bullet-}$), hydroxyl radicals (OH•), peroxyl radical (ROO•) and hydrogen peroxide (H_2O_2), are highly reactive compounds produced during aerobic growth, which in elevated amounts lead to damage to proteins, DNA, and membrane lipids (Wesche et al. 2009). *L. monocytogenes* inside macrophages is also exposed to oxidizing compounds, such as $O_2^{\bullet-}$, H_2O_2 and nitric oxide (NO). *Listeria*'s response to oxidative stress involves the activity of enzymes that prevent oxidative damage, such as catalases (Kat), superoxide dismutases (SOD) and alkyl hydroperoxidase reductases, and repairing enzymes (such as RecA) (Imlay 2002; Lungu et al. 2009). Stress regulators that modulate the expression of various antioxidant genes such as PerR (Rea et al. 2005) and σ^B (Zhang et al. 2011), are also essential to *L. monocytogenes'* response to oxidative stress.

L. monocytogenes possesses one manganese superoxide dismutase (MnSOD) that, when its encoding gene is deleted, the survival capacity of *L. monocytogenes* is impaired both in the presence of chemical oxidative agents and also within macrophages. Furthermore, *L. monocytogenes* growth and biofilm formation are affected even in the absence of oxidative stress (Archambauld et al. 2006; Suo et al. 2012). Interestingly, the exposure of an *Δsod* deletion mutant of *L. monocytogenes* strain 4b G to 1 mM paraquat (generates ROS in the cells) resulted in over-expression of the *recA* gene (activates SOS response, a general response to DNA damage), whereas in the absence of paraquat the expression of several stress-related genes (*perR*, σ^B, *kat*, *fri*, and *recA*) was inhibited compared to the wild type. Overall these results reveal that SOD has a crucial role in the protection of *L. monocytogenes* 4b G and the SOS response possibly influences *Δsod* survival when challenged with oxidative stress (Suo et al. 2014).

Once more these results highlight that *L. monocytogenes* uses various stress-associated mechanisms in a coordinated way in order to overcome adverse conditions.

Bile Stress

L. monocytogenes must overcome the bile environment encountered during passage through the gastrointestinal tract. Bile salts cause membrane injury by disrupting membrane proteins and phospholipids, and also inducing DNA mutations and oxidative stress, along with other effects, such as calcium and iron chelating activity (Begley et al. 2005). Bile resistance/tolerance has been detected in *L. monocytogenes* isolates from food processing, food and clinical cases (Barmpalia-Davis et al. 2008; Olier et al. 2004). For example *L. monocytogenes* LO28 is able to tolerate up to10% (w/v) of bile (Zhang et al. 2011). Several genes have been associated with the bile tolerance, including those encoding bile salt hydrolases (BSH) which degrade bile into less toxic forms. The bile exclusion system, "BilE," which participates in the removal of bile salts from cells, has also been identified (Begley et al. 2005; Dussurget et al. 2002; Watson et al. 2009). Furthermore, *bsh* genes are regulated by PrfA, suggesting its importance for the virulence of *L. monocytogenes* (Dussurget et al. 2002).

Begley et al. (2009) determined that the exposure of *L. monocytogenes* EGD to bile improves its ability to adhere to plastic surfaces, thereby enhancing its ability to colonize and persist in the gastrointestinal tract.

Cross-Adaptation

The concept of cross-adaptation is based on the fact that exposure of a bacterium to an individual stress can result in not only the acquisition of protection to that individual environmental stress following subsequent exposure to more

severe stress, but also the acquisition of protection to different adverse conditions. For example, exposure to a sublethal low pH environment protects *L. monocytogenes* from exposure to lethal pH values, but also provides cross-protection against other stressful challenges, such as salt, heat, ethanol and oxidative stress (Bonnet and Montville 2005; Faleiro et al. 2003; Ferreira et al. 2003b; Makariti et al. 2015; Skandamis et al. 2008). Additional examples of cross-adaptation include exposure to salt stress induces protection to hydrogen peroxide and to nisin (Bergholz et al. 2012, 2013), and oxygen limitation induces an ATR of *L. monocytogenes* J0161, a strain implicated in a listeriosis outbreak associated with the consumption of deli turkey (Sewell et al. 2015). Cross-adaptation is especially relevant for minimally processed ready-to-eat foods, particularly for those in which MAP and refrigeration are used to increase shelf life, as illustrated in a study by Francis et al. (2007) in which it was observed that the GAD system, an ATR system (see section "Systems that Sustain *L. monocytogenes* Acid Resistance"), enhanced *L. monocytogenes* LO28 survival on MAP food.

The cross-adaptation event results in expression of stress-related proteins that are induced by more than one stress challenge. For example, the transcriptome approach used in the study of Berghloz et al. (2012) revealed that exposure of *L. monocytogenes* to salt stress (10% NaCl) at 37 °C induced over-expression of the genes *ctc*, *opuCA*, *bsh* and *inlA*, which are known to have a role not only in salt stress, but are also involved in bile tolerance and general stress response. Berghloz et al. (2013) also determined that a salt stress challenge (6%) at 7 °C induced protection to nisin (2 mg/ml Nisaplin), which was modulated via LiaR, a response regulator that has a role in activating the cell envelope stress response of *L. monocytogenes* (Fritsch et al. 2011).

A study by Tang et al. (2015) using a RNAseq approach determined the transcriptome response of *L. monocytogenes* H7858 (a serotype 4b strain that was implicated in the listeriosis outbreak from 1998 to 1999 associated with the consumption of RTE meat [CDC, 1999]) inoculated in vacuum-packaged sliced smoked salmon stored at 7 °C. Results revealed that four genes of the agmatine deiminase system which is mainly involved in responses to acid-adaptation (see section "Systems that Sustain *L. monocytogenes* Acid Resistance"), up regulated. This stress response may have implications for *L. monocytogenes* during the exposure to gastric fluids. This study also revealed an up regulation of genes involved in virulence, in particular, *inlB*, *pclA*, *pclB*, *actA*, and *hly*, suggesting that a food matrix, such as cold-smoked salmon may have an influence on *L. monocytogenes* H7858 virulence.

Another example of foods that influence *L. monocytogenes* survival by providing cross-adaptation responses are dairy products, including cheese. In particular, soft cheeses can support the survival and growth of this pathogen by providing conditions of low pH and sufficient salt content to induce cross-adaptation to protect the pathogen during refrigerated storage (Cataldo et al. 2007; Ilhak et al. 2011). For a more detailed review of the importance of stress responses related to cheese and the dairy environment, see Melo et al. (2015).

Cross-adaptation is particularly important for food on which a contaminating pathogen is exposed to a number of environmental stresses related with the physicochemical properties of the food itself, the food process, and with refrigerated storage.

After ingestion, *L. monocytogenes* must circumvent the host's natural defences, which includes the gastric and bile fluids, high acid concentration, and the oxidative burst of reactive oxygen species occurring in the phagosome (Freitag et al. 2009).

Hence, both the food-processing environment and the food milieu influence the expression of stress adaptation responses of *L. monocytogenes* and its survival and virulence.

The Relationship Between the Food Matrix, Stress Response and Virulence of *L. monocytogenes*

After ingestion, *L. monocytogenes* is exposed to stresses, similar to those encountered in food matrices. Therefore, many genetic elements involved in assembling *Listeria*'s stress response to enable the pathogen to survive stresses it encounters in foods, are also involved in contributing to the pathogen's virulence. Many studies have addressed the influence of stress responses, enabling *L. monocytogenes* to survive in various food matrices. This approach provides a better understanding of the mechanisms used by *L. monocytogenes* to survive and grow in the adverse conditions presented by many food matrices, and also can provide insights for the development of strategies to better control this pathogen. Additionally, this approach can help evaluate the influence of exposure to food matrices on the virulence potential of the pathogen, in particular RTE foods, such as those that have been associated with listeriosis outbreaks.

In a study by Mujahid et al. (2008), the proteome profile of *L. monocytogenes* strain F2365 (serotype 4b) grown on smoked turkey breast slices was compared with the proteome profile of cells grown on BHI agar plates at 15 °C for 5 days. Results revealed that there were approximately thirty-four proteins expressed by bacteria grown on the RTE meat, and about 160 protein bands matched the proteome profile of the bacteria grown on the food matrix and in BHI. The proteins induced specifically by the growth in meat included proteins of several functional categories, namely energy metabolism, biosynthetic and degradation processes of fatty acid and phospholipid metabolism, protein synthesis, and various cellular processes, such as cell envelope biosynthesis and cell division. However, specific stress response protein was exclusively expressed by *L. monocytogenes* grown in turkey-meat, the ClpB-lmof2365_2239. Interestingly, among the proteins that were expressed in both meat and BHI, four were related to stress responses, ClpC, ClpP, cold-shock domain family protein-lmof2365_1381 and the universal stress protein family-lmof2365_1602. Participation of the ClpC protease in several virulence processes has been reported, including in the escape of listeriae from the phagosome (Rouquette et al. 1996), and in adhesion and invasion via modulation of expression of the virulence genes, *inlA*,

inlB and and *actA* (Nair et al. 2000b). The ClpP serine protease has also been associated with the virulence of *L. monocytogenes*, specifically it was determined that it is required for intracellular growth in macrophages and modulates the levels of listeriolysin O (LLO) (Gaillot et al. 2000, 2001). Interestingly, Olesen et al. (2010) studying expression of the virulence genes *prfA*, *inlA*, *sigB* and *clpC* of three *L. monocytogenes* strains (O57, a susceptible salt strain, 6896, a salt tolerant, and EGD as a reference strain) in liver pâtés with different salt levels determined that transcription of the *clpC* gene was induced in liver pâté with reduced salt and supplemented with organic acid salts (Ca-acetate and Ca-lactate) for all three strains, whereas in liver pâté with lower salt levels the transcription levels of *prfA*, *inlA*, *sigB* or *clpC* were not affected but *sigB* in strain 6896 and *prfA* in strain O57 had increased transcript levels. This study revealed how bacterial inhibitors, such as Ca-acetate and Ca-lactate, can influence the expression of *L. monocytogenes* virulence genes.

A study by Rantsiou et al. (2012) also revealed the influence that food matrix has on gene expression by *L. monocytogenes*. In this study, investigators determined responses of three strains (#3, serotype 3c isolated from fermented sausage, #12, serotype 4b isolated from milk, and EGD as a reference strain) to the acidic and osmotic stresses present in fermented sausage and minced meat juice at 4 °C, in comparison to their growth in BHI at the same temperature. The expression profile of the three strains was similar when they were grown in BHI. In contrast, differences in gene expression in the sausage and meat juices between the three strains were evident, particularly in virulence (*iap* and diverse internalin genes) and stress-related genes (*gadC* and *gadE*). Furthermore, the *iap* gene (see section "Osmotolerance Response") was the unique gene, differentially expressed, regardless of the conditions tested; and, differences between strains were observed, namely strain #12 had the lowest expression, in contrast to strain #3 that had the highest level, in comparison to strain EGD (Rantsiou et al. 2012).

Larsen and Jespersen (2015) investigated the effect of salt (<0.15% and 3.6%) on expression of virulence genes (*prfA*, *sigB*, *agrA*, *inlA*, *hly*, *actA*, *opuC*, *bsh*, *gadC*, *clpC*, *clpP*, *ami*) of two *L. monocytogenes* strains isolated from cheese (*L. monocytogenes* ATCC 51779 [serotype 1/2c] more salt susceptible and DSMZ 15675 [serotype 4b], a more salt-tolerant strain) and EGD (ATCC BAA-679 (serotype 1/2a) as reference strain, using Danish hard-cheese type Samsoe as a food matrix. The study revealed a greater expression of *prfA*, *inlA*, *hly* and *actA* genes for the more salt-susceptible strain (strain 51,779) compared to the more salt-tolerant strain (15675). Furthermore, expression of the *prfA* gene was about 2 to 3-fold greater in strain 51,779 during the 2 to 48-h incubation period on low salt cheese (< 1.5%) and also after 2 h on cheese with a higher concentration of salt (3.6%). This same strain at 3.6% salt had the greatest expression of gene *bsh*, which is associated with bile resistance (Begley et al. 2005) (see section "Bile Stress"), suggesting a greater capacity to overcome the gastrointestinal tract stress and successfully engage in the virulence process. These findings revealed that transcription of the virulence genes of *L. monocytogenes* grown on cheese matrix is dependent on both strain and salt content. Thus, the addition of salt may enhance the virulence potential of *L. monocytogenes*, but this is dependent on the strain's salt tolerance.

Use of alternative and affordable insect models to study the influence of the food matrix and stress-adaptation on virulence was addressed by Schrama et al. (2013). In this study, the virulence of two *L. monocytogenes* strains (A9 and T8) isolated from a cheese-processing environment, a cheese isolate (C882) and *L. monocytogenes* ScottA exposed to stress-adaptation conditions (pH 5.5 and 3.5% [w/v] NaCl) in a cheese-based medium was examined using the wax moth *Galleria mellonella* infection model. The results revealed that combined adaptation to both low pH and salt in the cheese-based medium influences *L. monocytogenes* virulence. However, the infectious capacity varied between the strains. The investigators determined that cheese isolate C882 was the most virulent strain, and both adapted and non-adapted cells caused similar larvae mortality, while Scott A was less virulent with no differences in infectious capacity between adapted and non-adapted cells. In contrast, adaptation caused a distinct effect on the virulence of the dairy isolates; A9 non-adapted cells (pH 7.0 and no added NaCl) had a higher infectivity in comparison to adapted cells, and T8 adapted cells displayed analogous results to A9 until 48 h, when thereafter their infectivity capacity returned to similar levels of the non-adapted cells. The requirement of *L. monocytogenes* EGD LLO (codified by *hly*) for virulence was previously determined in the *G. mellonella* model (Joyce and Gahan 2010). However, the authors were not able to explain the differences between the strains' virulence by analyzing expression of the *hly* gene, either in the cheese-simulated matrix or induced by the larvae. Overall, this study reveals the complexity of the influence of the food matrix (cheese-based) and stress responses (low pH and salt) on *L. monocytogenes* virulence.

The virulence process of *L. monocytogenes* involves the expression of many genes, and among them are *inlA* and *inlB* (these code for the internalins A and B, respectively) that are essential for entry into host cells. To evade the phagosome, LLO (codified by *hly*) and phosphatidylinositide phospholipase C (PI-PLC, codifyied by *plcA*) are crucial, and with the aid of ActA tails (codified by *actA*), intracellular motility can occur (Cossart and Lebreton 2014). Differences in virulence among strains of *L. monocytogenes* have been attributed to mutations in the *inlA* gene. Such mutations produce a premature stop codon (PMSC) that results in a truncated or non-secreted InlA that affects virulence (Kovacevic et al. 2013; Nightingale et al. 2008; Roldgaard et al. 2009; Van Stelten et al. 2010, 2011).

Kovacevic et al. (2013) determined there was a wide range of *inlA* genotypes among *L. monocytogenes* strains isolated from food and food processing environments. Approximately 57% of strains with no PMSCs could adapt more rapidly to a temperature switch from 37 to 4 °C, suggesting that those strains, due to their ability to attain critical levels at low temperatures, represent a particular hazard to the safety of refrigerated foods (Kovacevic et al. 2013). Besides the many studies that have examined the virulence potential of strains that carry *inlA* PMSCs both *in vivo* (Roldgaard et al. 2009; Van Stelten et al. 2011) and *in vitro* (Felicio et al. 2007; Kovacevic et al. 2013; Nightingale et al. 2008), the influence of different food matrices on the virulence of those strains (either *in vitro* or *in vivo*) needs further elucidation.

The ability of *L. monocytogenes* to pass through the gastrointestinal tract of the host is an essential step contributing to its virulence potential. Many studies have addressed the influence of the food matrix on the ability of *L. monocytogenes* to cope with the stress conditions of the gastrointestinal tract (Abdullah and Calicioglu 2013; Barmpalia-Davis et al. 2009; Ilhak et al. 2011; Melo et al. 2013b, c; Peterson et al. 2007). Several studies have determined the survival of *L. monocytogenes* strains during exposure to artificial digestion systems by previously growing the bacteria on laboratory media (Barbosa et al. 2012; Cheng et al. 2013; Barmpalia-Davis et al. 2008; Jiang et al. 2010; Ramalheira et al. 2010). This approach lacks the advantages that real food matrices present by mimicking the real environment that the foodborne pathogen encounters prior to being exposed to the gastrointestinal tract and entering the host infection process (Abdullah and Calicioglu 2013; Ilhak et al. 2011; Peterson et al. 2007).

Barmpalia-Davis et al. (2009) determined the impact of fat content (5% or 36%) of frankfurters (storage at 7 °C) on the survival of *L. monocytogenes* exposed to gastrointestinal fluids. Results revealed that the fat levels of frankfurters protected *L. monocytogenes* during the gastric challenge; – the fat had no effect for the intestinal challenge. Unfortunately, identification of the molecular elements that the foodborne pathogen uses for gastric protection was not determined.

Using a proteomic approach (two-dimensional electrophoresis), Melo and collaborators (2013b, c) examined the influence of a cheese-based medium on the protein components that assisted in stress protection of three *L. monocytogenes* dairy isolates challenged with gastrointestinal fluids. Adaptation of the listeriae in the cheese matrix induced a proteome pattern that differed among the three strains and the stress-related proteins that were over-produced did not overlap, suggesting there was strain variation in the adaptation machinery, and also implying that the pathogen can cope with food-related stresses by implementing different mechanisms of adaptation (Melo et al. 2013b). The ability of three dairy isolates to survive exposure to artificial gastric fluid (pH 2.5) after previous growth on a cheese-based medium at different salt concentrations and pH values (3.5% (w/v) NaCl and pH 5.5 [adapted cells] or no added NaCl and pH 7.0 [non-adapted cells]) was similar between the adapted and non-adapted cells, indicating these dairy isolates were inherently adapted to this food matrix. Analysis of the proteome that was induced by the gastric challenge however, revealed significant differences between the three dairy isolates, as each isolate over-produced distinctly different stress-related proteins (Melo et al. 2013b).

There were no differences in the ability of the three dairy isolates to overcome the intestinal stress following exposure to an artificial gastric fluid (Melo et al. 2013c). However, the proteomic analysis revealed that the isolates' response to the intestinal challenge was supported by the over-production of distinct stress-related proteins, and was more pronounced in non-adapted cells, indicating that exposure to the cheese matrix at sublethal conditions of salt and low pH triggered changes in the intestinal proteome. There were major differences in the proteome of all three strains in their response to intestinal stress; hence, there were many proteins involved in carbon metabolism and glycolysis, denoting a higher energy demand under

intestinal challenge. Both studies revealed strain variation in the proteome response to the food matrix and gastrointestinal milieu. These findings of strain-specific stress responses to food and host-related stresses (e.g., Larsen and Jespersen 2015; Rantsiou et al. 2012) highlight the importance of addressing several strains in such studies and to take into consideration their origin.

Altogether, these studies, using either transcriptomics or proteomics approaches, reveal the importance of using food matrices rather than laboratory culture media to obtain unbiased insights into the stress responses and the virulence potential of *L. monocytogenes* in real-world situations.

Conclusions

The analysis of studies on the investigation of acquisition of tolerance responses by *L. monocytogenes* has revealed that there are many molecular mechanisms involved in the pathogen's response to different stresses. These tolerance profiles can enable pathogen survival through the food chain as well as during its infection of the human host. The importance of using omics approaches, such as RNAseq, microarrays, and proteomics, to elucidate how the pathogen survives and overcomes the stress-challenges imposed by real food matrices, rather than growth in/on classical laboratory media, has become increasingly evident. Several studies have revealed the pathogen responses to food matrix and storage conditions are often strain specific, hence future studies addressing the influence of food matrices on stress tolerance responses should take this into consideration and include several strains of appropriate origin. Studies have also revealed that in different food matrices, the pathogen not only acquires a tolerance profile that enhances its survival ability in food, but also enables the pathogen to circumvent the stresses it encounters during passage through the gastrointestinal tract by undergoing modulation of virulence genes that prepare the pathogen for better competiveness in completing the infection process.

In the near future, a more robust number of studies using food matrices to elucidate how *L. monocytogenes* responds to real food challenges is anticipated, and this knowledge will be applied to create more effective control measures and provide safer food products, especially those that are minimally processed.

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Virulence Traits in the *Cronobacter* Genus

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Abstract Members of the *Cronobacter* genus are opportunistic pathogens within the bacterial family Enterobacteriaceae. They are associated with rare and sporadic infections in adults and associated with severe life threatening outbreaks of necrotizing enterocolitis, as well as causing meningitis and sepsis in neonates and infants. The organism is most commonly known for its infections of highly susceptible neonates through the consumption of contaminated reconstituted powdered infant formula; however, most infections are in the adult population and, therefore, wider sources of exposure and mechanisms of infection need to be better elucidated. This chapter will review the array of *Cronobacter's* virulence traits, which may aid the organisms' pathogenicity. In addition, environmental fitness will also be considered, as this may be crucial to understanding the prevalence of *Cronobacter* pathovars with particular infections in specific age groups.

Cronobacter has an array of virulence factors that facilitate tissue adhesion, invasion, and host cell injury. The outer membrane protein A (OmpA), is one such virulence marker, which also has importance in neonatal meningitic *E. coli* pathogenesis. Various plasmid-associated genes, such as those for filamentous hemagglutinin (*fhaBC*), *Cronobacter* plasminogen activator (*cpa*), as well as chromosomal genes responsible for iron acquisition (*eitCBAD*) and *iucABD/iutA*) and sialic acid utilization (*nanAKT*) have been reported. The possession of these virulence factors alone does not necessarily define a *Cronobacter* strain as being virulent, as there is also the aspect of environmental fitness. Physiological properties, such as biofilm formation and resistance to desiccation, may also contribute to the incidence of infection, as they increase its persistence in the environment and, hence, increase exposure. Capsular profiling has revealed an association between strains producing specific K-antigen, colanic acid and cellulose with neonatal meningitis, which could be

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linked to both host immune evasion as well as environmental survival. Recently, the use of whole genome comparative analysis with >200 strains has revealed the variation in the prevalence of virulence traits across the genus. In parallel is the recent recognition of specific pathovars, being linked to clonal lineages; for example, *C. sakazakii* CC4 with neonatal meningitis. These associations should lead to future virulence studies with well-chosen genetically characterized strains.

Keywords *Cronobacter* • Virulence factors • Human susceptibility • Pathogenicity • Neonates • Animal models

Abbreviations

CC	Clonal complex
CDC	U.S. Centers for Disease Control and Prevention
CNS	Central nervous system
FAO-WHO	Food and Agriculture Organization of the United Nations-World Health Organization
ICMSF	International Commission on Microbiological Specifications for Foods
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care units
PFGE	Pulsed-field gel electrophoresis
PIF	Powdered infant formula
ST	Sequence type
UTI	Urinary tract infection
WHO	World Health Organization

General Overview

The *Cronobacter* genus is composed of motile, non-sporeforming, peritrichous Gram-negative rods and is a member of the Enterobacteriaceae family. Within this family, *Cronobacter* are most closely related to the *Enterobacter* and the *Citrobacter* genera. *Cronobacter*, and in particular *C. sakazakii,* has been linked to a number of confirmed neonatal meningitis cases worldwide, in addition to cases of NEC and sepsis (Joker et al. 1965; Lai 2001; Joseph and Forsythe 2011). Bacterially-contaminated and reconstituted infant formula is associated with many neonatal outbreaks, which resulted in severe clinical outcomes (Muytjens et al. 1983; Biering et al. 1989; Himelright et al. 2002; Townsend et al. 2007b). Although *Cronobacter*

is a poorly understood genus, the Enterobacteriaceae family includes the better-known enteric bacterial pathogens, *E. coli, Salmonella*, *Shigella, Klebsiella* and *Yersinia,* which may serve as useful comparative organisms when determining virulence mechanisms.

Cronobacter spp. is present in a wide range of environments, including water, soil, and a variety of fresh and processed foods. It has been isolated from milk powder production lines, including powdered infant formula (PIF) manufacturing factories, and households. Potential sources of exposure include PIF, the hospital and home environment (Kandhai et al. 2004; Kilonzo-Nthenge et al. 2008), as well as food ingredients, retail foods (Friedemann 2007; Hochel et al. 2012), and water (Hariri et al. 2013; Liu et al. 2013). *Cronobacter* species are probably also part of the normal flora. For a fuller coverage of sources of *Cronobacter* see Forsythe et al. (2014).

In 2002, the International Commission on Microbiological Specifications for Foods (ICMSF) classified *Cronobacter* as pathogenic organisms to a restricted population, endangering their lives, and causing serious long-term consequences. In addition, the FAO-WHO recognized all *Cronobacter* species as microorganisms pathogenic for human beings. According to Minor et al. (2015), *Cronobacter* is the most expensive food-associated infection due to the loss of life and debilitation of survivors, each case being estimated at ca. $1 million USD.

The *Cronobacter* genus was first defined in 2007 and currently contains seven formally recognized species. Not all species have been linked to human infections, and only *Cronobacter sakazakii, C. turicensis*, and *C. malonaticus* have been associated with severe and fatal neonatal infections (Joseph and Forsythe 2011). Adult infections have been recorded for some of the remaining species, and no animal infections have been reported. Clinical isolates have been recovered from cerebrospinal fluid, blood, bone marrow, sputum, urine, and fecal samples. Although a number of virulence factors have been identified within *Cronobacter* strains, there is no comprehensive understanding of the pathogenesis of this organism. The severity of clinical presentation varies even within an age group, which suggested the existence of different types of pathogenicity. This variation is now better-recognized through the application of multilocus sequence typing (MLST), as being linked to specific clonal lineages. Nevertheless, better knowledge of the infection process would contribute to more effective control of exposure and a reduction in infection risk.

Background to *Cronobacter* spp.

The *Cronobacter* genus has received considerable interest, because of its association with rare but severe cases of neonatal infections, despite adult infections being more numerous. Low-birth-weight neonates are most at risk of severe illness and have been associated with life-threatening outbreaks of necrotizing enterocolitis, meningitis, and septicemia (Bar-Oz et al. 2001). Infections with these clinical presentations result in high mortality rates ranging from 40% to 80% (Bowen and Braden 2006 Friedemann 2007).

Urmenyi and Franklin (1961) are attributed with reporting the first cases of severe systemic neonatal infections by *Cronobacter.* Since then, there have been many cases reported worldwide. However, it was not until 2001 when there was a fatal meningitis outbreak at a neonatal intensive care unit (NICU) in Tennessee (USA) that the organism received considerable attention. At that time, the bacterium was known as *Enterobacter sakazakii*, as the organism had not been studied in detail since it had been defined as a species by Farmer et al. (1980). Later analysis by Iversen et al. (2007) of both partial 16S rDNA and *hsp60* sequences revealed that *E. sakazakii* isolates formed at least four distinct clusters, and it was proposed that these clusters could be unique species. This initial taxonomic reevaluation was confirmed using DNA-DNA hybridization and phenotyping, and subsequently *Enterobacter sakazakii* was reclassified as the new genus *Cronobacter* (Iversen et al. 2007). Initially, four species were defined as comprising the genus, and this has been further revised such that it currently contains the seven species: *C. sakazakii, C. malonaticus, C. turicensis, C. muytjensii, C. dublinensis, C. universalis*, and *C. condimenti.* This classification is supported by whole genome phylogeny, which confirmed the taxonomic positions of the seven species (Kucerova et al. 2010; Stephan et al. 2011; Joseph et al. 2012a, b).

Although *C. sakazakii*, *C. malonaticus*, and *C. turicensis* are reported to cause neonatal infections, the most common isolates are *C. sakazakii*. The reader may be aware of four additionally reported *Cronobacter* species in the literature: *C. pulveris*, *C. helveticus, C. colletis*, and *C. zurichenisis.* However, these species are now recognized as members of the genera *Franconibacter* and *Siccibacter.* They are outside the scope of this chapter, since they have not been associated with any cases of human infection. Thus, their omission, despite some reservation concerning possible misidentification, is not of clinical significance.

C. sakazakii and *C. malonaticus* are closely related and can be difficult to distinguish by 16S rDNA gene sequence analysis. The situation is exasperated by the lack of accurate curation of strains before depositing sequences in GenBank, and microheterogeneties within the multiple copies of the 16S rDNA gene sequence in the *Cronobacter* genome. Subsequently, Baldwin et al. (2009) proposed a 7-loci multilocus sequence typing (MLST) scheme, which clearly distinguished between these two species. This MLST scheme has now been established for the entire *Cronobacter* genus and is available with online Open Access at http://www.pubMLST.org/cronobacter. This manually-curated database currently contains >1400 profiled strains, and >230 whole genomes with metadata. The scheme is based on seven housekeeping genes (*atpD, fusA, glnS, gltB, gyrB, infB, ppsA*). Concatenating these genes gives a total length of 3036 nucleotides that can be used for phylogenetic studies by multilocus sequence analysis (MLSA). Whole genome phylogenetic analysis of the genomes has confirmed the use of *fusA* for *Cronobacter* speciation in place of the ambiguous 16S rDNA sequence. Other genome-based MLST schemes, such as ribosomal-MLST (53 loci) and core-genome MLST (1865 loci) also agree with the whole genome phylogeny, but are outside the scope of this chapter. For examples of their use, the reader should refer to Jackson et al. (2015a, b) and Forsythe et al. (2014).

Phylogenetic analysis estimates that the separate *Cronobacter* species evolved in the past, with *C. sakazakii* and *C. malonaticus* emerging as definable species (Joseph et al. 2012b). These two species are more associated with infant and adult infections than the remaining five species. Therefore, they may have evolved or acquired accessory genes that have enhanced their virulence capacity and host species adaptation, and thus augmented their overall pathogenicity.

The application of MLST to such a large and diverse collection of *Cronobacter* strains has revealed that there are stable clonal lineages in *C. sakazakii* and *C. malonaticus*. A clonal complex being a cluster of sequence types (ST) with five or more loci in common, and is named after the central sequence type. More importantly for this chapter, it is now recognized that there are certain associations between clonal complexes (CC) and clinical presentations, and therefore some clonal complexes can be regarded as pathovars. Joseph and Forsythe (2011) were the first to report the special significance of *C. sakazakii* CC4, which appears to have a high propensity for neonatal meningitis. This was a retrospective study covering strains from seven countries for over a 50-year period. Hariri et al. (2013) confirmed this association by determining the sequence type of *Cronobacter* strains submitted to the CDC (USA) in 2011. This information gives direction for further meningitis research with the bacterium as it guides the researcher with the selection of strains for analysis. Other associations between clonal lineages and source have been noted (Forsythe et al. 2014). *C. sakazakii* ST1 strains are primarily isolates from infant formula and clinical sources, whereas *C. sakazakii* ST8 is primarily composed of isolates from clinical sources, but none with severe cases of infant infection. *C. malonaticus* ST7 is associated with adult infections, though the source has not been identified. The reason for the predominance of *C. sakazakii* ST4 strains with neonatal meningitis could be due to greater environmental fitness, resulting in increased exposure, as well as the possible encoding of virulence genes.

The first reported age-profiled data was for 819 *Cronobacter* bacteremia cases in England and Wales between 1992 and 2007. The majority (91%) of bacteremia cases were patients >15 years in age. Holý and Forsythe (2014) reported the largest age-profile study to date, with incidence of *Cronobacter* (unspeciated) from >45,000 patients over a 7-year period from 2005–2011. The organism was isolated from every age group, with a higher frequency in children less than 14 years of age. Most *Cronobacter* spp. isolates were from throat swabs, followed by urine, tracheal aspirates, bronchoalveolar lavage, cannulae, and sputum samples. Patrick et al. (2014) reported an age profile for *Cronobacter* infections from an earlier period (2003–2009), which confirmed its prominence in the adult population, especially in urinary tract infections (UTIs). However, none of these earlier age-profiling studies speciated or genotyped the *Cronobacter* isolates.

More recently, Alsonosi et al. (2015) speciated and genotyped *Cronobacter* strains from age-profiled clinical isolates of Holý and Forsythe (2014). Of the 51 strains, most were *C. sakazakii* (65%) and *C. malonaticus* (33%). *C. sakazakii* ST4 was the predominant sequence type (32/51 strains), and these strains were all isolated from one hospital during a 1-year period. Seventeen *C. malonaticus* ST7 strains were isolated from two hospitals during the 6-year period from 2007 to 2013.

The two remaining strains were *C. sakazakii* ST64 and *C. muytjensii* ST28. The strains had been isolated from throat and sputum samples of all age groups, as well as rectal and fecal swabs. There was no apparent association between the age or sex of the patient and the *Cronobacter* species isolated. Pulsed-field gel electrophoresis (PFGE) is routinely used for epidemiological analysis to determine the source of infectious strains of *Salmonella*; however, many of the *Cronobacter* isolates from patients for whom there were no known links could not be further differentiated. For example, the *C. sakazakii* ST4 pulsotype strains were isolated from 15 adults (aged 27–85 years) between May 2012 and May 2013. This important observation could be due to the reported high clonality of sequence types within *C. sakazakii* and *C. malonaticus,* limiting the discriminatory power of PFGE.

The FAO-WHO reported that the annual incidence rate of *Cronobacter* infection in the USA among low birth-weight infants who weighed <2500 g and were <1 year old was 8.7 per 100,000 (FAO-WHO 2006). Although there is no international surveillance system for *Cronobacter* infections, the FAO-WHO (2008) collated 120 cases of infants (6–11 months) and children (12–36 months) between 1961 and 2008. It was reported by Stoll et al. (2004) that among 10,660 neonates in the United States, there was only one case of sepsis with mild illness diagnosed caused by *Cronobacter*. The infant received parenteral nutrition of both breast milk and ready-to-use premature infant formula. Overall, these data indicate that, apart from outbreaks, *Cronobacter* infection is very rare in very-low-birth-weight (VLBW) infants. There are different routes of transmission other than contaminated reconstituted PIF. Ravisankar et al. (2014) reported a case of a premature neonate who was fed exclusively with breast milk, had a *C. sakazakii* culture-positive sepsis, and developed clinical signs of meningitis at 18 days of life. Another case was reported by Broge and Lee (2013) of a 33-day-old infant who was breastfed and developed bacteremia as a result of *Cronobacter* infection. In fact, the type strain of *C. malonaticus* was isolated from a breast abscess. As always with such studies, these frequency studies underestimate the actual number of cases (CDC 2009; Friedemann 2009; Teramoto et al. 2010). It is highly probable that *Cronobacter* infections are often misidentified as *Enterobacter cloacae* infections due to their superficial similarity, according to commonly-used phenotyping identification methods in hospitals.

Although the reported incidence of *Cronobacter* infection in neonates is very low, the survivors often have permanent neurological sequelae and developmental disorders such as hydrocephalus, quadriplegia, and retarded neural development. It is probable that the greater susceptibility for *Cronobacter* infection following premature birth and/or low birth weight is because these neonates lack both a normal gut microflora and an established gut epithelial lining in their intestines, resulting in increased mucosal permeability.

An early hypothesized source of *Cronobacter* infection was the birth canal. However, this source now seems to be unlikely, as none of the vaginally-delivered infants developed signs of infection until several days after birth and it is also unlikely that *Cronobacter* spp. constitute part of the vaginal microflora (Yan et al. 2012; Hunter and Bean 2013). Instead, attention in recent years has focused on bacterially-contaminated reconstituted infant formula. This is because several

outbreaks of bacterial infection in NICUs have been traced to PIF contaminated with *Cronobacter* spp. (Biering et al. 1989; van Acker et al. 2001; Himelright et al. 2002; Teramoto et al. 2010). The recovery of *Cronobacter* in PIF varies but a general figure is ca.1 cell/100 g in 3% of samples (Forsythe 2005; FAO-WHO 2006). It is improbable that ingestion of such intrinsically-contaminated PIF with desiccated-stressed low levels of *Cronobacter* are a direct cause of outbreaks. It is more probable that such contaminated feeds are either temperature-abused after hydration (as per Caubilla-Barron et al. 2007), or outbreak-associated feeds were externally contaminated with high levels of metabolically-active *Cronobacter* cells. A precautionary clause to this statement is that the nasogastric feeding tubes are *in situ* for days and can act as loci for *Cronobacter* (and other bacteria) multiplication as a biofilm, followed by the ingestion of sloughed clumps of cells (Hurrell et al. 2009). The reader should see Holý and Forsythe (2014) for further consideration of unresolved issues with respect to sources and clinical relevance of *Cronobacter,* along with the risk management for the safe feeding of premature neonates.

The carriage of the organism by adults, including breast milk, and the high incidence of the bacterium in urinary tract infections (UTIs) indicate that *Cronobacter* exposure routes other than contaminated infant formula still need to be elucidated. The bacterium has also been isolated from breast abscess, and cases of exclusively breast-milk-fed infants occur (Stoll et al. 2004; Iversen et al. 2007; Broge and Lee 2013; Ravisankar et al. 2014). Also of note is that not all *Cronobacter* outbreaks have been accurately reported. Jackson et al. (2015a) reevaluated two cases from Mexico, originally reported by Flores et al. (2011). Despite the initial use of PCR probes for *Cronobacter*, ribosomal-MLST and whole genome phylogeny revealed the key isolates were in fact *E. hormaechei* and a currently-unspeciated *Enterobacter* spp., which had been isolated from both an unopened PIF tin and symptomatic infants.

Clinical Aspects of *Cronobacter* Infection

It is reasonable to propose that the pathogenic route of infection for *Cronobacter*, and in particular *C. sakazakii*, following ingestion, is that it adheres, invades and translocates through intestinal epithelium to the underlying tissue and blood stream. Then, it persists and may even multiply in macrophages as well as in the blood stream (being serum resistant). Consequently, it could disseminate throughout the body with clinical presentations of bacteremia. After that, it can attach, invade, and translocate through the brain endothelium and cross the blood brain barrier (BBB) to cause invasive meningitis. This process is summarized in Fig. 1, which will be further described in later Sections. The case-fatality rate of neonates due to *C. sakazakii* infection is 50%, with many patients dying within 1 week of diagnosis. Meningitis due to *Cronobacter* results in brain infarction and severe neurologic impairment (Jiménez and Giménez 1982; Howkins et al. 1991; Lai 2001). The host response to infection, such as nitric oxide secretion, apoptosis induction, and cytokine production might contribute to the pathogenesis leading to such severe outcomes in the host.

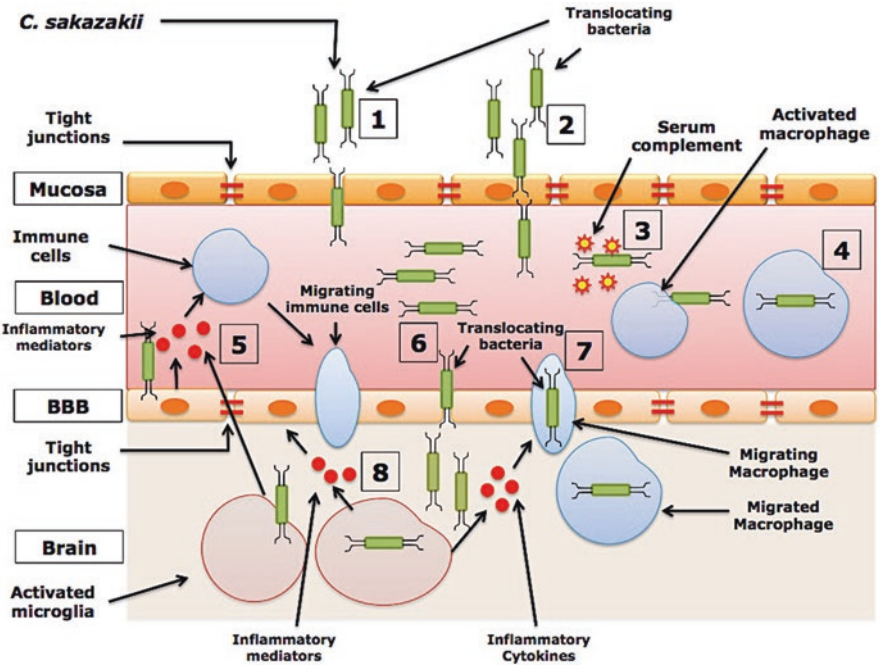

Fig. 1 Proposed sequential steps of *C. sakazakii*-host interactions during the pathogenesis of *C. sakazakii*-induced meningitis and variable behavior of *C. sakazakii* strains. (*1*) Paracellular translocation of bacterial cells through the disrupted tight junctions (of Caco-2 cells) that might have been affected by the cytotoxicity induced by *C. sakazakii*, the translocation of these strains was accompanied by a notable decrease in Transepithelial electrical resistance (TEER). (*2*) Transcellular translocation of bacterial cells by invasion of gut cells (Caco-2) and translocation through them without a notable change in TEER. OmpA might play an important role in this step. (*3*) *C. sakazakii* survival in the bloodstream by avoiding complement-dependent killing of serum – possibly mediated through cleaving complement components C3 and C4b, by the *cpa* gene product. Moreover, some other factors such as iron uptake could support the survival of *C. sakazakii* in the host environment. (*4*) Intracellular survival and multiplication of *C. sakazakii* in blood macrophages that help the bacterium to evade the immune response and work as a vehicle to transport the intracellular bacteria to other body sites. Gene products such as SodA might have an important role in this process. (*5*) *C. sakazakii* attachment and invasion of brain endothelium (HBMEC cells) may lead to the release of some inflammatory mediators such as IL-1β, TNF-α, IL.6, IL-8, GM-CSF, and iNOS (that could lead to increased NO production). These might contribute to cell line permeability and the migration of several immune cells such as neutrophils and macrophages (pleocytosis). The cell line permeability might facilitate the translocation of *C. sakazakii* into the brain. (*6*) Two ways of *C. sakazakii* translocation into the brain are suggested; paracellular translocation by altering the tight junctions through cytotoxicity and apoptosis (caspase-3) induction, and by (*7*) using the Trojan horse mechanism inside macrophages. (*8*) The translocated *C. sakazakii* strains could be phagocytized by microglia (HMGC cells) inside the brain and then survive and multiply. The activated microglia produce inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-8 and GM-CSF, which might contribute to brain endothelium (HBMEC cells) permeability and by attracting more immune cells to migrate to the brain. The inflammatory process inside the brain might lead to neuronal death, increased intracranial pressure, and severe inflammation of the meninges

Infection of the Gastrointestinal Tract: Necrotizing Enterocolitis (NEC)

It is generally accepted that *Cronobacter* colonizes and infects the human body through the gastrointestinal tract. Destruction of the intestinal cells leads to NEC (Liu et al. 2012b). Whether *Cronobacte*r causes the initial intestinal tissue injury or is more associated with NEC due to subsequent growth in the intestinal tract is unclear. The increased levels of mucosal inflammation could be attributed to the production of high levels of host inflammatory factors such as cytokines, platelet-activating factor, nitric oxide, and prostanoids. These will cause further damage to the epithelial cells.

NEC is the most common gastrointestinal condition in NICUs and primarily affects premature infants. Moreover, it is a common cause of death among 20–40% of neonates who have NEC and require surgical intervention (Holman et al. 2006). The earliest cases of NEC in the United States were reported in the early 1960s. The estimated fatality rate ranged from 10% to 50% among NEC cases. This disease has different stages (i.e., NEC I, II, and III, and could further lead at its final stages to marked abdominal distension, deterioration of vital signs, septic shock, gastrointestinal haemorrhage, and intestinal failure) (Bell et al. 1978; Henry and Lawrence Moss 2010; Iben and Rodriguez 2011).

Infection of the Central Nervous System; Bacterial Meningitis

For *Cronobacter* to establish a systemic infection, it must translocate to the underlying tissues, and then disseminate throughout the body. Therefore, the epithelium has an important role in protecting the body against bacterial invasion as once this layer losses its integrity, the invading organism will infect the tissue layer beneath (Wilson et al. 2002). The ability of *Cronobacter* to invade the intestinal epithelium and brain endothelium is, therefore, considered to be a crucial step for pathogenesis. Townsend et al. (2007a) reported that the presence of LPS (residual dead bacterial biomass) in PIF increased the permeability of tissue barriers leading to the translocation of intestinal bacteria, including *C. sakazakii*. Laboratory studies by Giri et al. (2011) revealed that invasive C. *sakazakii* strains were able to translocate through the intact monolayers of human intestinal (Caco-2) and brain (HBMEC) cell lines transcellularly. This suggests that the bacterium is able to overcome the physical host barriers in the intestines and central nervous system (CNS). Strains of *C. sakazakii* and *C. malonaticus* were more invasive of Caco-2 cells than other *Cronobacter* species (Townsend et al. 2008). Similarly, both *C. sakazakii* and *C. malonaticus* survive and replicate in macrophages inside phagosomes, whereas strains of *C. muytjensii* die and *C. dublinensis* is serum sensitive (Joseph et al. 2012b).

Once the organism has entered the blood stream, it has a tropism toward the CNS, thus increasing the propensity to cause meningitis among low-birth-weight

neonates and infants (Forsythe et al. 2014). The BBB is a highly specialized brain endothelial structure and represents a diffusion barrier that is important to biological functions of the CNS (Ballabh et al. 2004; Zlokovic 2008). Once these bacteria migrate across this barrier, they can multiply within the subarachnoid space concurrently with the release of bacterial components, such as cell wall fragments, which are highly immunogenic and can increase the inflammatory response of the host (Sellner et al. 2010; Barichello et al. 2013). This could lead to ventriculitis and the formation of cysts or brain abscesses, which later could develop into hydrocephalus, due to the accumulation of cerebrospinal fluid (CSF; Bowen and Braden 2006). The accumulation of CSF could increase the intracranial pressure that could affect the brain's tissues.

Mechanisms of Pathogenicity in *Cronobacter* spp.

Although there has been a significant increase in *Cronobacter* research over the last decade, the precise mechanism of *Cronobacter* pathogenicity is still unknown. One of the first attempts to understand this mechanism was by Pagotto et al. (2003) who investigated the production of enterotoxin among different *Cronobacter* strains. The study revealed that *Cronobacter* isolates are able to produce enterotoxins, and the toxin was lethal to suckling mice. Other candidate virulence determinants include superoxide dismutase (SodA) for macrophage survival, hemolysin, flagella, fimbriae, a metalloprotease (zpx), and plasmid-borne virulence factors such as *Cronobacter* plasminogen activator (Cpa) and type-six secretion systems (T6SS; Joseph et al. 2012b, Franco et al. 2011a, b). Adhesins (i.e., fimbriae) are presumably important, as the bacterium can attach to intestinal cells, but the specific receptors involved remain to be determined. Survival mechanisms in macrophages have not been identified. It was reported that the OmpA gene product of *Cronobacter* spp. has 88% similarity to that of *E. coli* K1 at the protein level. This protein promotes the invasion ability of *E. coli* K1 to human intestinal and brain mircovascular endothelial cells *in vitro* (Singamsetty et al. 2008; Nair et al. 2009). It was also determined that the expression of OmpA is critical for *Cronobacter* spp. invasion of HBMEC and is required for microtubule condensation, PI3-kinase, and PKC-α activation (Singamsetty et al. 2008). Kim et al. (2010) determined that OmpA and OmpX are essential for *Cronobacter* basolateral invasion of the host cell, including Caco-2 and INT407 cells, and the movement into deeper organs. OmpA and OmpX possibly have a role in the organism penetrating the blood-brain barrier, although the mechanism leading to the destruction of the brain cells is unknown and could, in part, be a host response. Cytopathogenicity could be attributable to toxins in outer membrane vesicles, LPS endotoxins and enterotoxins. The sequence of attachment through to brain cell invasion is shown in Fig. 1 for *C. sakazakii* and is explained further below.

Attachment, Invasion and Translocation

In vitro tissue culture assays have been applied to examine the bacterial-host interaction of *Cronobacter* with human intestinal (Caco-2, Hep-2, INT-407), macrophage (U937), rat brain microvascular endothelial cells (rBCEC4), and human brain microvascular endothelial cells (HBMEC). Understanding the attachment mechanisms are also pertinent.

Attachment

There has been very sparse laboratory investigation into *Cronobacter* adhesins for the attachment to intestinal epithelial cells; Fig. 1 steps 1 and 2. Mange et al. (2006) reported that neither mannose-sensitive type 1 fimbriae nor type 3 fimbriae were involved in the adhesion of *C. sakazakii* ES5 to human epithelial or brain cells. Fibronectin, a glycoprotein in the extracellular matrix of host cells has been postulated in bacterial adherence to intestinal epithelial or endothelial cells (Mittal et al. 2009b). Nair and Venkitanarayanan (2007) determined that inhibiting fibronectin binding reduced the attachment of *C. sakazakii* cells to INT-407 epithelial cells, yet did not significantly reduce their adherence to HBMECs. The implications of these findings in pathogenesis and virulence have not been fully understood. It is probable that *Cronobacter* attachment mechanisms are host cell-type specific. Hence a range of plausible adhesins such as OmpA, OmpX, and fimbriae warrant further investigation.

Whole genome analysis of *Cronobacter* strains has revealed ten putative fimbriae clusters (Joseph et al. 2012b). All fimbriae clusters were similar to the type I chaperone/usher-assembled pilus system. These were predicted to encode complete and functional pili due to their homology between the genes in the fimbriae clusters and the remaining components necessary for type-I pilus assembly (the minor tip fibrillum fimG and fimbrial adhesin fimH genes). Type 1 fimbriae have been associated with *E. coli* K1 invasion of human brain cells and are, therefore, of particular interest with respect to the mechanisms of severe neonatal infections by *Cronobacter*. Grim et al. (2013) reported that the *Cronobacter* genome contains genes for the type IV pili in addition to a P pilus homologous to that found in uropathogenic *E. coli* that causes meningitis in infants.

Interestingly, not all *Cronobacter* spp. encode for all ten fimbrial gene clusters. Of particular interest is the distribution of two fimbrial clusters; curli and β-fimbriae. Curli fimbriae have an important role in the adhesion and colonization of host cells by *E. coli* through binding to surface matrix components (i.e., fibronectin, laminin, and plasminogen). However, the *C. sakazakii* genomes lack the genes encoding for curli fimbriae. Instead, *C. sakazakii* were the only *Cronobacter* species to possess β-fimbriae. This may explain, in part, the variation in the host susceptibility range and pathogenicity observed among the different *Cronobacter* spp. (Kucerova et al. 2011; Joseph et al. 2012b, Holý and Forsythe 2014). The implications of these findings in virulence have not been fully elucidated, but presumably reflect differences in the ecosystem of *C. sakazakii* compared with the rest of the genus. Whether this is directly related to neonatal infection is unclear at present.

Invasion

The invasive ability of *Cronobacter* has been studied by several researchers and is summarized in steps 3–7 in Fig. 1. Singamsetty et al. (2008) determined a higher invasion frequency of HBMECs than endothelial and epithelial cells. Townsend et al. (2008) also revealed a variation in *Cronobacter* invasion capacity with human intestinal (Caco-2) and rat brain (rBCEC4) cell lines, indicating that *Cronobacter* invasion of rBCEC4 was strain-dependent, with no direct connection between the extent of bacterial attachment to Caco-2 cells and the amount of invasion. Kim and Loessner (2008) found that *Cronobacter* invasion of Caco-2 cells was an active process that required *de novo* protein synthesis, and was dependent on exposure time and the multiplicity of infection.

Translocation

Giri et al. (2011) determined that *C. sakazakii* food and environmental isolates were able to transcytose across tight monolayers of Caco-2 cells and HBMECs. Moreover, Almajed and Forsythe (2016) determined that *C. sakazakii* clinical isolates have the capacity to translocate paracellularly through the polarized monolayers of the Caco-2 and HBMEC cell lines, thus mimicking the *in vivo* ability to cross the intestine and subsequently the blood brain barrier to cause meningitis; Fig. 1 steps 6 and 7. In addition, the rate of transcytosis of some strains was equivalent to that of the neonatal meningitic *E. coli* K1 pathovar strain used as a comparative positive control. The rate of transcytosis varied significantly between bacterial strains and human cell type, with transcytosis of HBMECs requiring three times longer than Caco-2 cells.

Role of OmpA in *Cronobacter* Invasion of Mammalian Cells

OmpA is believed to have a major role in *Cronobacter* invasion of host cells using *in vitro* cell cultures with human intestinal epithelial cells and human brain endothelial cells (Fig. 1 step 2; Nair and Venkitanarayanan 2007; Kim and Loessner 2008; Singamsetty et al. 2008; Kim et al. 2010). There was 87% reduction in the invasion of INT-407 cells by a *C. sakazakii* ATCC 29544^{T} mutant that was not expressing OmpA (Kim et al. 2010). Similarly, Nair et al. (2009) determined that OmpA was required for *C. sakazakii* invasion of HBMECs, since the absence of OmpA reduced the amount of invasion by up to 83%. Adherence to INT-407 and Caco-2 cells was independent of OmpA, and therefore there are other surface determinants (i.e., fimbriae) that contribute to *C. sakazakii* attachment to host cells that may not have a role in the invasion (Kim et al. 2010).

Kim et al. (2010) determined that OmpA and OmpX are essential for *Cronobacter* basolateral invasion of host cells, including Caco-2 and INT407 cells, and the movement into deeper organs. Additionally, the invasion levels of Caco-2 cells by *ompA*–

and *ompX*− were less than the wild type. However, invasion by the *ompX*− mutant was not as much decreased as the *ompA*− mutant, suggesting that OmpA has a dominant role in Caco-2 invasion. Surprisingly, Jaradat et al. (2009) and Giri et al. (2012), using the primers described by Nair and Venkitanarayanan (2007), determined that not all *Cronobacter* spp. were positive for *ompA*. However these must be false-negative results due to primer design since the *ompA* is present according to BLAST searching of all *Cronobacter* genomes on the PubMLST database (http://pubmlst.org/cronobacter/). Therefore the authors advise that the curated genomes be searched for traits due to the possibility of false-negative virulence gene probes, which may be due the early design of the primers before the diversity of the genus was more fully understood.

Role of Cytoskeleton Rearrangement in *Cronobacter* Invasion

To facilitate smooth entry into the host cell cytoplasm, several pathogenic bacteria have the capacity to manipulate the host cell cytoskeleton either by secreting effector molecules or by interference with the phosphorylation cascades of the intracellular signal transduction pathways (Singamsetty et al. 2008; Kim and Loessner 2008; Mittal et al. 2009a). Several studies have revealed the role of microfilaments and microtubules in *Cronobacter* invasion of host cells.

Nair et al. (2009) determined that localization of both microfilaments and microtubules occurred in INT407 cells following *Cronobacter* infection. According to Kim and Loessner (2008), the entry of *C. sakazakii* ATCC 29544^T into Caco-2 cells could be receptor mediated, whereby the invasion process depends on bacterial *de novo* protein synthesis. Moreover, the organism can disrupt the tight junctions of the cells, which are important in host cell polarity and prevent molecules from passing freely through the gaps between cells. This disruption requires actin microfilaments, and, in turn, facilitates and enhances the invasion mechanism. Cytochalasin D (CyD) has the ability to prevent G-actin polymerization and disrupt F-actin in a concentration-dependent manner. This might inhibit bacterial entry or movement that requires F-actin. It was revealed that the invasion of *Cronobacter* was increased in CyD-treated Caco-2 monolayers. This invasion was due to the disruption of the tight junctions that requires, in the case of *Cronobacter*, actin filaments (Kim and Loessner 2008).

The expression of OmpA is of importance for *Cronobacter* spp. invasion of HBMEC and is essential for microtubule condensation, PI3-kinase (PI3K), and protein kinase C-alpha (PKC-α) activation (Singamsetty et al. 2008). Li et al. (2010) reported that *C. sakazakii* ATCC 29544^T invasion of HBMEC led to rearrangement of actin stress fibers and cortical actin fibers in addition to an increase in protein kinase B (Akt) phosphorylation. Liu et al. (2012a) determined that HBMEC cytosolic phospholipase A2 α (cPLA$_2$α), which acts downstream of Akt signaling pathway and is associated with the rearrangement of actin filaments, was promoted by *C. sakazakii* ATCC 29544^T infection. Furthermore, inhibiting cPLA$_2$α phosphorylation attenuated *C. sakazakii* invasion into HBMEC.

Tight Junction Disruption

Tight junctions are important in host cell polarity and prevent molecules from passing freely through gaps between cells. Kim and Loessner (2008) determined that the disruption of Caco-2 tight junctions substantially increased *C. sakazakii* invasion efficiency. Furthermore, Hunter et al. (2009) determined that attachment of the bacterium to enterocytes of the infected animal led to enterocyte apoptosis. They suggested that NO is a vital mediator in *Cronobacter*-mediated NEC, and its toxic metabolite $ONOO^-$ might contribute in the apoptosis induction of rat enterocytes that could lead to tight junction disruption.

Emami et al. (2011) determined the role of dendritic cells (DC) in *Cronobacter*-induced NEC. The study revealed the ability of *Cronobacter* to disrupt the tight junctions of Caco-2 cells and pass through the monolayers. This translocation depends on three factors; namely, (1) DC recruitment to lamina propria upon infection that is responsible for intestinal barrier dysfunction, (2) TGF-β secretion by DCs that is involved in tight junction disruption and apoptosis induction in enterocytes, and (3) OmpA expression in *Cronobacter* that is important in the pathogenesis of NEC. Almajed and Forsythe (2016) revealed the ability of *C. sakazakii* clinical isolates to disrupt the tight junctions of Caco-2 cells and HBMECs showing declined transepithelial electrical resistance levels.

Lipopolysaccharide (LPS) Endotoxin

Lipopolysaccaride (LPS) is a major component of the Gram-negative bacterial cell wall. It can interact with enterocytes through LPS-mediated binding to TLR4. It is pyrogenic and is also known as endotoxin. Using the Limulus amebocyte lysate assay, the amount of pyrogenic LPS in purchased PIF has been shown to vary by 1000-fold (Townsend et al. 2007a). As LPS is heat stable at 100 °C, it will persist in infant formula after commercial drying and after reconstitution, and may have a contributory role in enhancing the pathogenesis of *Cronobacter* in human infants (Townsend et al. 2007a). Townsend et al. (2007a) also provided evidence that endotoxins administered via intraperitoneal injection enhanced the translocation of *C. sakazakii* from the intestinal tract and dissemination within the body, being recovered from the mesentery, spleen, CSF, and blood of endotoxin-treated rats due to enhanced gut and blood brain barrier penetration. One possible mechanism of penetration could be disruption of the tight junctions by LPS, thus increasing the permeability of the intestinal wall to the organism (Kim and Loessner 2008). In addition, endotoxin impairs enterocyte migration and epithelial restitution, and, therefore, can inhibit tissue repair following bacterial infection, leading to the translocation of organisms from the gut into the blood and internal tissue (Cetin et al. 2004).

Different strains of *Cronobacter* have different LPS structures and this variation is responsible for serological diversity of the O-antigen. Whether the different serotypes differ in their immune response is unknown, although the genes encoding for

LPS synthesis do not follow the phylogeny of the genus and therefore do not correlate with the *Cronobacter* sequence-type pathovars (Ogrodzki and Forsythe 2015). To overcome the non-phylogeny of the O-antigen, the previous PCR-probe method has been replaced by *galF* and *gnd* allele sequencing (Ogrodzki and Forsythe 2015).

Enterotoxins and Type-Six Secretion Systems (T6SS)

Pagotto et al. (2003) were the first to describe putative enterotoxin activity in *Cronobacter*. They used a suckling mice assay and reported a minimum lethal dose of 10^8 colony-forming units (CFU) per mouse by intraperitoneal injection. They also determined an enterotoxin effect by four of the 18 *C. sakazakii* isolates tested. When tested on cultured mammalian cells, filtrates from one strain were toxic to Vero (African green monkey epithelial cells) and Y-1 (mouse adrenal cells) cells, causing rounding and cell lysis. Boiling the filtrates for 20 min did not affect the toxin activity in suckling mice, but it reduced the cytopathic effect of the toxin on Vero cells. Yang et al. (2009) determined that filtrates from two out of eight strains studied were toxic to Vero cells. This study further revealed the possibility of an enterotoxin analogue in infections. The function of this toxin may act in a parallel fashion to lipopolysaccharide (LPS), mediating Toll-like 1 receptor (TLR1) activation and stimulating a host inflammatory response.

Raghav and Aggarwal (2007) were the first to purify and characterize a putative enterotoxin from *Cronobacter* isolates. The thermostable putative toxin had a molecular mass of 66 kDa, which is similar in size to the 62-kDa toxin of *Shigella dysenteriae* 1 and to the 65-kDa toxin of *Pseudomonas aeruginosa*, with optimum activity observed at pH 6. Additionally, the activity of the toxin was unaffected by holding it at 50 or 70 °C, and it was stable at 90 °C for 30 min, implying that it is a moderately heat-stable toxin. This indicates that the enterotoxin is resistant to commercial milk pasteurization treatments and thus remains active in PIF. The toxin had a LD_{50} of 56 pg. The contribution of the enterotoxin to the organism's pathogenesis is uncertain, as the genes encoding the putative toxin and the protein structure have not yet been identified. It is anticipated that future molecular studies will further characterize of this enterotoxin.

The Type VI secretion system (T6SS) is a newly-described system that may be involved in competing with other bacteria in adherence, cytotoxicity, host-cell invasion, growth inside macrophages, and survival within the host. This system can export proteinaceous toxins such as enterotoxins, which are potentially important virulence factors. Six T6SS have been identified in the chromosome of *Cronobacter*, and can be plasmid-borne (pESA2/pCTU2) (Kucerova et al. 2010; Franco et al. 2011a, Joseph et al. 2012b). These are putative T6SS clusters, as it remains to be determined whether they encode functional secretion systems. One T6SS-associated gene is *vgrG*, encoding a lipoprotein and is found in most *Cronobacter* strains. Although several T6SS are found in all *Cronobacter* species, none is unique to *C. sakazakii* CC4 strains but may have a contributory role in neonatal meningitis.

Outer Membrane Vesicles (OMVs)

OMV may have a role in activating the cytopathogenic and host cell responses on human intestinal epithelial cells. Alzahrani et al. (2015) determined that the OMVs of *C. sakazakii* strain 767 (isolated from fatal outbreak of neonatal meningitis and necrotizing enterocolitis) were internalized by Caco-2 cells, increased host cell proliferation, and stimulated the host's innate proinflammatory responses, without inducing overt cytotoxicity. They identified and evaluated the potential pathogenicity roles of 18 OMV-associated proteins using mass spectrometry. It was concluded that *C. sakazakii* OMV may play a role in the pathogenesis via bacterial toxin delivery into host epithelial cells, and driving proliferative and proinflammatory responses.

iNOS Production

Nitric oxide (NO) is a small, free-radical gas that promptly diffuses within cells and cell membranes where it interacts with molecular targets (Gross and Wolin 1995). It is synthesized primarily by inducible nitric oxide synthase (iNOS) that is present in fully activated mammalian cells. The cytotoxicity is usually linked to the NO product of iNOS and not to the products of other NO synthases (Kröncke et al. 1997; Aktan 2004). The expression of iNOS during inflammation increases the production of NO significantly (Hunter et al. 2009). NO has a major role in pathophysiological conditions, including neurodegeneration and chronic inflammation. Moreover, it is a cytotoxic effector molecule that displays cytotoxic activity *in vivo*. It is utilized by the immune system to fight invading pathogens, it can be, however, toxic to host tissues if overproduced and may contribute in cell damage or death (Gross and Wolin 1995; Kröncke et al. 1997; Mayer and Hemmens 1997; Aktan 2004).

C. muytjensii ATCC 51329^{T} induces iNOS expression in human and mice intestinal cells, leading to increased NO production and triggering apoptosis that could alter the tight junctions addressed above (Hunter et al. 2009; Emami et al. 2012; Liu et al. 2012b).

Apoptosis

Apoptosis is programmed cell death that is characterized by morphological alterations and a set of cellular changes, including cell shrinkage, chromatin condensation, nuclear DNA cleavage, and membrane blebbing (Cohen et al. 1992; Dimmeler and Zeiher 1997). Inducing cell death, via apoptosis in human cell lines, may contribute to the permeability of these cell lines by the disruption of the tight junctions. It has a major impact on the integrity of the gut barrier, as it is a

characteristic feature of NEC that would lead to the translocation of organisms through the permeable infected layer (Hunter et al. 2008; Liu et al. 2012b, Hunter and Bean 2013). According to Liu et al. (2012b), *C. sakazakii* strains were able to induce apoptosis to human Caco-2 cells and rat IEC-6 cells. Moreover, it was reported by Emami et al. (2012) that *C. muytjensii* ATCC 51329^T had the ability to induce cell death via apoptosis to the intestinal epithelial cell line IEC-6. Hunter et al. (2008) determined the same strain-induced apoptosis in IEC-6 significantly *in vitro* and in an animal model of NEC. This suggests the ability of *Cronobacter* to induce apoptosis which could lead to overcoming the host barrier and facilitate the dissemination of the organism. It should be noted, however, that the *C. muytjensii* species is not associated with neonatal infections and therefore the direct relevance of these studies to neonatal infection is unclear.

Cronobacter infection triggers a group of immune responses, such as cytokine and chemokine production; Fig. 1 step 5. Different inflammatory molecules are involved in the pathogenesis, including TNF-α and IL-6 (Hunter et al. 2008). Studies by Townsend et al. (2007b) revealed that *C. sakazakii* strains, isolated from a NICU outbreak, were able to stimulate the human macrophage cell line U937 to produce TNF-α, IL-6, and IL-10; however, they did not suggest the role of these cytokines in infection. Hunter et al. (2008) determined that *C. muytjensii* triggered IEC-6 cells to produce IL-6. Moreover, Emami et al. (2012) applied a cytokine assay on harvested mice intestinal tissue infected with *C. muytjensii* and determined that *C. muytjensii* infection triggered TNF-α, IL-1β, IL6, and IL-12 secretion. Cruz-Córdova et al. (2012) reported that the flagella of *C. sakazakii* play an important role in triggering the host immune response and the activation of cytokine production such as IL-8 and TNF-α by macrophage and human embryonic kidney (HEK293) cell lines.

Cronobacter Plasminogen Activator (Cpa)

Invasive microorganisms have protective mechanisms against serum-mediated killing; Fig. 1 step 3. Franco et al. (2011b) were the first to determine that the *Cronobacter* outer membrane protease Cpa is a plasminogen activator, which plays an essential role in serum resistance. Cpa has significant identity to proteins that belong to the Pla subfamily of omptins (Franco et al. 2011b). This protease of *Cronobacter* provides resistance against complement-dependent killing of serum by cleaving complement components C3 and C4b. Moreover, it has a major role in converting plasminogen to plasmin, which leads to the activation of other proteolytic enzymes, including matrix metalloproteinases, resulting in degradation of the tight junctions of microvascular endothelial cells. This will allow the bacteria to migrate to peripheral tissue and invade the CNS (Lähteenmäki et al. 2005; Franco et al. 2011b), suggesting that this protein is a significant virulence factor for *C. sakazakii*. Analysis of *C. sakazakii* BAA-894 revealed that Cpa is encoded on a large (130 kbps) plasmid (pESA3) (Kucerova et al. 2010). More recent searching of the *Cronobacter* PubMLST database (>1000 strains) revealed that the gene is found

in most strains of *C. sakazakii* and *C. universalis*, but not in other *Cronobacter* species (Forsythe et al. 2014). Therefore, it may be a contributory virulence factor to host susceptibility, but does not account for the pathogenicity of *C. malonaticus* and *C. turicensis*.

Sialic Acid Utilization

Sialic acid is found in human milk in the form of sialyloligosaccharides and is an ingredient in infant formula. Human milk oligosaccharides are weakly digested and are substrates for intestinal bacteria, encouraging bacterial development in the intestinal tract. As a site of bacterial attachment, the intestinal microvilli of neonates have increased sialic acid and N-acetylglucosamine residues, whereas adults have more glucose, mannose, and fucose residues. Recently, Joseph et al. (2013) described a plausible linkage between the recent evolution of sialic acid metabolism by *C. sakazakii* and its pathogenicity. They reported that *C. sakazakii* is the only *Cronobacter* species that has the *nanAKT* gene cluster encoding for sialic acid utilization as a carbon source. The trait is also found in one branch of the *C. turicensis* species. The unique utilization of sialic acid from breast milk, infant formula, milk oligosaccharides, mucins lining the intestinal wall, and even gangliosides in the brain after passing through the blood–brain barrier could have a possible role as additional virulence factors. Hence, it is possible that the ability of *C. sakazakii* to utilize sialic acid will enhance its pathogenicity for neonates and young infants.

Iron Acquisition Gene System

Iron is an important microelement and acts as an electron acceptor/donor for many important redox reactions; hence, it is essential for many cellular processes (Tanaka et al. 1994; Yoshida et al. 1995; Bishop et al. 2011). Iron also has a vital role in bacterial pathogenesis.

As part of acute-phase response (APR), which is initiated by innate immunity in response to infection, the human body reduces the iron availability to decrease free iron levels to limit bacterial growth (Parrow et al. 2013). During APR, the intracellular storage of iron is increased as ferritin in some cells, including microglia and macrophages. However in such iron-limited environments, bacteria produce high-affinity iron-binding molecules, such as siderophores and hemophores, to scavenge any available iron (Yoshida et al. 1995; Franco et al. 2011a, Parrow et al. 2013). Hence, iron is essential for bacterial pathogenesis, and *Cronobacter* does produce siderophores to survive in iron-limited environments (Franco et al. 2011a, Grim et al. 2012). Moreover, this might facilitate bacterial uptake of iron from the iron-rich cells in the human brain such as microglia (Barron 1995).

According to Tanaka et al. (1994) macrophages and microglia are important iron storage cells. Moreover, at the time of infection the redox-active iron decreases extracellularly and intracellularly, increasing the retention and storage of iron in macrophages as ferritin (Birgegard and Caro 1984). As *Cronobacter* can survive and even multiply within these phagocytic cells, they could be utilizing this intracellular source of iron via siderophores. Moreover, ferritin can be converted to free iron as a result of superoxide production (Bishop et al. 2011). *Cronobacter* could use all these strategies for iron acquisition, and survive in iron-limited environments. Hence, this trait may contribute to the systemic survival of *C. sakazakii* in neonates and the subsequent invasion of the CNS.

Whole genome sequencing has provided considerable insight into *Cronobacter* virulence traits, especially iron-acquisition. The first *Cronobacter* genome sequenced was *C. sakazakii* BAA-894, the strain associated with the fatal NICU outbreak in Tennessee (Himelright et al. 2002; Kucerova et al. 2010). The strain carries three plasmids (pESA1–3), of which, two have been further annotated and characterized by Kucerova et al. (2010). There are many virulence gene clusters encoded on the large plasmid (pESA3, a RepFIB-type plasmid), including two iron acquisition loci; a homologue of an ABC transport-mediated iron uptake and siderophore system (*eitCBAD* operon), and a siderophore-mediated iron acquisition system (*iucABCD/iutA* operon). These have been described in detail by Franco et al. (2011a) and Grim et al. (2013). There are two genes upstream of the *iucA* gene, named *viuB* and *shiF*. The putative protein encoded by *viuB* has a significant similarity to ViuB and YqjH encoded by *Vibrio cholerae* and *E. coli*. These two proteins are accountable for reducing iron from the ferric to ferrous state, leading to the loss of affinity of the ferrous iron for the siderophore (Bogard and Oliver 2007; Grim et al. 2012). Franco et al. (2011b) determined that the *iucABCD/iutA* siderophore was the only functional siderophore possessed by *Cronobacter*. However, Grim et al. (2013) identified both the *feo* and *efe* systems for acquisition of ferrous iron. Cruz et al. (2011) reported that *C. sakazakii* isolates harbor a siderophore-interacting protein (*sip*) gene. The *sip* gene has a ferrodoxin-reductase domain with binding sites to FAD and NAD(P), capable of transferring electrons from reduced ferrodoxin to FAD and then reducing $NADP^+$ to NADPH. But as iron utilization genes are found in all *Cronobacter* species and not just *C. sakazakii* and *C. malonaticus*, its role in virulence is still not understood (Joseph et al. 2012b).

Hemolysins

Cruz et al. (2011) reported type III hemolysins (*hly*) in *Cronobacter* isolated from human and nonhuman sources. The type III hemolysin, a virulence factor in frequently associated with pathogenesis, is an integral outer membrane protein with hemolytic activity. Joseph et al. (2012b) reported that all genomes sequenced of

Cronobacter strains encoded for various hemolysin and hemolysin-related genes, and that these were scattered across the genome. There was some strain to strain variation, with one strain of *C. sakazakii* and *C. malonaticus* that lacked the 21-kDa hemolysin precursor gene.

Capsule Production

Ogrodzki and Forsythe (2015) have determined that *C. sakazakii* and *C. malonaticus* isolates with capsular type [K2:CA2:Cell^{+}] were associated with neonatal meningitis and necrotizing enterocolitis. Their capsular profiling scheme is based on sequence analysis of K-antigen, colanic acid, and cellulose, respectively. This profiling approach is more physiologically relevant than the original 7- housekeeping-gene MLST scheme since the capsule layer can be involved in several physiological and virulence attributes. As well as conferring possible serum resistance and macrophage evasion, capsule production contributes to biofilm formation, and, hence, its persistence on work surfaces, desiccation persistence, and ability to colonize enteral feeding tubes.

Flagella

Flagella are not only responsible for bacterial motility, but may also aid in adherence and translocation of host cells. Cruz-Córdova et al. (2012) reported that *C. sakazakii* flagella have a crucial role in triggering the host immune response and the activation of cytokine production such as IL-8 and TNF-α. According to Hartmann et al. (2010), the absence of flagella in *Cronobacter* mutant strains was associated with reduction in its adhesion to the human intestinal Caco-2 cell line. This suggests that flagella may also have an important role in *Cronobacter* pathogenesis.

Superoxide Dismutase (SOD)

SOD protects the bacterial cell against phagocytosis at the surface of phagocytic cells according to Beaman and Beaman (1984); Fig. 1 step 4. However, its prolonged survival might be a result of other bacterial virulence mechanisms such as inducing apoptosis for phagocytic cells. Townsend et al. (2007b) linked the expression of SOD by *Cronobacter* strains to its survival within human macrophages. Although some of the SOD-expression results correlated with the survival results, results of one of the strains did not correlate, having high SOD activity and low survival. This, however, cannot totally discount the role of *sodA*, as this gene also varied in its expression.

Evading the Immune Response

Cronobacter may evade the host's immune system by persisting, and even replicating intracellularly within macrophages; Fig. 1 step 4. Townsend et al. (2007b) determined that *C. sakazakii* strains from a NICU outbreak could persist *in vitro* for up to 96 h and multiply within human U937 macrophages. This survival and multiplication could help the organism use macrophages as a vehicle to invade the other body organs. This mechanism is called the 'Trojan horse' whereby the organism translocates through tissues inside macrophages; Fig. 1 step 7. This mechanism allows the bacterium to hide inside the phagocytic cells, escape from the immune response, and reach other body organs such as the brain. Some cytokines secreted by the infected tissues attract phagocytic cells and make these tissues permeable and leaky to allow the immune cells to migrate to the site of infection, and help increase the number of the invading organism.

Studies by Mittal et al. (2009a) using both scanning and transmission electron microscopy revealed that *Cronobacter* were taken up by dendritic cells through a conventional phagocytic mechanism and the internalized bacterial cells were enclosed within membrane-bound compartments of the dendritic cells. Also, the *Cronobacter* could interfere with the maturation of dendritic cells and exploit them as a replication-permissive econiche. *Cronobacter* target Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) to survive in myeloid dendritic cells. Emami et al. (2011) determined that dendritic cells are recruited to the intestine upon infection with *C. muytjensii* ATCC 51329^{T}, which is responsible for the intestinal barrier dysfunction. Dendritic cells infected with OmpA^{+} *C. muytjensii* ATCC 51329^{T} produced more IL-10 and transforming growth factor (TGF)-b, and very low levels of proinflammatory cytokines, a phenotype associated with tolerogenic dendritic cells. Thus, *Cronobacter* could exploit IL-10- and TGF-b-producing tolerogenic dendritic cells to escape temporarily potent host immune defence mechanisms (Mittal et al. 2009a, Emami et al. 2011).

Various Putative Virulence Factors

There are a number of remaining putative virulence factors which have been reported, although often the studies only used single strains and, therefore, the range of bacterial strains needs to be expanded in order to determine the relevance of the proposed virulence trait at the whole genus, species, or particular sequence types level.

In addition to enterotoxins (section "Enterotoxins and Type-Six Secretion Systems (T6SS)"), *Cronobacter* also secrete proteolytic enzymes that lyse cells and create tissue damage at the site of infection in mice (Pagotto et al. 2003). Kothary et al. (2007) reported the presence of a zinc metalloprotease (zpx) caused CHO cells to change their morphology, rounding and leading to cell damage. However *zpx* is encoded in all *Cronobacter* strains and species analyzed to date (>230 whole genomes), and, therefore, does not directly relate to the prevalence of *C. sakazakii* and *C. malonaticus* in clinical isolates.

Kim et al. (2015) proposed that the RNA chaperone Hfq has a role in virulence. In the absence of *hfq*, *C. sakazakii* ATCC 29544^T was highly attenuated in dissemination *in vivo*, showed decrease in invasion (threefold) into animal cells and survival (1000-fold) within host cells, and exhibited low resistance to hydrogen peroxide (100-fold). It was also notable that the loss of *hfq* led to hypermotility on soft agar.

Choi et al. (2015) generated a random transposon insertion mutant library, and showed the plasmid-borne methyl-accepting chemotaxis protein gene (*mcp*) had a role in virulence in *C. sakazakii* ATCC 29544^T. The *mcp* gene is carried on the strain's plasmid pCSA2. The *mcp*− mutant was reduced in motility and biofilm formation. It also had reduced ability to adhere and invasion of epithelial cells, and in its virulence of rat pups.

Suppiger et al. (2016) revealed that *C. turicensis* possesses a functional RpfF/R system, which is involved in the regulation of several phenotypes, including biofilm formation, colony morphology, and swarming motility. The RpfF/R system is known to be involved in sensing and responding to diffusible signal factor (DFS)-type quorum sensing signals that are produced by bacterial pathogens to control virulence and biofilm formation. The study used the Zebrafish infection model and it revealed that the *C. turicensis rpfF* mutant showed reduced virulence when it infected the larvae (Suppiger et al. 2016).

Conclusions and Future Perspectives

Cronobacter spp. is a newly classified genus and more research has yet to be completed for a better understanding of this unique group of organisms. As the virulent members cause high mortalities in neonates, it is important to understand which gene products are responsible for its pathogenicity and how expression of these virulence factors is regulated. More research is needed to develop a better understanding of the progression and pathogenesis of *Cronobacter* spp.-related diseases, and, particularly, using *in vitro* cell-based assays, combined with animal models. Nevertheless, considerable progress has been made in the past 10 years through the work of several international laboratories, and the application of whole genome sequencing will serve to enhance this.

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Factors Affecting Variation in *Salmonella* Virulence

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Abstract *Salmonella* annually causes a substantial number of illnesses associated with the consumption of fecally-contaminated meats, eggs, milk, and produce. Whether someone becomes ill following the consumption of *Salmonella*-tainted foods is dependent on: (1) the host's susceptibility to infection (age, immune status, etc.); (2) pathogen concentration (population); (3) the amount of the adulterated product consumed; (4) nature of the food matrix; and (5) *Salmonella* serovar or strain type. *Salmonella* primarily causes self-limiting gastroenteritis, and its pathogenesis is associated with a type 3 secretion system involved in cell invasion and inflammation. The evolution of *Salmonella* as a pathogen is attributed to acquisition of large virulence gene clusters, referred to as pathogenicity islands that are responsible for cell invasion, inflammation, colonization, and macrophage survival. While *Salmonella* is genetically diverse, as evident in 2500 plus serovars, there is a significant disparity in the serovars and strain types associated with human illness. This disparity is in part due to the distribution of mobile genetic elements (prophages and plasmids) and resident virulence genes. Several of these plasmid/phage-borne virulence genes are type 3 secretion system effectors associated with *Salmonella* pathogenicity islands responsible for cell invasion and macrophage survival. Gene polymorphisms (alleles) and alterations in virulence gene expression play an important role in shaping the virulence of *Salmonella* serovars and strains. Transitioning towards whole genome sequencing as an epidemiologic, investigative tool, will reveal more about *Salmonella* virulence and provide markers for rapid identification of virulent serovar/strain types in foods.

Keywords *Salmonella* • Virulence • Pathogenicity islands • Type 3 secretion systems

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Introduction

A recent estimate of the incidence of *Salmonella* illnesses in the USA is 68/100,000 persons each year (or 1 in 1470 people) (Curtis et al. 2014), accounting for 217,600 cases each year. However, this may be an underestimate of the true burden of disease due to under reporting and the true number of cases may actually be one million annually (or 1 in 320 people) (Scallan et al. 2011). The economic burden of salmonellosis is estimated to be $3.3 billion annually, due to missed work days, hospitalizations, and valuation associated with premature mortality (Hoffman et al. 2012). *Salmonella* can cause gastroenteritis, septicemia, or enteric fevers, including typhoid fever. Typhoid fever is a serious, life-threatening illness, requiring medical intervention; symptoms include high fever, weakness, headache, abdominal pain, loss of appetite, and rash, characterized by low flat, rose-colored spots (http://www.cdc.gov/nczved/divisions/dfbmd/diseases/typhoid_fever/). Most patients with typhoid fever do not present signs of gastroenteritis, despite the small intestine being the initial site of infection. This disease is geographically limited to regionally-endemic areas (Mogasale et al. 2014). Gastroenteritis, the disease syndrome most commonly associated with *Salmonella,* is characterized by nausea, vomiting, diarrhea, fever, and abdominal cramps. This infection is generally self-limiting and lasts 4–7 days. Medical intervention is sometimes required due to marked dehydration or septicemia that sometimes follows the initial symptoms of gastroenteritis. The incubation period for *Salmonella*-induced gastroenteritis is 12–36 h and ranges from 6 to 72 h (http://www.cdc.gov/salmonella/).

Most *Salmonella* infections in humans are attributed to the consumption of contaminated foods. Foods most commonly associated with *Salmonella* outbreaks are varied and include, but are not limited to, milk, meats, eggs, and produce (fruits, vegetables, and nuts). Other sources for human infection include contact exposure to pets and wildlife infected with *Salmonella*, as well as the environment (e.g. water) contaminated with this pathogen. Because *Salmonella* can colonize a variety of animal species, most human infections are attributed to consumption of food or water directly or indirectly contaminated with feces or contact exposure with a *Salmonella*-infected animal. Water is frequently a vehicle of *Salmonella* contamination of fresh produce, from irrigation of crops to its use in processing fruits and vegetables.

Whether a person will become ill following exposure to *Salmonella* depends on several factors. Most *Salmonella*-associated illnesses occur in children ≤5 years of age (http://www.cdc.gov/foodnet/reports/annual-reports-2013.html). Individuals who are immunocompromised are also more susceptible to *Salmonella* infections (Gordon 2008). Gastric acid-suppressing medications, such as antacids and proton pump inhibitors, also increase a patient's likelihood of contracting a *Salmonella*-associated gastrointestinal infection (Schmid et al. 1996; Brophy et al. 2013). The food matrix itself also significantly influences the minimum infective dose for *Salmonella*, incubation period, as well as the severity of illness (Bollaerts et al. 2008). Outside of general food safety deficiencies in food processing and handling (refrigeration, holding temperature, etc.), which can increase *Salmonella* populations in

contaminated foods, a processing step(s) may also inadvertently affect the pathogen's physiology (e.g., acid tolerance response) and thus decrease its infective dose (Leyer and Johnson 1992). Finally, there is significant variation in *Salmonella* virulence. This review will explore the genetic basis behind the variation in *Salmonella* virulence and pathogenesis.

Evolution of *Salmonella* Genus, Species and Subspecies

Salmonella belongs to the phyla γ-proteobacteria and the family *Enterobacteriaceae*. There are two species within this genus, *S. enterica* and *S. bongori,* which are generally associated with warm-blooded and cold-blooded animals, respectively. Most human cases of salmonellosis are attributed to the species, *S. enterica*. Within *Salmonella enterica*, there are six subspecies; *S. enterica* subspecies I accounts for most human cases of salmonellosis. *Salmonella* is evolutionarily-related to *Escherichia coli* as reflected by its 16S rRNA, other house-keeping genes and conserved order of common core genes in the chromosome, referred to here as synteny. While there is significant conservation spanning an alignment of *Salmonella* and *E. coli* chromosomes, there are regions within the chromosomes that differ between the genera *Escherichia* and *Salmonella* (Fig. 1). These genetic differences can vary between one to few consecutive gene differences, to large gene clusters (≥10 genes). Two loci stand out that are dedicated to O-antigen and distal core lipopolysaccharide (LPS) synthesis from this genomic comparison. Within each genus and species, there are additional genetic differences within each locus that account for antigenic differences within the LPS (Fig. 2). There are, in addition, significant metabolic differences between the two genera, as well as within species, attributed to gain and loss of several metabolic genes (Fig. 1) (e.g., *Salmonella* and tetrathionate respiration) that gives *Salmonella* a selective advantage. Tetrathionate, for example, is produced during inflammation of the small intestine and, thus, gives *Salmonella* a growth advantage over other bacterial species (Winter et al. 2010). Several gene clusters identified in this chromosome to chromosome comparison relate to the ability of the microorganism to cause disease, disease syndrome associated with the pathogen, and disease severity; referred to here as pathogenicity islands (PAI) (see Fig. 1). Pathogenicity islands have several features in common: (1) they are adjacent to tRNA gene; (2) the %GC content is different in comparison to typical housekeeping genes; and (3) the presence of remnant phage genes (Hacker and Kaper 2000; Hansen-Wester and Hensel 2002). The evolutionarily event that separates *Salmonella* from *E. coli* is assumed to be the acquisition of the *Salmonella* pathogenicity island 1 (SPI1) (Mills et al. 1995; Fookes et al. 2011) (see Fig. 3). SPI1 encodes a type 3 secretion system (Galan et al. 1992; Collazo et al. 1995) and iron transport system (Janakiraman and Slauch 2000). The Type III secretion system (T3SS) is a protein export system that produces a needle complex (Kubori et al. 1998) responsible for the injection of bacterial proteins (T3SS effectors) that affect signal transduction in eukaryotic cells (Pace et al. 1993; Kaniga et al. 1995; Zierler

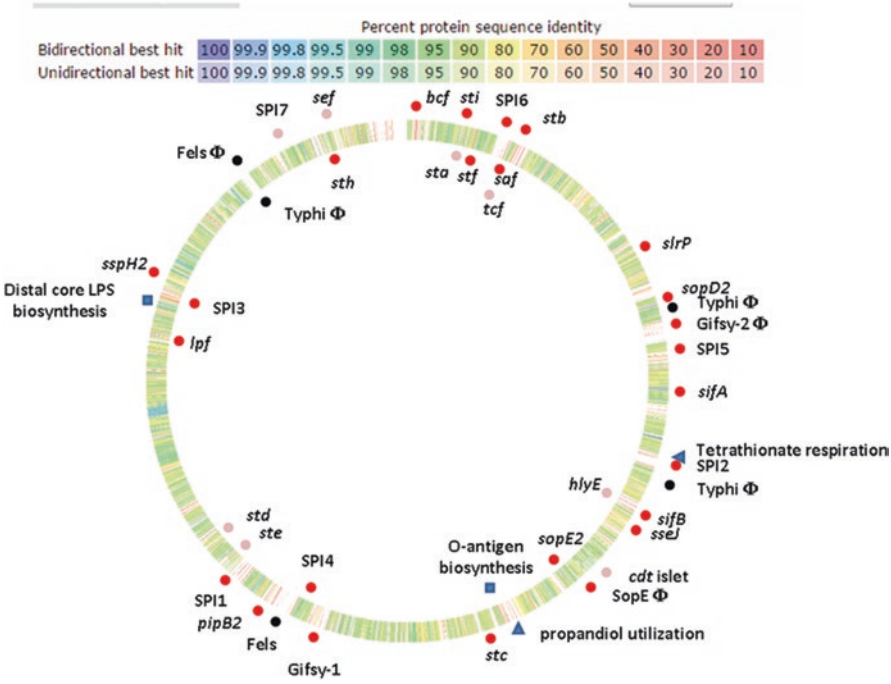

Fig. 1 Genomic Comparison of *Salmonella* to Escherichia coli K12. Rapid Annotations using Subsystems Technology (RAST) (Aziz et al. 2008) was used to compare the *S.* Typhimurium LT2 genome vs. the *E. coli* K12 genome, order genes common to both, and display % similarity (color coded: *blue* (100% identity) to *orange* (10% identity)) of common core genes present in both organisms. *Gaps* represent gene(s) absent in *E. coli* K12. A similar genomic comparison was performed between *S.* Typhi Ty2 and *E. coli* K12. *S.* Typhi-specific gene(s) (*salmon color*) were mapped to the *S.* Typhimurium chromosome. *Salmonella* prophages (*black circles*), virulence (*circles*; *red or salmon colored*), metabolic (*blue triangles*), and LPS biosynthesis (*blue squares*) gene(s) were mapped to the *S.* Typhimurium LT2 chromosome. Several of these virulence genes are contained within: seven *Salmonella* pathogenicity islands (SPI1–7); and three of six prophages (Gifsy-1, Gifsy-2, and SopE Φ). In addition, fimbrial operons (*bcf, lpf, saf, sef, sta, stb, stc, std, ste, stf, sth, sti, tcf*), type 3 secretion system (T3SS) effectors (*sifA, sifB, slrP, sopE, sopE2, sseI, sseJ, sspH2*), and toxins (*cdt* islet, *hlyE*) were mapped to the *Salmonella*/*E. coli* core genome

and Galan 1995; Chen et al. 1996; Collazo and Galan 1997). T3SS are present in many Gram-negative pathogens of plants as well as animals (Hueck 1998). The SPI T3SS induces intestinal epithelial cells to phagocytize *Salmonella* cells via rearrangement of the cytoskeleton in the microvilli of the intestinal enterocyte (Ginocchio et al. 1992). This alteration of the microvilli cytoskeleton is temporary and reassembles after the invading bacterium enters the infected epithelial cell (cell invasion) (Ginocchio et al. 1992). The SPI1 T3SS is a primary characterizing feature that distinguishes *Salmonella* from other member species of the family *Enterobacteriaceae* and it is the target of several *Salmonella*-specific PCR tests (Rahn et al. 1992; Rodriguez-Lazaro et al. 2003; Suo et al. 2010). Several T3SS effectors responsible for cell invasion are located within the SPI1 locus (Collazo

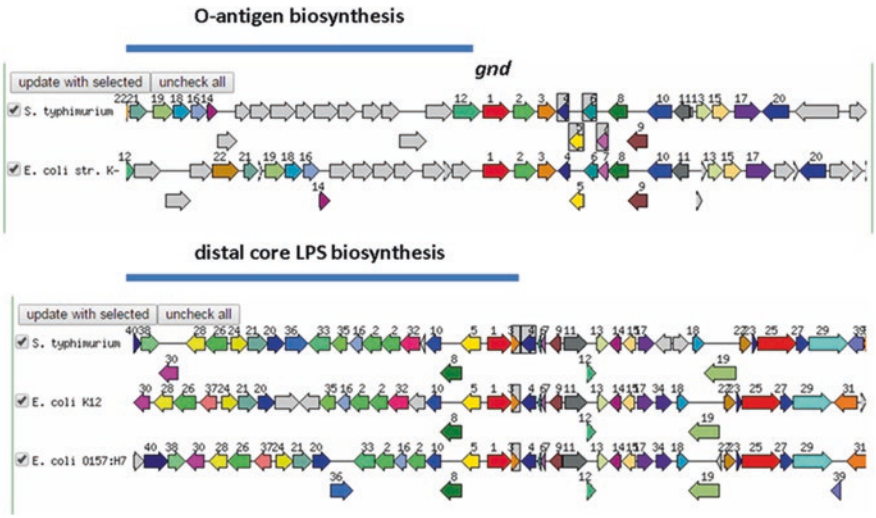

Fig. 2 LPS Biosynthesis Loci. RAST (Aziz et al. 2008) genome browser was used to display genetic differences within O-antigen biosynthesis and distal core LPS biosynthesis operons in S. Typhimurium LT2, *E. coli* K12, and *E. coli* O157:H7

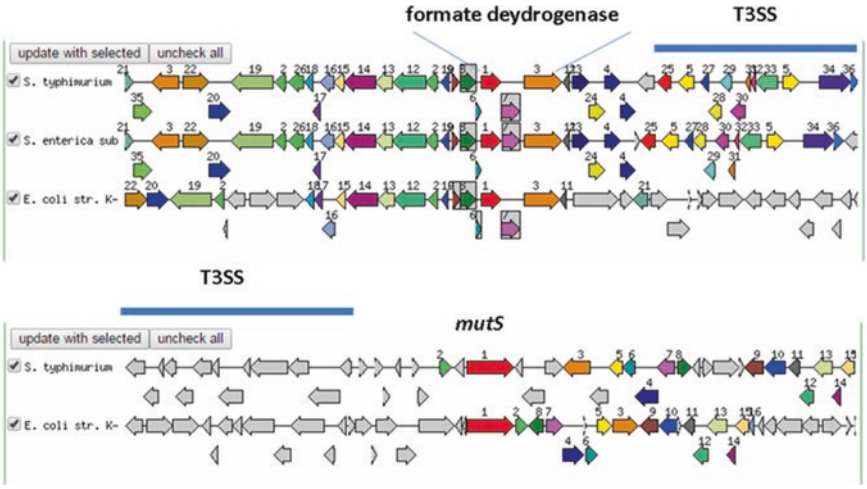

Fig. 3 *Salmonella* Pathogenicity Island 1 (SPI1) Cell Invasion Locus. RAST (Aziz et al. 2008) genome browser was used to display the position of SPI1 locus of S. Typhimurium LT2 relative to *E. coli* K12. SPI1 is positioned between formate dehydrogenase genes and *mutS* in *Salmonella* and *E. coli*

and Galan 1997). There are, however, additional cell invasion T3SS effectors that map outside the SPI1 locus (Hardt et al. 1998; Bakshi et al. 2000; Mirold et al. 2001a; Brown et al. 2011; Luo et al. 2011) (Fig. 1). In addition to eliciting cell invasion, SPI1 also causes the production of inflammatory cytokines and chemokines in

the *Salmonella*-infected epithelial cell (Jung et al. 1995; Hobbie et al. 1997; Wall et al. 2007). Therefore, SPI1 is important in *Salmonella* pathogenesis as related to gastroenteritis (Watson et al. 1998).

Salmonella enterica has a second T3SS (Shea et al. 1996) responsible for macrophage survival (Ochman et al. 1996), referred to here as SPI2. This 2nd PAI locus alters macrophage function by preventing phagosome-lysosome fusion of phagocytized *Salmonella* by the macrophage (Uchiya et al. 1999). This 2nd PAI separates *Salmonella* species *S. enterica* from *S. bongori* (Chan et al. 2003). Like SPI1, several T3SS effectors are located within and outside the SPI2 locus in *Salmonella* (Knodler et al. 2003; Miao et al. 2003; Rajashekar et al. 2014) (Fig. 1). In addition to SPI2, horizontal gene transfer of PAIs, SPI3–5 also phylogeneticly separates *S. enterica* from *S. bongori* (Desai et al. 2013). However, additional horizontal gene transfer events have played a significant role in shaping *S. bongori* (Fookes et al. 2011). The gain and loss of several fimbrial operons were important contributors to the evolution of the genus *Salmonella*, species *S. enterica* and *S. bongori*, subspecies, and *Salmonella* serovars and strains (Yue et al. 2012; Desai et al. 2013). Pathogenicity islands SPI2 and SPI3 are responsible for *Salmonella* survival within macrophages. SPI4 mediates *Salmonella* attachment to epithelial cells (Wagner et al. 2014), whereas SPI5 is involved in enteritis associated with *Salmonella* infection (Wood et al. 1998).

Genetic Diversity within *S. enterica* Subspecies I, Serovars and Strains

Like *E. coli*, there is substantial significant antigenic diversity in salmonellae LPS, specifically the O-antigen and flagella, to account for the greater than 2500 serovars within the genus *Salmonella* (CDC 2013. National *Salmonella* surveillance annual report). These genetic differences are a reflection of the genetic diversity within the O-antigen biosynthesis gene cluster and flagellin genes, *fliC* and *fljB*. While the antigenic differences in O antigen or O serogroups are rather limited compared to the antigenic diversity of the flagellin, horizontal gene transfer of O-antigen gene cluster and flagellin alleles are responsible for the 2500 plus *Salmonella* serovars. Phage conversion is also responsible for O-antigen alterations that account for additional antigenic differences in the *Salmonella* LPS. Some *Salmonella* express two antigenicly distinct flagellins, referred to as biphasic variants, which express variations of the variable phase 1 (*fliC*) and phase 2 (*fljB*) antigens. The phage 2 antigen locus consists of an invertase, Hin protein that alters promoter orientation; turning on and off the genes *fljB* and repressor *fljA*, of *fliC* (Silverman et al. 1979; Simon et al. 1980; Inoue et al. 1989). The O-antigen biosynthesis gene cluster is complex, encoding for many enzymes responsible for the synthesis of the unique sugars, nucleosugars, sugar linkages (glycosyltransferases),

O-antigen polymerase, export machinery and enzymes for linking the O-antigen to the core LPS molecule (Samuel and Reeves 2003). Certain lysogenic bacteriophages carry enzymes that alter the O-antigen via resident genes, which acetylate or glycosylate the LPS; thus, altering the O-antigen and protecting the lysogen from phage infection by the same or similar bacteriophages (Wollin et al. 1987; Vander Byl and Kropinski 2000). The antigenic formula for each *Salmonella* serovar is based on antigenic differences in the O, phase 1 and phase 2 antigens. For example, *S. enterica* subspecies 1 serovar Typhimurium's antigenic formula is 4,5,12 (O serogroup B): i (phase 1 antigen): 1, 2 (phase 2 antigen). *Salmonella enterica* Enteritidis antigenic formula is 9, 12 (O serogroup D1): g, m:-. *Salmonella* serovars with the designation "-"for phase 2 antigen indicates monophasic flagella. There are many *Salmonella* serovars, which are naturally monophasic, having only the *fliC* gene. However, there has recently been the emergence of the *Salmonella* serovar with the antigenic formula 4,5,12,:i:-. Detailed genetic analyses of these isolates revealed that they are *S*. Typhimurium with a large chromosome deletion within the region containing the *fljB* locus (Soyer et al. 2009). *Salmonella* serovars Pullorum and Gallinarum are nonmotile, O serogroup D1 (antigenic formula 9, 12: -: -). These two *Salmonella* serovars contain the same *fliC* allele as *S*. Enteritidis (Kilger and Grimont 1993) but do not express the flagellin when using the culture methodology typically used to serotype *Salmonella* (Holt and Chaubal 1997). This relationship in serology is purportedly the factor that led to an increase of *S.* Enteritidis in laying hens following mandatory vaccination schemes for *S.* Gallinarum and *S.* Pullorum in the United States (Baumler et al. 2000).

Salmonella appear to have further evolved with the emergence of each serovar. Phylogenetic analyses of several housekeeping genes support unique lineages (monophyletic) for several *Salmonella* serovars and evolutionary-relatedness of serovars based on the common O serogroup (Selander et al. 1990; Smith et al. 1990; Beltran et al. 1991). However, this same analysis also revealed several phylogenetic lineages (polyphyletic) within certain *Salmonella* serovars, indicating separate horizontal gene transfer events rather than the expansion of that serovar (Smith et al. 1990). These serovar-specific and diverse lineages are reflected in both common and diverse DNA fingerprints generated by pulsed-field gel electrophoresis (PFGE) (Zou et al. 2013; Maurer et al. 2015). PFGE can discern genetic differences within a *Salmonella* serovar; therefore, becoming a useful epidemiologic tool (Swaminathan et al. 2001). However, there are *Salmonella* serovars and strain types for which PFGE is incapable of discerning sufficient genetic differences for the purpose of epidemiologic investigations (Hudson et al. 2001). Despite genetic commonalities associated with *Salmonella* pathogenesis, not all *Salmonella* are equal in their ability to cause disease. This becomes evident when one looks at *Salmonella* serovars associated with human infections reported by public health agencies such as the Centers for Disease Control and Prevention (CDC) (CDC 2013. National Salmonella surveillance annual report) and the World Health Organization (WHO) (Galanis et al. 2006), and disparities in the distribution of these same *Salmonella* serovars in various food animal species (Sarwari et al. 2001; Anonymous 2010). The *Salmonella* infective dose in humans varies with regard to *Salmonella* serovar or strain in

question (Blaser and Newman 1982). In addition, many *S. enterica* serovars and strains vary in their virulence in several animal models (Poppe and Gyles 1987; Berndt et al. 2007; Paulin et al. 2007; Swearingen et al. 2012; Suez et al. 2013).

While *S. enterica* subspecies I causes most human infections, not all serovars are equivalent in the disease syndromes and severity of illness associated with them (CDC. 2013. National Salmonella surveillance annual report; Galanis et al. 2006). There are also differences among *S. enterica* subspecies I serovars in the animal species they infect and illnesses they cause (Baumler et al. 1998). *Salmonella* enterica Typhi causes typhoid fever in humans, and its distribution in nature is limited to humans. *S. enterica* Typhimurium, however, causes primarily gastroenteritis and sometimes septicemia in many animal species, including humans. *Salmonella* serovar Typhimurium colonizes many animal species; many of whom may not exhibit apparent signs of disease and thus pose a significant food safety concern (Baumler et al. 1998). A better understanding behind the genetic basis attributed to this disparity is being developed from whole genome sequencing.

Pathogenicity islands, fimbrial operons, prophages and plasmids have played a major role in the evolution of *S. enterica* subspecies I serovars. *S.* Typhi's evolution is attributed to gene polymorphisms, gains and losses in genes and gene function. Similar genetic trends are evident in many other *Salmonella* serovars. While *S.* Typhi virulence is attributed in part to the collective acquisition of the Vi capsule locus (SPI7), fimbrial operons, and toxin islet, only a few of these genes are unique to *S.* Typhi (Hashimoto et al. 1993; Pickard et al. 2003; Nair et al. 2004; Suez et al. 2013) (Fig. 1). The conditions that lead to septic *Salmonella* infections are multifactorial, involving age, complement deficiencies, and immune function. Some *Salmonella* serovars and strains are more likely than others to be associated with septicemia. Several *S.* Typhi virulence genes (*cdtB*, *hlyE*, *tcf*, and *taiA*) have been linked to other *Salmonella* serovars associated with invasive disease in humans (Suez et al. 2013). However, *Salmonella*'s ability to cause invasive disease in humans is complex due to the underlying genetic and phenotypic heterogeneity in this pathogen.

Salmonella enterica serovars Enteritidis and Typhimurium account for approximately 50% of *Salmonella* infections in humans worldwide (Galanis et al. 2006). While there are distinctive genetic and serotype-specific differences (Thomson et al. 2008), *S.* Enteritidis and *S.* Typhimurium have several virulence genes in common, associated with their invasive nature. Prophages and plasmids are important in disseminating virulence genes in *Salmonella* and other pathogenic species of the family *Enterobacteriaceae* (Gulig 1990; Brussow et al. 2004; Johnson and Nolan 2009). Many *S.* Enteritidis and *S.* Typhimurium strains possess a large incFI/II plasmid (Tinge and Curtiss 1990a, b) that contain a SPI2 T3SS effector, *spvB* (Browne et al. 2008) and the fimbrial operon *pef* (Baumler et al. 1996). SpvB is an ADP-ribosylating toxin that targets F-actin in macrophages (Lesnick et al. 2001), killing the *Salmonella*-infected cell (Lesnick et al. 2001). Pef fimbriae mediate *Salmonella* colonization of the small intestine (Baumler et al. 1996). The *spvB*-virulence plasmid is present in other *Salmonella* serovars, including *S.* Choleraesuis, *S.* Derby, *S.* Dublin, *S.* Gallinarum, *S.* Paratyphi C, and *S.* Pullorum, associated with invasive,

fulminant infections in their animal host (Tinge and Curtiss 1990a, b; Boyd and Hartl 1998). While plasmid-borne in the aforementioned *Salmonella* serovars, the *spv* locus is also present in the chromosome of other *S. enterica* subspecies (*spv*- S. enterica subspecies: II, IIIa, Iv, and VII (Boyd and Hartl 1998). There are considerable genetic differences in the *spvB*-virulence plasmid among these *Salmonella* serovars, as reflected in gene deletions (S. Enteritidis, S. Choleraesuis, S. Dublin; (Hong et al. 2008)) and acquisition of ancillary genes (e.g., the *fae* fimbrial operon in *S*. Dublin (Rychlik et al. 1998)). As more *Salmonella* genomes have become annotated, new plasmids are being discovered (Moreno Switt et al. 2012), with some harboring virulence-related genes (e.g., *S*. Heidelberg) (Hoffmann et al. 2014).

Prophages are important drivers in the evolution of bacterial pathogens. *Salmonella* Typhimurium LT2, the first *Salmonella* genome to be sequenced and annotated, contains four prophages (Gifsy-1, Gifsy-2, Fels-1, and Fels-2) (Fig. 1). The *S.* Typhimurium LT2 Gifsy prophages contain several putative virulence genes, including the SPI2 T3SS effector *sseI* (Miao et al. 2003) (also referred to as *sfrH*). There exist polymorphisms in *sseI* that affect *S.* Typhimurium invasiveness, and thus the virulence of these strain types (Thornbrough and Worley 2012). Genomic analyses of other *Salmonella* genomes have revealed additional prophages with ancillary virulence genes integrated into their prophage genome (Bacciu et al. 2004; Pang et al. 2013). There is significant genetic diversity in *Salmonella* virulence genes associated with phage classes – virulence gene assortment associated with specific prophage, and prophage distribution in *Salmonella* (Mirold et al. 2001b; Hopkins and Threlfall 2004; Pang et al. 2013; Hiley et al. 2014) (Figs. 4 and 5). Many of these prophages are restricted in their distribution to specific *Salmonella* serovars or strains (Chan et al. 2003; Thomson et al. 2004; Saitoh et al. 2005; Scaria et al. 2008; Santiviago et al. 2010; Pang et al. 2013; Hoffmann et al. 2014). Several of these phage-associated virulence genes are secreted effectors of SPI1 (Hardt et al. 1998) and SPI2 (Miao et al. 2003; Rajashekar et al. 2014). SPI1 and SPI2 T3SS effectors are important in shaping the virulence *Salmonella* serovars and strains (Dhanani et al. 2015). Two lysogenic phages harboring *sopE* (SPI1 T322

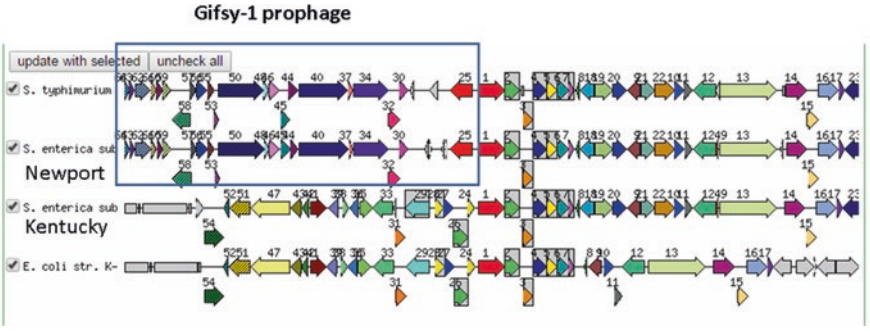

Fig. 4 *Salmonella* Gifsy-1 Prophage. RAST (Aziz et al. 2008) genome browser was used to identify Gifsy-1 prophage integration within the *Salmonella*/*E. coli* core genome and presence (*S.* Newport, *S*. Typhimurium)/absence (*S*. Kentucky) of this prophage in select *Salmonella* genomes

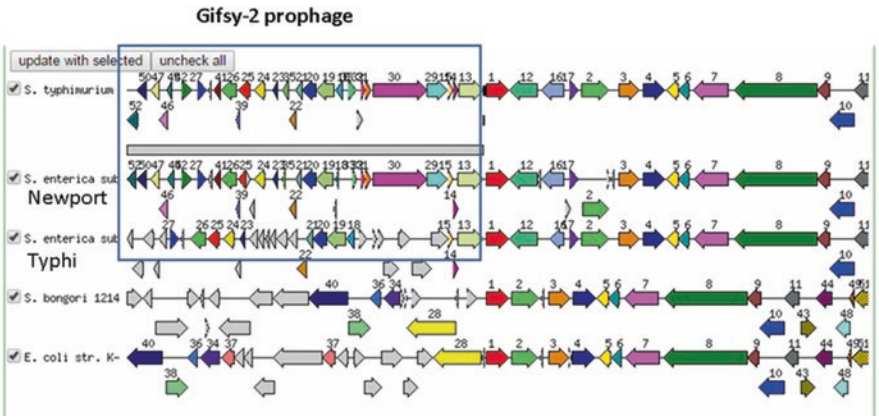

Fig. 5 *Salmonella* Gifsy-2 Prophage. RAST (Aziz et al. 2008) genome browser was used to identify Gifsy-1 prophage integration within the *Salmonella*/*E. coli* core genome and genetic differences within the prophage genomes of *S* Newport/*S*. Typhimurium vs. *S*. Typhi

effector) have a rather restricted distribution to *S*. Enteritidis, *S*. Typhimurium, and a few other *Salmonella* serovars (Hopkins and Threlfall 2004). One explanation for the ascendency of *S.* Enteritidis and *S.* Typhimurium in the top 10 causes of human salmonellosis is their acquisition of the *spvB*-virulence plasmid, and *sseI* and *sopE1* prophages. Possession of SPI2 T3SS effectors *spvB* and *sseI* may explain the virulence and invasive nature of these two *Salmonella* serovars in humans relative to other *S. enterica* subspecies I serovars (Suez et al. 2013; Cheng et al. 2015).

Even within *Salmonella* serovars *S*. Enteritidis and *S.* Typhimurium, there is a spectrum in the disease severity, differences in disease syndromes (gastroenteritis vs. septicemia), and animal hosts affected by these strains (Hernandez et al. 2012; Suez et al. 2013). The genetic basis behind this strain variability in virulence is complex, involving gene polymorphisms (Wilmes-Riesenberg et al. 1997; Robbe-Saule et al. 2003; Hopkins and Threlfall 2004; Guo et al. 2009; Dwyer et al. 2011; Kuzminska-Bajor et al. 2012; Thornbrough and Worley 2012), gain or loss of gene(s) (Luo et al. 2011; Suez et al. 2013), gene function (Wilmes-Riesenberg et al. 1997; Robbe-Saule et al. 2003), or changes in global gene regulation (Shah 2014).

Gene Expression, Pathogenesis, and Virulence

Salmonella pathogenesis involves coordinated and programmed global regulation of virulence genes (Saini et al. 2010). *Salmonella* colonization and virulence in other animal species also involves coordinated expression of many core virulence and metabolic genes (Morgan et al. 2004; Harvey et al. 2011; Chaudhuri et al. 2013). Virulence gene regulation in *Salmonella* is complex, involving several global gene regulators (*rpoS*, *envZ*/*ompR*, *phoPQ*) that respond to different environmental stimuli during the course of infection (Dorman et al. 1989; Miller et al. 1989; Fang

et al. 1992; Sheikh et al. 2011). Differences in a *Salmonella* strain's virulence have been tied to the expression level of these regulatory genes (e.g., *phoP/Q* (Heithoff et al. 2012). Point mutations affecting promoter strength is one explanation for variable expression of these regulatory genes. DNA methylation also affects global regulation of *Salmonella* loci by silencing or activating genes important to virulence (Heithoff et al. 1999). Differences in DNA methylation patterns may explain variability in *Salmonella* virulence (Pirone-Davies et al. 2015). It is important to note that many plasmids and phages harbor DNA methylases (Naderer et al. 2002; Kropinski et al. 2007) that can alter the bacterium's regulatory network, and thus these mobile genetic elements may be important to the *Salmonella* serovar or strain in question.

Concluding Comments

The likelihood of becoming ill, the severity of illness, disease symptoms manifested, and incubation period following exposure to *Salmonella* is multifactorial, related to the host's immune status, implicated food matrix, *Salmonella* numbers ingested, and *Salmonella* serovar/strain type. Another contributing factor to *Salmonella* disease not discussed, but worthy of consideration, is the composition of the intestinal microbiome (Xu and Gordon 2003). It has been determined that the intestinal microflora can affect an enteropathogen's ability to colonize the gastrointestinal tract (Nurmi et al. 1992). The mechanism behind this competitive exclusion is currently not known. While an animal model may not necessarily be predictive of a *Salmonella* serovar's or strain's virulence in humans, due to the limitation of the model to mimic human disease (Suez et al. 2013), detection of a specific combination of virulence genes (e.g., *cdtB, hlyE, spvB, sseI, sopE, taiA, tcf*) or alleles (e.g., *sseI*), or expression of aforementioned genes or alleles may prove to be a better predictor of disease potential. As whole genome sequencing eventually replaces PFGE as an epidemiologic tool to investigate foodborne outbreaks, candidate genes or alleles associated with salmonellae virulent for humans are likely to be identified. That realization is likely only a few years away.

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Shigella: Virulence Factors and Pathogenicity

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Abstract Each year, *Shigella* species are responsible for an estimated 100–150 million infections worldwide, with nearly 500,000 of these infections occurring in the United States, where approximately 30% of cases can be attributed to the ingestion of contaminated foods. Clinical symptoms associated with shigellosis (otherwise known as bacillary dysentery) include diarrhea, sometimes bloody; fever; stomach cramps; and tenesmus. Possible sequellae of *Shigella* infection can include hemolytic uremic syndrome (HUS) or rheumatoid arthritis. This pathogen possesses a repertoire of virulence factors that modulate the host innate/adaptive immune systems and enable the bacteria to invade colonic epithelial cells, escape from phagocytic vesicles, and finally destroy mucosal cells lining the intestinal tract. One of the hallmarks of *Shigella* pathogenesis is utilization of the type three secretion system (T3SS) to insert bacterial effector proteins into host cells. Most of the genetic factors necessary for pathogenicity reside on a large virulence plasmid (pINV), while a few of these genes are in chromosomal pathogenicity islands.

Keywords *Shigella* • Virulence factors • Pathogenicity • Type three secretion system • T3SS

Introduction

Shigella is a Gram-negative, rod-shaped bacterium that causes shigellosis, a severe form of bacillary dysentery characterized by cramps, diarrhea (occasionally bloody), and fever. This pathogen is extremely effective at spreading via the fecal-oral route,

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with ingestion of as few as 10–100 organisms required to cause disease (DuPont et al. 1989). In the United States alone, the annual number of *Shigella* cases is estimated to be 500,000 (Centers for Disease Control and Prevention 2011). Scallan et al. suggest that approximately 30% of these are the result of eating contaminated food (Centers for Disease Control 2011; Scallan et al. 2011). Globally, approximately 164.7 million diarrheal episodes are attributed to *Shigella* infection per year (Kotloff et al. 1999). These infections result in 1.1 million fatalities, most of whom are children under the age of five. While outbreaks continue to occur in developed nations, *Shigella* puts a much greater burden on developing nations, where poor hygiene and water sanitation methods contribute to transmission. To compound this problem, persistent socioeconomic issues, such as childhood malnutrition, have been linked to an increased frequency and severity of diarrheal disease (Guerrant et al. 1992). Currently, there is no protective vaccine to prevent *Shigella* infection.

The *Shigella* genus is divided into four species or serogroups, each defined by a specific antigen type: *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) (Ewing 1949). These organisms can be further differentiated into serotypes based on specific structural differences of the O-antigen lipopolysaccharide (Allison and Verma 2000; Levine et al. 2007). There are at least 43 distinct serotypes of *Shigella*, including 8 serotypes of *S. flexneri*, 19 of *S. boydii*, 15 of *S. dysenteriae*, and a single serotype of *S. sonnei*, which is considered to be clonal (Centers for Disease Control and Prevention 2011; Karaolis et al. 1994).

Globally, *Shigella* species are unevenly distributed between the industrialized and developing world. In industrialized countries, *S. sonnei* contributes to 77% of all *Shigella* infections; in developing nations, *S. flexneri* is responsible for 60% of infections (Kotloff et al. 1999). Children in day care settings, or military/civilian travelers to endemic countries, are among the most at-risk populations for *Shigella* in industrialized countries (Kotloff et al. 1999; Pickering et al. 1981; Hyams et al. 1991). In contrast, displaced populations, driven by war, famine or persecution, are at highest risk for shigellosis in developing nations (Kotloff et al. 1999).

Shigella is distinguished from *Escherichia coli* only for medical and historical purposes; taxonomically, genomic analysis classifies these as members of the same genus (Pupo et al. 1997). Pathogenic *Shigella* strains are postulated to have evolved directly from commensal *E. coli* lineages between 35,000 and 270,000 years ago, and this evolutionary leap likely took place multiple times, each branch-off corresponding to the independent acquisition of a large 230-kB virulence plasmid (Pupo et al. 2000; Yang et al. 2007). Using comparative genomics, Lan et al. (2004) suggest that there are three main *Shigella* clusters, with clusters 1 and 2 containing strains which branched from *E. coli* 50,000 to 270,000 years ago, and cluster 3 emerging more recently, between 35,000 and 170,000 years ago (Lan et al. 2004). From this analysis, several outlier strains were also described, which possibly emerged independently of the three primary lineages. *S. flexneri* and *E. coli* K-12 genomes still share a common chromosomal backbone, which has undergone a number of large (>5 kb) rearrangements, such as inversions and translocations (Wei et al. 2003). Because most *Shigella* virulence determinants are plasmid-encoded, novel chromosomal ORFs (i.e., chromosomal genes unique to *Shigella* which are not present in *E. coli*) are scarce. In *S. flexneri* 2a strain 2457 T,

only 175 ORFs are unique, whereas the remaining 3030 ORFs are shared with *E. coli* K-12 strain MG1655 (Wei et al. 2003).

Two major events have driven the niche adaptation of *Shigella*: acquisition of the large virulence plasmid (pINV), encoding a type three secretion system (T3SS), and concomitant inactivation or loss of chromosomal factors. The addition of a functional T3SS drove these previously extracellular, commensal bacteria into the human colonic epithelial cell to exploit a novel intracellular niche (Sansonetti et al. 1982; Menard et al. 1993). In addition, several pathogenicity islands have been horizontally transferred to the chromosome, promoting virulence via genes that encode for aerobactin (iron) transport, O-antigen modification, and antibiotic resistance, to name a few (Schmitt and Payne 1988; Huan et al. 1997; Luck et al. 2001). These acquired virulence factors will be discussed in detail later in the chapter.

Conversely, genes no longer necessary for survival within the host cell were either lost or forced into a state of decay, resulting in a significantly smaller genome, and a remarkably large number of pseudogenes still remain in the *Shigella* spp. (Wei et al. 2003; Feng et al. 2011). Feng et al. examined five *Shigella* genomes and determined that 85 genes were commonly lost in all strains, whereas a staggering 1456 genes were pseudogenized in at least one strain (Feng et al. 2011). Although a number of different mutational events are responsible for disrupting the *Shigella* genome, most of the pseudogenes arose from either nonsense mutations or insertional inactivation of genes (Wei et al. 2003; Lerat and Ochman 2004; Zaghloul et al. 2007). Insertion sequence (IS) elements are abundant in the genomes of *Shigella* spp.: *S. flexneri* 2a alone has 314 IS elements, which is more than sevenfold that of related *E. coli* K-12 strains (Wei et al. 2003; Zaghloul et al. 2007; Jin et al. 2002). Many of the inactivated or missing genes of *Shigella* spp. are from catabolic pathways (Monk et al. 2013). Most strains have lost the ability to grow on D-alantoin, D-malate, and xanthine as sole carbon sources, or inosine as a nitrogen source, even though the majority of *E. coli* strains readily utilize these nutrients, suggesting that the catabolic pathways to break down and utilize these substrates as energy sources have been inactivated. An example of a non-metabolic pathway in decay in the *Shigella* spp. is the flagellar system; as a result of deletions or inactivation events of flagellar genes, *Shigella* strains are universally non-motile (Feng et al. 2011).

As *Shigella* evolved into a pathogen from *E. coli, Shigella* genes inhibitory to the new pathogenic lifestyle, known as antivirulence genes (AVGs), were also discarded (Maurelli et al. 1998; Bliven and Maurelli 2012). The AVGs of *Shigella* include the genes that encode lysine decarboxylase (*cadA*); quinolinate synthetase A/L-aspartate oxidase (*nadA*/*nadB*); spermidine acetyltransferase (*speG*); and an outer membrane protease (*ompT*) (Maurelli et al. 1998; McCormick et al. 1999; Fernandez et al. 2001; Barbagallo et al. 2011; Prunier et al. 2007a; Nakata et al. 1993). Expression of these genes in a wild-type *Shigella* strain attenuates different virulence phenotypes, such as cell-to-cell-spread (*ompT*) or macrophage survival (*speG*). Several of these genes encode for enzymes which catalyze an inhibitory end product. CadA, for example, catalyzes the conversion of lysine to the end product cadaverine, which, when added exogenously during *Shigella* infection, blocks PMN transepithelial migration, phagosome escape, and ShET enterotoxin activity (Maurelli et al.

1998; McCormick et al. 1999; Fernandez et al. 2001). NadA/NadB, enzymes in the NAD biosynthetic pathway, produce quinolinic acid, which blocks invasion, cell-to-cell spread, and PMN transepithelial migration (Prunier et al. 2007a). Alternatively, SpeG, a spermidine-acetyltransferase, depletes the bacterial cell of the beneficial substrate spermidine, which is critical for macrophage survival (Barbagallo et al. 2011). In order to optimize pathogen fitness in the human host, the genes encoding these enzymes were inactivated or lost from all *Shigella* spp. (Maurelli et al. 1998; Bliven and Maurelli 2012; Barbagallo et al. 2011; Day et al. 2001; Prunier et al. 2007b).

Enteroinvasive *E. coli* (EIEC), which also harbors the large virulence plasmid encoding the T3SS, is often grouped with *Shigella* as a distinct pathovar within *E. coli* (Peng et al. 2009). Although EIEC strains are also invasive, they are considered less pathogenic, since they require a higher infectious dose, show a lower efficiency of cell-to-cell spread, and generate a milder host inflammatory response following infection (DuPont et al. 1971; Moreno et al. 2009). EIEC are characteristically non-motile and have lost or inactivated many of the AVGs common to *Shigella*, including *cadA,* although some of the AVGs, such as *nadA/nadB* and *speG,* remain intact in at least a few EIEC strains (Casalino et al. 2003; Di Martino et al. 2013; Campilongo et al. 2014). Moreover, EIEC share several biochemical properties with commensal *E. coli* that the *Shigella* spp. have lost, including mucate and acetate production (Silva et al. 1980). These characteristics suggest that the EIEC pathotype represents an intermediate phenotype between commensal *E. coli* and pathogenic *Shigella* (Lan et al. 2004). However, EIEC strains are genetically less diverse than *Shigella*, and are thought to be more recent ancestors of commensal *E. coli*, suggesting that EIEC strains did not give rise to the *Shigella* spp. (Lan et al. 2004). Instead, shared niche adaptation is driving evolution in the EIEC and *Shigella* lineages.

Type III Secretion System

Unique to Gram-negative bacteria, type three secretion systems (T3SSs) are specialized protein export systems utilized by bacteria to effectively exploit eukaryotic hosts (Cornelis 2006; Coburn et al. 2007; Blocker et al. 2008). Bacterial adherence, invasion, and manipulation of the host's intracellular trafficking and immune systems are some of the diverse functions attributed to bacterial T3SSs. These processes are mediated by proteins known as effectors, which are translocated through the needle pore of the T3SS and deposited directly into the cytosol of the host cell, where they can interact with a target host protein(s). The targets of the T3SS proteins are incredibly diverse, and include host cell actin/tubulin, caspases, Rho GTPases, kinases, NF-kB, and cell cycle proteins, just to name a few (Abe et al. 2005). In *Shigella*, the T3SS facilitates bacterial invasion of the human colonic epithelium, enables subsequent cell-to-cell spread, and controls the host inflammatory response for the benefit of the bacterium (Schroeder and Hilbi 2008) (Fig. 1).

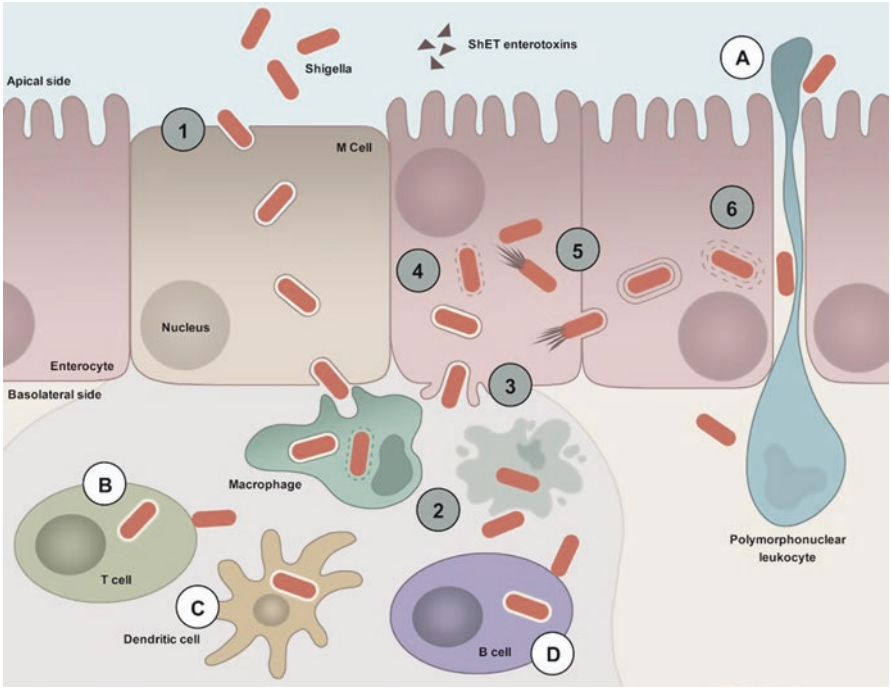

Fig. 1 *Shigella* pathogenesis. Following ingestion, *Shigella* travels through the gastrointestinal tract to the colon, where it invades into the host epithelium. This multi-step process includes (1) transcytosis through M cells; (2) phagocytosis by macrophages, phagosome escape, and induction of pyroptosis; (3) basolateral invasion of enterocytes; (4) phagosome escape; (5) actin polymerization and cell-to-cell spread; and (6) phagosome lysis of the double membrane. Additionally, *Shigella* subverts the host immune system. (**a**) *Shigella* induces a massive polymorphonuclear leukocyte (PMN) influx, which is thought to disrupt tight junctions and provide bacteria an alternative route to access the basolateral membrane. (**b**–**d**) *Shigella* also invades cells of the adaptive immune system, including T cells, dendritic cells, and B cells, to induce apoptosis. In T cells and B cells, T3SS needle contact with the host cell is sufficient for cell death. Details on these processes are in the text

The T3SS needle apparatus is composed of 20–25 different proteins, which form three main components: a basal body with a multi-ring base to anchor the T3SS to the bacterial inner and outer membranes; a long needle-like portion that extends out from the bacteria and into the surrounding environment; and a translocon complex that creates pores in the eukaryotic cell membrane (Erhardt et al. 2010). Intriguingly, the T3SS structure bears a striking resemblance to the bacterial flagellum, and the core genes that form the T3SS needle share significant homology with core flagellar genes (Erhardt et al. 2010; Van Gijsegem et al. 1995; Abby and Rocha 2012; Gophna et al. 2003). Both the T3SS and flagellum are complex structures which span the cytoplasmic and outer membrane of the bacterium, and both systems are driven by an F_0F_1-type ATPase (Abby and Rocha 2012; Pallen et al. 2006). Although it is widely accepted that these systems share an evolutionary heritage, debates continue

over which system arose first, or whether they diverged from a common ancestor (Abby and Rocha 2012; Gophna et al. 2003). However, there are some obvious distinctions between the two systems. While the T3SS needle secretes bacterial proteins directly into eukaryotic host cells, the flagellar system functions as a key component of bacterial motility. Furthermore, T3SS genes are almost always clustered on single pathogenicity islands within the chromosome or on plasmids, which indicates a history of acquisition by lateral transfer (Abby and Rocha 2012). *Chlamydia* is a rare exception to this rule, as the *Chlamydia* T3SS genes appear to be distributed into at least four separate loci (Abby and Rocha 2012). Likewise, flagellar genes are always scattered in multiple operons throughout their respective bacterial genomes, making horizontal transfer of the system much less likely (Liu and Ochman 2007).

Genetics of the T3SS

The T3SS of *Shigella* is encoded on a large 230-kB virulence plasmid (pINV) present in all *Shigella* and EIEC (Sansonetti et al. 1982; Maurelli et al. 1984). Distinct variances of G/C content throughout the plasmid, coupled with the presence of three separate plasmid partitioning systems (two of which remain functional), suggest that the pINV is actually a compilation of several ancestral plasmids (Buchrieser et al. 2000). In addition to the T3SS structural, effector, and regulatory genes, this megaplasmid is also composed of over 90 insertion elements, more than 10 putative open reading frames (ORFs), and a host of plasmid maintenance, replication, segregation, and transfer genes (Lan et al. 2003). All virulent *Shigella* and EIEC strains carry one of two major variants of this plasmid: pINV A or pINV B (Lan et al. 2003). There are over 500 nucleotide polymorphisms between the two variants, with over 300 of these changes resulting in an amino acid difference. It is not yet known if these variants are responsible for differences in pathogenesis between strains.

The genes which encode the T3SS structural needle are grouped together on pINV in a 32-kB cluster known as the *Shigella* Entry Region (SER) (Maurelli et al. 1985; Sasakawa et al. 1988). This cluster contains the *ipa*, *mxi*, and *spa* operons necessary and sufficient for construction of the T3SS needle and the initial invasion process. Outside of the SER, there are over 20 genes that encode effectors known or suspected to be secreted through the T3SS.

Structure of the T3SS and Recognition of Secreted Effectors

As mentioned above, the *Shigella* T3SS consists of three components: the basal body, the needle, and the translocon tip complex. The portion of the basal body embedded in the outer membrane of the bacteria consists of the MxiD ring and the stabilization protein MxiM; MxiJ and MxiG form a ring through the inner membrane (Schuch and

Maurelli 2001a). The cytoplasmic portion of the basal body includes a C-ring platform (Spa33, Spa47, and MxiN) essential for the recognition, sorting, and secretion of effectors through the type three needle (Hu et al. 2015; Lara-Tejero et al. 2011). One of these C-ring proteins, Spa47, functions as the T3SS ATPase, and provides the energy necessary for effector transport across the bacterial membrane (Eichelberg et al. 1994). A nonameric ring MxiA sits between the T3SS needle pore and the ATPase, and facilitates the secretion of T3SS effectors, potentially with the assistance of Spa13 and MxiC (Andrews and Maurelli 1992; Abrusci et al. 2013; Cherradi et al. 2014; Martinez-Argudo and Blocker 2010). Spa32, which controls the length of the needle MxiH and interacts with the assembly protein Spa40, is likely located near the base of the needle complex (Tamano et al. 2002; Botteaux et al. 2010). Other T3SS proteins, including Spa9, Spa24, and Spa29 are also proposed components of the basal body, although their structural locations and functions have not yet been determined (Blocker et al. 2003).

The T3SS needle is comprised of the major MxiH subunits, which assemble in a helical fashion to form the cylindrical needle, and the minor subunit MxiI, which is hypothesized to form the T3SS needle cap comparable to the flagellar hook cap FlgD (Blocker et al. 2001; Cordes et al. 2003). The precise transport and assembly of MxiH and MxiI appear to be dependent on the basal body proteins MxiK, MxiN, and Spa47, as mutants defective in any of these three genes produce non-functional T3SSs lacking the needle component (Jouihri et al. 2003).

At the needle tip, the translocon complex (IpaD, IpaB, and IpaC) mediates the recognition of environmental sensors required for activation of the T3SS, and participates in the insertion of IpaB and IpaC into host membranes (Veenendaal et al. 2007). IpaD is a hydrophilic protein bound via its C-terminal end to the tips of T3SS needles (Espina et al. 2006). Four IpaD units are thought to bind to each tip; in the inactive state, these proteins block secretion through the needle pore (Veenendaal et al. 2007; Dickenson et al. 2011). IpaB and IpaC are only recruited to the tip complex following the interactions of IpaD with a secretion trigger, such as exposure to bile salts (Dickenson et al. 2011).

An N-terminal signal (~20 residues of the protein sequence) is utilized to direct T3SS effectors to the base of the needle for subsequent secretion, although a strong consensus sequence or structure has not yet been identified and these signals may be specific to certain T3SSs (Harrington et al. 2003; Samudrala et al. 2009). Because of the narrow confines of the needle pore (~2–3 nm diameter), T3SS effectors are shuttled through in a partially unfolded state, and must be re-folded when they reach the host cell cytoplasm (Blocker et al. 1999, 2001; Akeda and Galan 2005). Certain chaperone proteins help preserve the unfolded state of these proteins as they pass through the T3SS; however, not all effectors have chaperones (Stebbins and Galan 2001). The *Pseudomonas* effector AvrPto, for example, utilizes a pH-sensitive mechanism for proper unfolding and re-folding (Dawson et al. 2009).

In *Shigella*, IpgA, IpgC, IpgE, and Spa15 function as the T3SS chaperone proteins. IpgA chaperones IcsB; IpgC chaperones IpaB and IpaC; IpgE chaperones IpgD; and Spa15 chaperones at least IpgB1, IpgB2, IpaA, OspC3, and OspD1, and possibly OspB, OspC2, and MxiE as well (Ogawa et al. 2003; Page et al. 2001, 2002; Parsot et al. 2005; Niebuhr et al. 2000; Hachani et al. 2008).

Regulation of the T3SS

Optimal expression of the *Shigella* T3SS is dependent on several factors which are likely to prevent expression of this system outside the host and ultimately conserve energy. One of these components of regulation is temperature (Maurelli and Sansonetti 1988). H-NS, a global regulator in *Escherchia* and one of the main components of bacterial chromatin, is a critical repressor of many of the T3SS genes in *Shigella*, including the two transcriptional activators, *virF* and *virB* (Tobe et al. 1993; Dorman and Porter 1998). In conditions designed to mimic temperature outside the host (30 °C), H-NS binds weakly to curved sections of DNA in the promoter regions of numerous T3SS operons, including *virF*, *virB*, *ipgD*, *spa*, *icsP*, *icsA*, and *virA*, repressing transcription (Prosseda et al. 1998; Castellanos et al. 2009; Colonna et al. 1995; Beloin and Dorman 2003). Upon exposure to host temperature (37 °C), H-NS binding is postulated to weaken due to thermally-induced changes in the DNA supercoiling of these promoter regions, and the repressor can be displaced by other transcriptional activators, such as the factor for inversion stimulation (FIS) and the integration host factor (IHF), which bind to and activate the *virF* promoter (Tobe et al. 1995; Falconi et al. 2001; Porter and Dorman 1997).

Once activated, expression of the T3SS is dependent upon a cascade of events (Dorman and Porter 1998). VirF, the primary transcriptional activator of the system, is a member of the AraC family of transcriptional regulators (Gallegos et al. 1997). Once expressed, VirF binds to and activates the promoters of *virB* and *icsA* to initiate this regulatory cascade (Tobe et al. 1993). VirB, a secondary regulator of the *Shigella* T3SS, is then available to activate the *ipa*/*mxi*/*spa* promoters (Watanabe et al. 1990). The *ipa* (invasion plasmid antigens) operon consists of *ipaBCDA*, several *ipg* genes (*ipgCBA*), *icsB*, and a putative acyl carrier-encoding gene, *acp*; the *mxi* (membrane expression of ipa) operon contains the remaining *ipg* (*ipgDEF*) and *mxi* genes (*mxiGHIJKLMEDCA*); and the *spa* (surface presentation of ipa) operon carries the nine *spa* genes (*spa15*, *spa47*, *spa13*, *spa32*, *spa33*, *spa24*, *spa9*, *spa29*, and *spa40*). As mentioned previously, expression of these three operons is both necessary and sufficient for the assembly of the *Shigella* T3SS and initial entry into the host cell. VirB also transcriptionally activates the first set of T3SS effector genes, including *ospC2*, *ospC3*, *ospC4*, *ospD1*, *ospD2*, *ospZ*, and *icsP* (Castellanos et al. 2009; Basta et al. 2013; Le Gall et al. 2005). Presumably, these effectors must be stored prior to secretion so that the bacteria are primed for the early stages of host cell survival and immune system modulation. MxiE activation, which drives the expression of a second set of T3SS effector genes, is blocked by the antiactivator complex OspD1 and Spa15 until secretion is induced (Parsot et al. 2005). This second set of genes includes *ospD3*, *ospE1*, *ospE2*, *ospG*, *ipaH1.4*, *ipaH4.5*, *ipaH7.8*, and *ipaH9.8* (Le Gall et al. 2005). Finally, in the absence of secretion, *ospF*, *ospC1*, *ospB*, *phoN2*, and *virA* are driven by VirB activation; in contrast, during secretion, these genes are activated in a MxiE-dependent fashion.

Osmotic stress, pH, and the availability of oxygen also affect T3SS expression (Mitobe et al. 2009; Marteyn et al. 2010; Nakayama and Watanabe 1995).

In conditions of low osmolarity, the RNA chaperone Hfq binds to and decreases the stability of *virB* mRNA, and corresponding T3SS expression is decreased (Mitobe et al. 2009). In response to environmental pH, expression of *virF* is attenuated at pH 6.0 and activated at pH 7.4 (Nakayama and Watanabe 1995). The role of a two-component regulatory sensor protein (CpxR) in this regulation is recognized but not well understood. Lastly, oxygen also modulates regulation; in the presence of oxygen, FNR, a regulator of anaerobic metabolism, dissociates from the promoters of the T3SS genes *spa32* and *spa33*, allowing transcription to proceed (Marteyn et al. 2010). Spa32, a component of the T3SS critical for controlling needle length, and Spa33, a regulator of Ipa translocation, are both critical for effector secretion (Tamano et al. 2002; Schuch and Maurelli 2001b). Levels of oxygen in the intestinal tract vary depending upon site, and it is likely that sufficient oxygen required for FNR disassociation would only be available close to the mucosal surface, promoting T3SS secretion and priming the bacterium for impending invasion (Marteyn et al. 2010; Torres Filho et al. 1994).

Adherence and Invasion

The human colonic epithelium presents a significant challenge to adhesive and invasive pathogens. This barrier consists of a thick layer of gastric mucosa which covers a single layer of epithelial cells knit closely together by tight junctions, with a limited repertoire of ligands present on the apical surface for bacteria to bind. To circumvent this problem, *Shigella* preferentially invade host epithelial cells through the basolateral surface, and employs at least two methods to gain access to this membrane (Mounier et al. 1992; Perdomo et al. 1994; Zychlinsky et al. 1994a). First, *Shigella* transcytose through specialized microfold cells (M cells), which overlay organized colonic lymphoid follicles that harbor B lymphocytes, macrophages, and follicular dendritic cells. In the normal course of sampling intestinal flora, M cells endocytose or phagocytose viruses, macromolecules or even entire microbes from the intestinal lumen and trancytose them to the lymphoid tissue underneath. Subsequently, the antigens are engulfed by resident macrophages or dendritic cells and then presented to T cells to stimulate either tolerance or immunity (Jung et al. 2010). Because of their vital immunological role in routine antigen-sampling, M cells express a variety of unique ligands that are absent on the apical surface of other intestinal epithelial cells, including glycoprotein 2, uromodulin, and ANXA5 (Mabbott et al. 2013). Several of these receptors directly bind bacterial adhesins; glycoprotein 2, for example, associates with FimH of type I pili and is essential for M cell transcytosis in certain Gram-negative bacteria (Hase et al. 2009). Consequently, while M cells are clearly necessary for passive immunological surveillance of the intestine, they actually represent a weak point in the host defense that can be exploited by any bacterial pathogen able to avoid or overcome the host immune responses of the underlying lymphoid tissue (Jones et al. 1995).

During infection with *Shigella*, or other pathogens such as *Salmonella* and *Yersinia*, M cell transcytosis is actively initiated by the bacterium, as mutants defective in adhesion or invasion are attenuated in M cell transport (Sansonetti et al. 1996; Jones et al. 1994; Marra and Isberg 1997). *Shigella* ligands are hypothesized to bind specific M cell glycoprotein receptors in order to facilitate uptake, although specific receptors have not yet been identified (Sansonetti and Phalipon 1999). Following M cell entry, *Shigella* does not lyse the endocytic vacuole nor induce immediate cytotoxicity of the cell; instead, the bacterium is released directly into the intercellular space of the intraepithelial pocket (Sansonetti et al. 1996).

Shigella are then engulfed by resident macrophages and dendritic cells of the intestinal lymphoid tissue, and quickly escape into the host cytoplasm (Zychlinsky et al. 1992; Edgeworth et al. 2002). In macrophages, the *Shigella* T3SS effector IpaB binds to caspase-1 (previously known as interleukin-1 beta converting enzyme), which is responsible for processing the pro-inflammatory cytokine interleukin-1 beta (IL-1β) to its biologically active form, rapidly inducing cell pyroptosis (Zychlinsky et al. 1994b; Chen et al. 1996; Thirumalai et al. 1997). In dendritic cells, *Shigella* also induce cell death, but the mechanisms involved are less clear, partially involving caspase-1 and as-yet-unidentified cysteine protease(s) (Edgeworth et al. 2002). Additionally, *Shigella* may invade B lymphocytes and induce cell death, while extracellular *Shigella* kill B lymphocytes shortly after interaction of the needle tip complex protein IpaD with the TLR2 receptor (Nothelfer et al. 2014). Following pyroptosis, the bacteria are free to invade the basolateral membrane of the colonic epithelium. Once inside, the bacteria hijack host cell pathways to amplify PMN influx across the intestinal barrier, further destabilizing the epithelium and promoting bacterial translocation (Phalipon and Sansonetti 2007).

The *Shigella* invasive process is T3SS-dependent. First, the needle contacts host cell lipid rafts, cholesterol/sphingolipid-rich regions that cluster closely together in the eukaryotic membrane (Lafont et al. 2002; van der Goot et al. 2004). The formation of the needle tip complex and membrane insertion of the *Shigella* effectors required for invasion occur in a stepwise manner. IpaD, at the T3SS needle tip, is thought to act as the environmental sensor for invasion. Following stimulation with deoxycholate or other bile salts during bacterial passage through the small intestine, IpaD recruits IpaB to the T3SS needle complex (Dickenson et al. 2011; Dickenson and Picking 2012). During initial contact with the host cell, IpaB is inserted into the phospholipid membrane where it binds directly to the transmembrane hyaluronan receptor, CD44. On the cytoplasmic side, CD44 is associated with the actin cytoskeleton, and therefore may be critical for bacterial hijacking of host cell signaling cascades (Lafont et al. 2002; Hirao et al. 1996; Adam et al. 2014). *Shigella* T3SS adherence also initiates further accumulation of cholesterol and raft-associated proteins at the site of entry, likely drawing in the necessary components and cytoskeletal elements required for invasion, such as the sorting protein p4.1 (Lafont et al. 2002; Ruetz et al. 2012). Finally, IpaC is recruited to the needle tip following IpaB interaction with host lipid rafts, and then inserted into the host membrane (Epler et al. 2009). An actual trigger mechanism for effector translocation remains poorly understood, however. Congo Red, a synthetic sulfonated azo dye, induces type three

secretion *in vitro*, and it is hypothesized that interactions between the needle and either Congo Red or sphingolipid rafts will destabilize the needle tip complex to open the pore for secretion (Bahrani et al. 1997).

Reorganization of the host cytoskeleton induces the cell to engulf the bacterium, a process known as membrane ruffling (Nhieu et al. 2005). Following secretion, the released IpaB, IpaC, and IpaD effectors bind $\alpha_5\beta_1$ integrin, which prompts additional actin cytoskeleton rearrangement (Watarai et al. 1996). The *Shigella* effector IpaC triggers actin polymerization and formation of filopodial and lamellipodial extensions by activating the small GTPases Cdc42 and Rac1, respectively. This process is likely initiated through induction of the Abl/Arg tyrosine kinase signaling pathway (Nhieu et al. 2005; Tran Van Nhieu et al. 2000; Hall 1998). Other T3SS effectors also play a role in the activation of Cdc42 and Rac1. IpgB1 and IpgB2 directly drive the activation of both small GTPases by stimulating the dissociation of guanosine disphosphate (GDP) to allow for guanosine triphosphate (GTP) binding (Ohya et al. 2005; Klink et al. 2010; Ehsani et al. 2012). Activated Cdc42 and Rac1 induce members of the WASP verpolin-homologous protein family to recruit the actin-nucleating complex Arp2/Arp3 for cytoskeletal remodeling (Ehsani et al. 2012). To uncouple the eukaryotic plasma membrane from the actin cytoskeleton and facilitate the underlying rearrangements, the *Shigella* inositol 4-phosphatase IpgD dephosphorylates phosphatidylinositol 4,5-bisphosphate into the lipid phosphatidylinositol 5-monophosphate, resulting in destabilization of the plasma membrane composition (Niebuhr et al. 2002). Finally, IpaA directly binds to the N-terminal residues of a focal adhesion protein, vinculin, and stimulates an association between vinculin and F-actin (Park et al. 2011; Tran Van Nhieu et al. 1997). The interactions between vinculin, IpaA, and F-actin promote actin depolymerization, further organizing the cell surface for efficient invasion (Bourdet-Sicard et al. 1999). Unlike IpaB/C/D, however, IpaA is not essential for cell entry; a mutant deficient in IpaA is still invasive, although its invasion efficiency is slightly attenuated compared to the wild-type parent (Tran Van Nhieu et al. 1997).

Once inside the epithelial cell, the bacterium must lyse the endocytic vacuole to escape into the host cytoplasm for subsequent bacterial replication and dissemination, and to avoid death following lysosome fusion with the endocytic vacuole. Thus far, studies on *Shigella* endocytic/phagocytic vacuole escape have focused on the phenotype in macrophages rather than epithelial cells, although the mechanisms are likely similar. Within 30 min following infection, almost half of *Shigella*-infected macrophages have undergone endocytic vacuole rupture (Senerovic et al. 2012). Using a tightly-regulated expression system, Page et al. (1999) were able to uncouple *Shigella* invasion from post-invasion processes to reveal that the T3SS effectors IpaB and IpaC, in addition to the cytoplasmic chaperone IpgC, are essential during bacterial escape from the phagosome in macrophages (Page et al. 1999). IpaC binds to and destabilizes phospholipid membranes, whereas IpaB forms cation pores that promote potassium flux into endosomes; the resulting disruption is likely the primary mechanism for endosomal/phagosomal lysis (Senerovic et al. 2012; De Geyter et al. 1997). Intriguingly, IpaC may have two separate mechanisms during invasion and phagosomal escape (Paetzold et al. 2007). In *Salmonella*, the IpaC

homolog, SipC, is essential for invasion; however, in contrast to IpaC, SipC possesses no endocytic lysis activity (Osiecki et al. 2001). When *ipaC* and *sipC* are swapped in *Shigella* and *Salmonella* strains, *Shigella* expressing *sipC* lose the ability to escape the phagocytic vacuole, which the *ipaC*-expressing *Salmonella* lyse. Furthermore, insertional mutagenesis studies of IpaC by Bârzu et al. (1997) suggest that different domains of IpaC may be responsible for the distinct phenotypes of invasion and vacuole escape (Barzu et al. 1997).

Intracellular Motility and Intercellular Spread

Motility of *Shigella* is mediated by IcsA, an outer membrane protein exported across the cytoplasmic membrane via the Sec secretion system, and auto transported across the outer membrane (Bernardini et al. 1989; Goldberg and Theriot 1995; Brandon et al. 2003; Gouin et al. 2005). Following secretion, IcsA localizes to a single pole on the bacterium, a process which is aided by the protease IcsP (formerly known as SopA), which cleaves IcsA from the entire bacterial surface (Egile et al. 1997; Steinhauer et al. 1999). Steinhauer et al. propose that IcsA is targeted to the pole for insertion, but diffuses towards the septum as it accumulates (Steinhauer et al. 1999). IcsP, meanwhile, slowly cleaves IcsA equally across the bacterial surface. The disparity in IcsA accumulation between the pole and the remainder of the bacterium, coupled with the slow catalytic activity of IcsP, ensures that IcsA is prevented from accumulating at non-pole regions. The chaperones DegP, Skp, and SurA, as well as the periplasmic apyrase PhoN2, are also important for proper IcsA localization, although the mechanisms involved are not yet clear (Scribano et al. 2014; Purdy et al. 2007).

Once at the pole, IcsA recruits and activates the neuronal Wiskott-Aldrich syndrome protein (N-WASP), enhancing N-WASP affinity for the Arp2/Arp3 complex, which then switches to its active form (Goldberg et al. 1993; Suzuki et al. 2002; Egile et al. 1999). The activated Arp2/3 complex binds G-actin, and an actin tail is polymerized at the bacterial pole to allow for motility. Similar to *Listeria monocytogenes*, *Shigella* bacteria can move through the host cytoplasm at rates of approximately 26 μm per minute (Gouin et al. 1999). Additionally, host protein kinase C (PKC) activation is essential for the organization of actin stress fibers to promote cell-to-cell spread during *Shigella* infection; the *Shigella* T3SS effectors OspE1 and OspE2 interact with host PDZ/LIM domain protein 7 (PDLIM7) to activate PKC (Yi et al. 2014; Defilippi et al. 1997).

Shigella specifically targets tight junctions between host cells for spread (Fukumatsu et al. 2012). Host cell pseudopodia containing *Shigella* are engulfed by neighboring cells through a clathrin-mediated endocytic pathway dependent upon host clathrin, Epsin-1, and Dynamin-2. Tricellulin, a tight junction integrity protein which localizes primarily to tricellular tight junctions, is an essential component of this process. Finally, the Vps/VacJ ABC transporter system encoded on the bacterial chromosome also appears to play a role in vacuole lysis and/or cell-to-cell spread,

possibly through maintaining lipid asymmetry in the outer membrane or by helping to target the tricellulin-containing epithelial cell junctions, although the exact mechanism has not yet been elucidated (Carpenter et al. 2014). Once inside the new host cell, the bacterium must lyse the double host membrane, a process dependent upon the T3SS effector proteins IpaB, IpaC, and IpaD (Schuch et al. 1999).

Host Immune System Manipulation and Evasion

Humans are the only known host for *Shigella* species, although there have been reports of higher primates infected with the pathogen after close contact with zookeepers (Banish et al. 1993). Patients with shigellosis typically recover within several days, but if the infection goes untreated, severe cases can progress, and patients may present with bloody, mucoid stools. Patients infected by Shiga toxin-producing strains may also be forced to endure additional complications such as hemolytic uremic syndrome, hemorrhagic colitis, or rheumatoid arthritis (Bennish 1991). Sometimes, the infection is fatal.

Following ingestion, *Shigella* must cope with a number of innate host defenses, such as the acidic environment of the stomach, the competing commensal microbiota of the gastrointestinal tract, and the nearly impenetrable barrier established by colonic epithelial cells. Moreover, subsequent bacterial invasion of the host epithelium provokes a number of host innate and adaptive immune responses. A classic and impressive example of a *Shigella*-induced host defense is the substantial polymorphonuclear leukocyte (PMN) influx to the site of infection, which eventually leads to tissue destruction and concomitant shedding of the pathogen to clear the infection (Perdomo et al. 1994; Mathan and Mathan 1991). Host adaptive immune responses also assist in recognizing and destroying the pathogen (Raymond et al. 2013). In an attempt to circumvent these outcomes, *Shigella* has developed mechanisms to manipulate or evade the host immune system.

Innate Immune System Manipulation and Evasion

Successful manipulation of the innate immune system is critical to the survival of *Shigella* within the host. As previously mentioned, *Shigella* directly induces the PMN influx, a massive inflammatory response that is one of the hallmarks of *Shigella* infection in the gut (Phalipon and Sansonetti 2007). This influx is likely a critical factor to destabilize the integrity of the colonic epithelium, and eventually provide bacteria with a route to the basolateral membrane, where they can preferentially invade (Perdomo et al. 1994). However, PMNs are also detrimental to bacterial infection, as they can devour and kill extracellular *Shigella* (Lowell et al. 1980; Weinrauch et al. 2002). Invading bacteria must therefore carefully manage the host inflammatory response: too little inflammation, and translocation of *Shigella* across

the intestinal epithelium might be attenuated; too much inflammation, and *Shigella* might be overwhelmed and killed by the immune system. An optimal level of inflammation, therefore, is critical for successful colonization and dissemination. A number of early and late *Shigella* T3SS effectors are secreted into the host cell with the sole purpose of supporting this complex interplay.

The rapid induction of inflammation is due to several key *Shigella* proteins, including the T3SS effectors OspB and IpaH7.8, and SepA, a serine protease (Ambrosi et al. 2015; Suzuki et al. 2014; Benjelloun-Touimi et al. 1995). In *Shigella*-infected macrophages, IpaH7.8 targets the host protein glomulin for degradation, activating the inflammasome and inducing pyroptosis within 2–3 h post-infection (Suzuki et al. 2014). The bacterial-induced macrophage death releases the cell's stores of mature IL-1β, which instigates a massive influx of PMNs to the tissue underlying the intestinal epithelium, although hepoxilin A_3 is required for the final induction of PMNs across the epithelium itself (Sansonetti et al. 2000; Mrsny et al. 2004). OspB activates host ERK1/2 and p38 mitogen-activated protein kinases (MAPKs), which in turn phosphorylate and activate cytosolic phospholipase A2 (cPLA2), an enzyme that releases arachidonic acid from phospholipid membranes (Ambrosi et al. 2015; Mrsny et al. 2004; Mumy et al. 2008). Arachidonic acid is converted to hepoxilin A_3, the chemoattractant required during *Shigella* infection to draw PMNs through the colonic epithelium (Mrsny et al. 2004). The autotransporter SepA functions as a cysteine protease to induce tissue destruction and inflammation in a rabbit ileal loop model of *Shigella* infection (Benjelloun-Touimi et al. 1995).

Meanwhile, other T3SS effectors, including members of the Osp and IpaH families, play critical roles in dampening or tempering the host inflammatory response (Kim et al. 2014). The IpaH proteins are a class of E3 ubiquitin ligases, and this family includes five genes on the virulence plasmid (*ipaH1.4*, *ipaH2.5*, *ipaH4.5*, *ipaH7.8*, and *ipaH9.8*), and seven additional *ipaH* family genes (*ipaH0722*, *ipaH0887*, *ipaH1383*, *ipaH1880*, *ipaH2022*, *ipaH2202*, and *ipaH2610*) on the bacterial chromosome (Rohde et al. 2007; Singer et al. 2008; Ashida et al. 2007). Not all *Shigella* strains carry a complete set of functional *ipaH* genes, however; for example, in *S. flexneri* YSH6000, *ipaH1.4* and *ipaH2.5* are pseudogenes (Ashida et al. 2007). Furthermore, of the chromosomal *ipaH* genes, only four are presumed intact in *S. flexneri* strain 2457 T, as the remaining three are disrupted by insertion elements or frameshift mutations (Wei et al. 2003). Each of these genes shares a highly conserved C-terminal region required for E3 ligase activity, as well as a more variable N-terminal region containing a common leucine-rich repeat motif essential for substrate recognition (Singer et al. 2008; Zhu et al. 2008). Therefore, although all IpaH proteins presumably function as ubiquitin ligases to target certain host cell proteins for degradation, each has a specific target; IpaH7.8 targets glomulin; IpaH9.8 targets NEMO; IpaH4.5 targets the p65 subunit of NF-κB; and IpaH0722 targets TRAF2 (Suzuki et al. 2014; Ashida et al. 2010, 2013). IpaH9.8, IpaH4.5, and IpaH0722, which tag proteins of the NF-κB signaling cascade for degradation, contribute to the attenuation of NF-κB activation and suppress the host inflammatory response. In addition to the IpaH proteins, members of the Osp (outer *Shigella* protein) family also contribute to the attenuation of the NF-κB signaling pathway.

OspF is a phosphothreonine lyase that irreversibly dephosphorylates host ERK1/2, p38, and JNK MAPKs; the activity of this T3SS effector directly opposes that of OspB (Reiterer et al. 2011; Li et al. 2007). Intriguingly, although OspF and OspB appear to follow the same initial time course of expression and secretion, expression of *ospB* decreases 1 h post-infection, with *ospF* expression remaining more stable, indicating that perhaps OspB plays a predominantly early role with OspF tempering the inflammatory response following initial infection (Ambrosi et al. 2015). OspI binds host Ubc13, an E2 enzyme, and deamidates Gln100 to a glutamic acid residue (Sanada et al. 2012; Fu et al. 2013). The altered Ubc13 is unable to polyubiquitinate TRAF6, and downstream NF-κB activation is attenuated. OspG functions as a serine-threonine kinase which binds to E2-ubiquitin complexes such as Ub-UbcH5b, which is an important factor for the ubiquitination and degradation of phospho-IκBα (Kim et al. 2005). Although the exact mechanism has not been elucidated, OspG binding to Ub-UbcH5b prevents phospho-IκBα degradation and corresponding NF-κB activation. Finally, OspZ, like its homologs NleE and NleB in enteropathogenic *E. coli* (EPEC), also attenuates IκB degradation (Newton et al. 2010).

Inflammation is not the only host process influenced by *Shigella* proteins. The OspC family of proteins (OspC1, OspC2, OspC3, and OspC4) are thought to play roles in the inhibition of different caspases within the host cell, likely delaying epithelial cell death and prolonging time for bacterial replication and spread (Kobayashi et al. 2013). OspC3, the only one of these effectors to be characterized thus far, binds directly to the p19 subunit of caspase-4, inhibiting the interaction of the p19 and p10 subunits required for heterodimerization and activation of caspase-4. Another T3SS effector, IcsB, binds cholesterol to protect the actin-polymerization protein IcsA from stimulating host autophagy (Kayath et al. 2010).

Adaptive Immune System Manipulation and Evasion

In addition to subverting the innate immune system, *Shigella* also molds adaptive immune responses to promote pathogenesis. Both T cells and B cells are manipulated to help *Shigella* evade clearance by the host immune system.

Salgado-Pabón et al. recently reviewed the literature on the role of T lymphocytes during *Shigella* infection (Salgado-Pabón et al. 2014). T cells locally present at the site of infection may be hijacked in one of two ways: *Shigella* can either invade T lymphocytes or utilize the T3SS to inject effector proteins directly into these cells in the absence of bacterial invasion (Konradt et al. 2011). As part of *Shigella*'s defense mechanism, both *in vitro* and *in vivo* experiments indicate that the pathogen restricts the migration of activated $CD4^+$ T cells. The PAI-encoded ShiA protein plays a role in attenuating this process although the mechanism is not yet known (Ingersoll et al. 2003). The T3SS effector, IpgD, has also been identified as a critical component of this inhibition (Konradt et al. 2011). IpgD mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 5-monophosphate (PI5P) at the plasma membrane, decreasing the concentration of

phosphorylated ERMs (ezrin, radixin, and meosin proteins). In the absence of activated (phosphorylated) ERMs, T cells are unable to migrate in response to chemoattractants, such as the pro-inflammatory cytokine surge resulting from *Shigella* invasion. In addition, the ability of $CD8^+$ T cells to stimulate the adaptive immune system is muted during invasion by *Shigella* species through an unknown mechanism (Jehl et al. 2011). Lastly, dendritic cells, which act as antigen-presenting cells during infection, have a reduced level of recruitment upon *Shigella* invasion (Sperandio et al. 2008).

As mentioned previously, Nothelfer et al. (2014) observed B lymphocyte death, both *in vitro* and *in vivo*, that were dependent upon the presence of IpaD (Nothelfer et al. 2014). Toll like receptors (TLR), specifically TLR2, can interact with IpgD and as yet unidentified bacterial co-signals to induce B cell apoptosis.

Shiga Toxin

Shiga toxin (Stx) was named after Dr. Kiyoshi Shiga, who identified *Shigella* as the causative agent of bacillary dysentery in 1898 (Trofa et al. 1999). Shortly after this discovery, Conradi and Shiga/Neisser independently described the deleterious effects of Stx in a rabbit model (Flexner and Sweet 1906). Another century would pass before Stx, and the related Stx-like enterotoxins of enterohemorrhagic *E. coli*, would be epidemiologically linked to and considered necessary for the development of hemorrhagic colitis and hemolytic uremic syndrome (HUS) in humans (Kaper and O'Brien 2014; Keusch et al. 1972; Fontaine et al. 1988; Karmali et al. 1983; O'Brien et al. 1983; Rasko et al. 2011).

For decades, Stx was thought to be confined to strains of *S. dysenteriae* type 1, only rarely appearing in isolates of *S. dysenteriae* type 4 or *S. sonnei* (Gupta et al. 2007; Beutin et al. 1999; Strauch et al. 2001). Between 2001–2003, however, 26 *S. flexneri* isolates harboring Stx were cultured from patients around the globe, many with a history of recent travel to Haiti or the Dominican Republic (Gray et al. 2014). The emergence of Stx-producing non-*S. dysenteriae* 1 *Shigella* strains has become a serious health concern, particularly because the bacteriophage carrying the Stx genes in *S. flexneri*, ΦPOC-J13, is novel among Stx-carrying bacteriophages. In *S. dysenteriae* 1, the functional *stx* genes are nestled within a defective lamboid prophage that was likely inactivated through numerous IS element insertions and rearrangements (McDonough and Butterton 1999). In the Shiga toxin-producing *E. coli* (STEC) strains, the Stx-like toxins Stx1 and Stx2 are carried on a diverse number of lambdoid phages (Muniesa et al. 2004). One of these phages, 933 W, appears to be closely related to the 7888 phage carrying *stx* in *S. sonnei* (Strauch et al. 2001; Plunkett et al. 1999). The emergence of bacterial strains carrying novel toxin-encoding phages suggest that there may be an environmental reservoir for such phages, and integration events into new *Shigella*/*E. coli* strains may continue to occur.

The two structural genes encoding Stx subunits, *stxA* and *stxB*, are expressed as a single transcript, although there is an additional promoter directly upstream of *stxB* which suggests this gene may also be independently transcribed (O'Loughlin and Robins-Browne 2001; Habib and Jackson 1992). Although toxin regulation in STEC has been well studied, considerably less attention has been given to *stx* regulation in *S. dysenteriae* 1 (Muhldorfer et al. 1996; de Sablet et al. 2008; Pacheco and Sperandio 2012). The promoter of the *stxA*/*stxB* operon is negatively regulated by the ferric uptake regulatory protein (Fur); in the presence of iron, the Fur-iron complex binds upstream of *stxA*/*stxB* to repress expression, which suggests that this operon is likely expressed in the host tissues, where free iron availability is limited during infection (Svinarich and Palchaudhuri 1992; Weinberg 1978; Carpenter et al. 2009).

Shiga toxin is an A_1B_5 holotoxin; the enzymatically active 32-kDa A-subunits are C-terminally anchored to a pentameric ring of 7.7-kDa B-subunits, each of which have three binding sites for the host receptor, which is predominantly the glycosphingolipid receptor Gb3 (Bergan et al. 2012; Jacewicz et al. 1986). Stx toxin export or release from the bacteria is not yet well understood, although outer membrane vesicle formation may be important for this process (Dutta et al. 2004).

Gb3 is highly expressed in vascular endothelial cells and the kidney, two prominent sites of Shiga toxin damage. Several renal cell types, including proximal/distal renal tubule cells, mesangial cells, and glomerular epithelial cells, carry the receptor (Meyers and Kaplan 2000). Once internalized into the host cell, the enzymatically active A-subunit is cleaved by host furin protease under low pH conditions into A1 and A2 fragments, which remain attached by a single disulfide bond (Garred et al. 1995). Following cleavage, the fragments dissociate in the endoplasmic reticulum and the A1 subunit is transported to the cytosol, where it removes a single adenine base from the host 28S rRNA within the 60S ribosomal subunit (Endo et al. 1988). Disruption of host ribosomal activity renders protein synthesis impossible, and cell death shortly follows.

Shigella Pathogenicity Islands

The acquisition by horizontal gene transfer of pathogenicity islands (PAIs) is a significant factor driving microbial evolution, producing pathogens from commensals and pushing pathogens into novel niches or helping them overcome host defenses or antibiotic treatment (Schmidt and Hensel 2004). These genetic islands are typically characterized by the presence of one or more associated virulence genes, G + C content atypical of the chromosome, and a close proximity to mobile genetic elements, such as integrases or insertion sequence (IS) elements, which suggest horizontal transfer of the DNA from an outside source (Schmidt and Hensel 2004; Hacker et al. 1990).

Although *Shigella* virulence is largely attributable to the T3SS of the large virulence plasmid, there are several *Shigella* PAIs that have been identified on the chromosome: SHI-1, SHI-2, SHI-3, SHI-O, and SRL (Schroeder and Hilbi 2008). It is important to note that these PAIs are not universally present in *Shigella*. The

SHI-1 locus, for example, is entirely absent from *S. dystenteriae* strain Sd197 and *S. flexneri* strain Sf8401, and other *S. boydii* and *S. sonnei* strains are missing the *pic*/*set1A*/*set1B* genes of the PAI (Yang et al. 2005; Nie et al. 2006).

SHI-1 Locus

SHI-1, previously known as the *she* locus, is flanked by a complete *pheV* tRNA gene directly upstream and a 22 bp repeat of *pheV* downstream (Al-Hasani et al. 2000). This is perhaps unsurprising, as the *pheV* gene is a common site of insertion for PAIs and transposons in both *E. coli* and *Salmonella*, and tRNA genes are effective insertion points (Swenson et al. 1996; Hacker et al. 1997). Four characterized genes (*set1A*, *set1B*, *sigA*, and *pic)* and 28 putative open reading frames (ORFs) form this locus. All four characterized genes encode virulence factors that are optimally active at 37 °C, mimicking *Shigella* T3SS temperature-dependent expression (Al-Hasani et al. 2000; Behrens et al. 2002; Henderson et al. 1999).

The *set1A/set1B* genes encode an A_1B_5 enterotoxin, ShET1, which is highly homologous (99%) to the virulence plasmid-encoded ShET2 and also contributes to the onset of watery diarrhea during shigellosis (Fasano et al. 1995, 1997). The other two characterized genes of the SHI-1 locus, *sigA* and *pic*, encode autotransporters. SigA, an immunoglobulin A-like cytopathic protease, is cytotoxic to HEp-2 cells and can degrade casein *in vitro*, although its target substrate in the host is unknown (Al-Hasani et al. 2000; Rajakumar et al. 1997). A Δ*sigA S. flexneri* 2a isolate is significantly impaired in its ability to induce fluid accumulation in the rabbit ileal loop model, indicating that SigA likely plays a role during the watery phase of diarrhea (Al-Hasani et al. 2000). Finally, *pic* encodes a serine protease that binds host mucin to catalyze its degradation (Henderson et al. 1999; Gutierrez-Jimenez et al. 2008). This breakdown process aids in destroying the thick mucus layer that lines the intestine, likely promoting *Shigella* colonization and invasion.

Of the remaining 28 putative open reading frames (ORFs) in SHI-1, most share sequence homology with hypothetical bacteriophage-associated genes, and their functions have yet to be elucidated. However, one of the ORFs, termed *sap*, bears high sequence homology to the previously characterized Ag43, an autotransporter surface protein responsible for autoaggregation in *E. coli* (Al-Hasani et al. 2001).

SHI-2 Locus

The SHI-2 locus encompasses a 30-kB region, including several genes associated with aerobactin iron transport (*iucA-D, iutA*), colicin immunity (*shiD*), and dampening the host inflammatory response (*shiA*) (Vokes et al. 1999; Moss et al. 1999). This locus is located 16 bp downstream of another tRNA gene, *selC*, and directly upstream of a disrupted *nlpA* gene. Similar to the SHI-1 insertion site, the region

surrounding the SHI-2 locus is also a common insertion site for *E. coli* and *Salmonella* PAIs (Moss et al. 1999). SHI-2 is not entirely conserved in all *Shigella* strains, however. In the *S. flexneri* 5a strain M90 T, the locus downstream of *iutA* is altered: it contains an IS600 element instead of IS2, and lacks several hypothetical ORFs, resulting in a 23.8-kB locus rather than the typical 30-kB locus (Moss et al. 1999). In *S. flexneri* serotypes 1a and 2b, *shiD* appears to be inactive, likely due to genetic polymorphisms within this gene (Vokes et al. 1999). Strains of *S. boydii* do not appear to harbor the SHI-2 locus at all (Purdy and Payne 2001).

In the microbial pursuit to acquire valuable iron from the host environment, two siderophores are commonly produced by members of the *Escherichia/Shigella* genus: aerobactin and enterobactin (Rogers 1973). All wild-type *Shigella* appear to synthesize and transport at least one of these two iron chelators (Lawlor et al. 1987). The aerobactin operon, *iuc* (iron uptake chelate), consists of four genes that synthesize this siderophore: *iucA*, *iucB, iucC*, and *iucD* (Bagg and Neilands 1987). *iutA* (iron uptake transport), a SHI-2 gene outside the aerobactin operon, encodes the outer membrane receptor for ferric aerobactin (Van Tiel-Menkveld et al. 1982). In iron-limited environments, aerobactin is released to scavenge free or bound iron, such as transferrin or lactoferrin. The iron-bound aerobactin is then recognized by the IutA receptor and shuttled back into the bacterium (Lawlor et al. 1987). The presence of aerobactin provides *Shigella* with an *in vivo* growth advantage when extracellular (i.e., prior to invasion); within the epithelial cell, however, host hemin or hematin can be directly transported into the cell to serve as the primary iron source, and a siderophore is no longer required (Lawlor et al. 1987; Nassif et al. 1987).

Colicins are molecules produced by enteric bacteria that target and kill neighboring bacteria, a process designed to circumvent competition for valuable resources (Cascales et al. 2007). These toxic peptides bind to specific receptors on a sensitive bacterium, are subsequently translocated via Tol or TonB machinery, and then initiate death in a manner dependent on the colicin; nucleotides and phospholipid bilayers are common targets. To avoid death, bacteria synthesize immunity proteins, which typically either block pore-forming colicins from reaching their target, or bind directly to nuclease colicins to prevent their catalytic activity (Cascales et al. 2007; Weaver et al. 1981; Jakes et al. 1974). The SHI-2 locus contains the gene *shiD*, which encodes for immunity against colicins V, Ib, and an uncharacterized colicin produced by *S. flexneri* strain 2a SA100, all of which bind to the Cir protein (Vokes et al. 1999). Interestingly, although *shiD* has been determined to produce functional protein, it shares no significant sequence similarity with any known colicin immunity genes, leaving its mechanism of action unknown (Moss et al. 1999).

shiA, located upstream of the aerobactin operon in SHI-2, plays a role in attenuating *Shigella* inflammation (Ingersoll et al. 2003). To study the impact of SHI-2 on inflammation, a SHI-2 deletion mutant was constructed in *S. flexneri* 5a strain M90 T and rabbit ligated ileal loops were infected with either the resulting mutant or the wild-type parent strain. Infection with the mutant resulted in significantly more blunted villi, a hallmark of increased inflammation in intestinal tissue. *shiA* alone was sufficient to repress this hyper-inflammatory phenotype. Conversely, a Δ*shiA* mutant increased the number of apoptotic cells and PMNs within infected villi.

Taken together, this evidence suggests that ShiA plays a role in dampening the immune response, although its mode of action remains unclear as it does not appear to regulate the common mediators of pro-inflammation (Ingersoll et al. 2003).

Of the remaining proteins encoded by genes in the SHI-2 locus, several share sequence homology to characterized proteins: ShiB to a DNA helicase (95% identity); ShiC to a member of the major facilitator superfamily transporters (56% identity); and ShiF to a tetracycline transporter (34% identity) (Vokes et al. 1999; Hillen and Schollmeier 1983) (BLAST analysis conducted on 01/13/15 (Altschul et al. 1990)). The remaining SHI-2 locus consists of either insertion/transposition elements or novel ORFs with no sequence similarity to characterized genes. There is also a single gene (*int2*) at the beginning of SHI-2 which shares homology with the bacteriophage P4-like integrase, suggesting that this PAI was possibly acquired by bacteriophage integration (Vokes et al. 1999).

SHI-3 Locus

The 21-kb SHI-3 locus is found exclusively in *S. boydii*, which lacks the SHI-2 PAI of *S. flexneri* and *S. sonnei*. The SHI-3 locus encodes a fully functioning aerobactin system that shares 97% nucleotide identity with the SHI-2 aerobactin system; however, it is located between *lysU* and the *pheU* tRNA gene rather than downstream of *selC* (Purdy and Payne 2001). Intriguingly, genome comparisons between *S. boydii* and *E. coli* K-12 indicate that the insertion of SHI-3 corresponded with the loss of a 6-kb region that included *cadA*, a known antivirulence gene in *Shigella* (Maurelli et al. 1998; McCormick et al. 1999; Fernandez et al. 2001; Purdy and Payne 2001). It is possible that insertion of the PAI resulted in the immediate deletion of *cadA*, and therefore increased *Shigella* pathogenicity not only through acquisition of a valuable siderophore system but also through loss of an antivirulence gene. However, there is no way to determine whether or not the *cadA* gene was present directly prior to the insertion event. If the genes in this region were already in decay, the *lysU-pheU* junction may have been an ideal insertion site for SHI-3, as interruptions in regions with essential genes can be deleterious to the organism. SHI-3 also contains numerous insertion/transposition elements and an integrase gene (*int3*), which, like *int2* from SHI-2, is a member of the P4 bacteriophage integrase family.

SRL Locus

SRL (*Shigella* resistance locus) is a 66-kb pathogenicity island that carries a functional ferric dicitrate uptake system in addition to multiple antibiotic resistance cassettes (Luck et al. 2001). Located in the chromosome, the SRL begins 161 bp upstream of *int* and ends at the 3′ terminus of *serX*, and contains 59 ORFs. Although not universally present in *Shigella*, 35 out of 55 *Shigella* strains tested by Luck et al.

linked the SRL genes *tet* and *fec,* suggesting that this PAI is widespread throughout the genus (Luck et al. 2001). Moreover, the SRL has been identified in strains of each of the four species.

The *fec* iron transport locus contains three operons: *fecI*, *fecR*, and *fecABCDE* (Luck et al. 2001). *fecI* and *fecR* encode the positive regulatory elements of the system, and the *fecABCDE* operon encodes FecA, the receptor involved in binding ferric di-citrate, as well as structural genes required for the transporter (Staudenmaier et al. 1989; Wagegg and Braun 1981). Although the mechanisms involved in ferric dicitrate uptake are still unclear, the structural apparatus for this system consists of a periplasmic protein (FecB), two nonpolar integral membrane proteins (FecC/FecD), and a membrane-bound protein which is thought to bind ATP (FecE) (Staudenmaier et al. 1989). In *Shigella*, the *fec* locus shares 99% nucleotide identity with *E. coli* K-12 genes, and complements an *E. coli* Δ*fec* strain for growth under iron-limited conditions (Luck et al. 2001). Furthermore, the *fec* locus is transcribed in *Shigella*; however, this system may be redundant in the presence of other iron uptake systems, as a strain lacking the *fecABCDE* locus shows no growth defect under iron-limiting conditions compared to an isogenic strain carrying the locus. Because the ferric dicitrate uptake system is commonly expressed in commensal *E. coli*, its presence in *Shigella*, like the aerobactin system, may confer a survival advantage within the intestine rather than within the host cell (Luck et al. 2001).

Many antibiotic resistance genes are also on the SRL, including several which encode resistance to streptomycin (*aadA1*), ampicillin (*oxa-1*), chloramphenicol (*cat*), and tetracycline (*tetA*) (Luck et al. 2001). Many of the remaining ORFs of SRL are homologous to the genes of the prophages CP4-44, CP4-57, and 933 L (Luck et al. 2001). A number of IS elements, both intact and disrupted, also litter the PAI.

SHI-O Loci

Serotyping in *Shigella* is dependent on the O polysaccharide side chain of the cell envelope lipopolysaccharide (LPS) (Simmons and Romanowska 1987). A linear tetrasaccharide repeat forms the backbone of the O side chain in *S. flexneri,* and, in the absence of modifications, is considered the parent serotype Y: -3)-*β*-D-L-Glc*p*Nac-(1-2)-*α*-L-Rha*p*I-(1-2)-*α*-L-Rha*p*II-(1-3)-*α*-L-Rha*p*III-(1- (Guan et al. 1999). At least three temperate bacteriophages (SfX, SfII, and SfV) are responsible for the glucosylation of different rhamnose residues along this backbone (Huan et al. 1997; Guan et al. 1999; Mavris et al. 1997). The resulting O-antigen variants produce unique *S. flexneri* serotypes. Such a mechanism is mutually beneficial for both phage and bacteria; certain bacteriophages induce specific O-antigen modifications as a defense to exclude homologous phages from infecting the same bacterial host, and diverse O-antigen variation can help the bacteria to evade or delay host immune recognition (Pajunen et al. 2000; Phalipon et al. 1995). Collectively, these bacteriophages form the SHI-O loci.

The SfX bacteriophage encodes three O-antigen modification genes: *gtrX*, a glucosyltransferase; *gtrA*, a small hydrophobic protein of unknown function; and *gtrB*, a bactoprenol glucosyltransferase (Guan et al. 1999; Verma et al. 1993). First, GtrB transfers a glycosyl group from UDP glucose to the lipid carrier undecaprenyl phosphate (UndP) (Guan et al. 1999) Although the exact function of GtrA is unclear, it is thought to play a role in the subsequent translocation of lipid-linked glucose across the cytoplasmic membrane. Finally, GtrX attaches the glucosyl group to the first rhamnose residue of the growing O side chain, altering the *S. flexneri* serotype from Y to X.

The remaining two bacteriophages, SfII and SfV, share similar gene organization to SfX in regard to their respective O-antigen modification genes. In SfII, *orf2* and *bgt* share high homology to *gtrA* and *gtrB* respectively, and likely produce proteins of similar functions (Mavris et al. 1997). The amino acid sequence of GtrII, the glucosyltransferase of SfII, is divergent from GtrX, however, likely due to differences in target specificity. GtrII mediates the attachment of a glycosyl group to the third rhamnose unit of the O side chain, producing *S. flexneri* serotype II. Three serotype conversion genes (*orf4*, *orf5*, and *gtrV*) are likewise present on the temperate bacteriophage SfV (Huan et al. 1997). Like both SfX and SfII, *orf4* and *orf5* encode proteins with high identity to GtrA and GtrB; only *gtrV*, the putative glucosyltransferase, is divergent. GtrV catalyzes the transfer of a glucosyl group to the second rhamnose unit, resulting in *S. flexneri* serotype V.

Drug Resistance

Shigella, like many bacterial pathogens, has become more resistant to antimicrobials over the past few decades. The rise of multidrug-resistant (MDR) *Shigella* strains poses critical health concerns for future treatment of infected individuals, particularly in the absence of an effective vaccine. While most MDR *Shigella* outbreaks in the last few years have occurred in southern Asia (India, Bangladesh, and China), there have also been reports of MDR outbreaks in South America (Chile) and the Middle East (Iran) (Zaidi and Estrada-Garcia 2014; Bhattacharya et al. 2003; Pazhani et al. 2004; Ud-Din et al. 2013; Zhang et al. 2014; Tajbakhsh et al. 2012; Toro et al. 2005).

One possible explanation for the rapid worldwide distribution of MDR *Shigella* strains is a substantial flux in the epidemiologic landscape of this pathogen over the course of the last century. International travelers are an underappreciated and significant factor for dissemination of *Shigella* species across the globe (Arai et al. 2008; Hirose et al. 2005). Travelers may introduce novel MDR strains to naïve populations; these strains may then become endemic to the region, particularly if they are more resistant to eradication by antibiotics than native circulating strains. Asymptomatic carriage of *Shigella* isolates may also account for persistence of these MDR strains, as an environmental reservoir for the bacteria has not yet been identified (Bovee et al. 2012). In developing nations, which share the largest burden

of *Shigella* infections, antibiotic use is often unregulated, which is a circumstance that can lead to the misuse and overuse of antibiotics (Hart and Kariuki 1998). Asymptomatic carriers of *Shigella* strains may be exposed to broad spectrum antibiotic drug treatment meant to target other infections, which could provide ample selective pressure for keeping these MDR strains circulating in the population.

Traditional first-line antibiotic treatment for *Shigella* includes ampicillin, trimethoprim-sulfamethoxazole (co-trimoxazole), and nalidixic acid (World Health Organization 2005). However, many of these first-line drugs have become ineffective and are no longer recommended for therapy due to widespread resistance. Ciprofloxacin has become the antibiotic of choice for MDR strains; however, increasing resistance to this antimicrobial has also been reported (Folster et al. 2011). Second-line antibiotics include other fluoroquinolones, pivmecillinam (amdinocillin pivoxil) and ceftriaxone (World Health Organization 2005). Azithromycin can also be used to treat adults and children (World Health Organization 2005).

The mechanisms for antimicrobial resistance in *Shigella* have been well characterized. Ampicillin resistance is mediated by beta-lactamases, which are encoded by the *bla* genes, such as bla_{TEM}, bla_{OXA}, and bla_{SHV} in *Shigella* (Toro et al. 2005; Ahmed et al. 2013). In *E. coli*, acquisition of the alternative dihydropteroate synthase (DHPS) genes (*sul1*, *sul2*, or *sul3*) results in trimethoprim-sulfamethoxazole resistance (Perreten and Boerlin 2003). All three of these genes have been identified in isolates of *Shigella* (Chang et al. 2011; Peirano et al. 2005). Finally, the mechanism for quinolone resistance (ciprofloxacin and nalidixic acid) involves the accumulation of one or more specific mutations in the chromosomal quinolone resistance determining regions (QRDR) or the plasmid-mediated quinolone resistance determining regions (PMQR). Target genes include the DNA gyrase (*gyrA*), topoisomerase IV (*parC*), aminoglycoside acetyltransferase (*aac(6′)-Ib-cr*), an efflux pump (*qepA*), and a pentapeptide-repeat family protein (*qnr*) (Tavio et al. 1999). The subsequent dissemination of mobile genetic elements, such as plasmids, transposons, and integrons, are common routes for the rapid spread of antimicrobial resistance genes among circulating strains (Balcazar 2014; Gillings 2014; Marti et al. 2014).

Concluding Remarks

The ability of *Shigella* to survive the hostile conditions of the gastrointestinal tract, invade the colonic epithelium, and manipulate the immune system of the human host requires a full complement of diverse virulence factors, including an impressive array of T3SS effectors and, in certain strains, PAI-associated factors, Shiga toxin, and/or antibiotic resistance genes. Although the virulence phenotypes of *Shigella* have been extensively studied thus far, additional research is needed to fully elucidate the mechanics of this organism. The facets of *Shigella* pathogenesis that remain the least understood include, but are not limited to, the T3SS-mediated manipulation of the host immune response; the complete characterization of the

Shigella PAIs; and the determination of specific mechanisms involved in T3SS effector activities. Future investigations of these topics will continue to reveal the complex interactions of this versatile pathogen with its host. This work is critical to not only advance our knowledge of *Shigella* virulence, but also to develop new treatments or preventions against this pathogen. To date, no effective vaccine has been developed to protect susceptible populations against *Shigella*, and the number of people who contract shigellosis annually is staggering. To complicate matters, the emergence of MDR strains hampers the effective treatment of patients with bacillary dysentery.

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Alterations in Shiga Toxin-Producing *E. coli* Colonization and Virulence Following Dietary Modulation and Administration of Antimicrobials

Cheleste M. Thorpe

Abstract Shiga toxin-producing *E. coli* (STEC) infection is primarily a foodborne zoonosis and can result in severe disease, with long-term health effects, or even death. Despite knowing the major STEC reservoir, how the human host is typically exposed, and primary virulence factors that contribute to pathogenesis, little progress has been made in certain areas that are important for human disease prevention. We have not yet been able to eliminate STEC from the cattle reservoir, to prevent infection after human exposure, or to prevent the serious and sometimes tragic sequelae of infection. This chapter will discuss dietary and antimicrobial effects on STEC colonization and virulence. We will review some of the dietary manipulations that have been tried to limit cattle STEC colonization as a way to prevent its subsequent spread to humans. The use of dietary supplements and probiotics for prevention or mitigation of human disease has also been studied, and these efforts will be addressed as well. Antimicrobial treatment of human STEC infection remains controversial, as some studies have associated antibiotic treatment with worse patient outcomes. This chapter will describe the mechanism(s) by which antimicrobial treatment may alter STEC pathogenicity. In addition, a recent outbreak of HUS caused by a Stx-producing *E. coli* O104 strain with an enteroaggregative phenotype challenged traditional thinking about the use of antimicrobials in the setting of STEC infection, and this will be reviewed.

Keywords Shiga toxin-producing *E. coli* • O157:H7 • Dietary modulation • HUS • Virulence • Foodborne illness • Probiotics • Super-shedder

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Introduction

Shiga toxin-producing *E. coli*, or STEC, are a family of organisms defined by the ability to produce one or more types of Shiga toxin (Stx). Human infection with STEC of the O157:H7 serotype was first associated with hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS) more than three decades ago. Since then, over 100 different O-group serotypes have been associated with human disease (Johnson et al. 2006; Mathusa et al. 2010). Infection with these organisms can result in illness ranging from self-limited, watery diarrhea to hemorrhagic colitis (HC) and/or development of hemolytic uremic syndrome (HUS), and can result in permanent renal failure, brain damage, and/or death (Bitzan et al. 2010). Shiga toxins (Stxs) are the causative virulence factor for both HC and HUS. HC can progress to gangrenous colitis, bowel perforation, peritonitis, and/or sepsis. HUS is a clinical syndrome manifested by a triad of acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia.

As nomenclature of STEC harboring different virulence factors can be confusing, in this chapter the term STEC will be used rather than using the generic terms "enterohemorrhagic *E. coli* (EHEC)" or "non-O157 STEC". *E. coli* O157:H7 will be specified when studies are done specifically with that organism and, if known, the strain studied.

Human infection with STEC is primarily a food- and waterborne zoonosis, although infection can be acquired through direct livestock contact, via person-to-person spread, or from household animals. Minimal bacterial exposures can result in infection, as some strains have an estimated infectious dose of fewer than 100 organisms (Paton and Paton 1998). The largest reservoir is cattle, which are asymptomatically colonized and shed the organism in their stools. Other healthy domestic animals can harbor STEC, such as sheep, goats, cats and dogs (Beutin et al. 1993). Wild animals can also harbor STEC. Human exposures to STEC-colonized deer and elk feces, and contaminated venison, have been linked to disease (Franklin et al. 2013; Laidler et al. 2013; Rounds et al. 2012). Young pigs can suffer from STEC infection, but healthy swine can carry STEC asymptomatically (Tseng et al. 2014).

Fecal shedding can result in contamination of beef and beef products, as exemplified by an early outbreak of HUS in which humans, primarily children, were exposed via undercooked ground beef in hamburger patties from a fast food restaurant in Washington state (Anonymous 1993). Produce and water can become contaminated through exposure to animal feces, subsequently resulting in human exposure. Examples of large-scale produce contamination include the US spinach outbreak of 2006 (Maki 2006), as well as the Japanese radish sprout outbreak of 1996. In the latter, radish sprouts in school lunches resulted in thousands of schoolchildren becoming ill, with 12 deaths reported (Michino et al. 1999). A waterborne outbreak occurred in Canada in 2000, resulting in approximately 2300 cases of infection and seven deaths (Salvadori et al. 2009). Eliminating STEC from the cattle reservoir has been a subject of intense study, as it is thought to be a critical public health measure in mitigating human exposure.

The "classic" serotype associated with HUS is O157:H7, but there are many other serotypes known to cause disease and are being increasingly recognized (Johnson et al. 2006; Gould et al. 2013; Luna-Gierke et al. 2014). From 2001 to 2010, the estimated incidence of non-O157 infection in the US increased from 0.12 cases per 100,000 to 0.95 cases per 100,000, while the incidence of O157 infections decreased by more than half (2.17 cases per 100,000 to 0.95 cases per 100,000). Preliminary FoodNet data reveal that this trend continues (Crim et al. 2015).

Genetic variability amongst the STEC family affects virulence of the STEC strain. The STEC family is quite heterogeneous with respect to virulence factors harbored by different strains. For example, STEC strains vary in which Stx type(s) they produce (Paton and Paton 1998; Scheutz et al. 2012). All Stx family members areAB5 toxins, with a binding pentamer of identical "B" subunits, non-covalently associated with the "A" subunit, where the enzymatic activity is located. All Stxs target the alpha-sarcin/ricin loop of the 28S rRNA, causing inhibition of protein synthesis. The major receptor for most Stxs is globotriaosylceramide (Gb3), though other receptors exist. Variants in the Stx1 family are very similar to Stx from *Shigella dysenteriae* type 1. Stx2 variants are homologous to Stx, but immunologically distinct. A number of different Stx1 and Stx2 variants have been described. Strains can make one or more Stx variants, and certain Stx2 variants are more likely to be associated with hemolytic uremic syndrome (HUS).

STEC of the O157:H7 serotype almost invariably harbor a pathogenicity island called the "locus of enterocyte effacement", or LEE (Paton and Paton 1998). The LEE region encodes virulence factors required for the bacteria to form lesions on the host epithelial cell and are called "attaching-and effacing", or A/E lesions. These genes include a type 3 secretion system (T3SS), an outer membrane adhesin called "intimin" that mediates bacterial attachment to host cells, and the translocated intimin receptor ("Tir"). Tir is injected via the T3SS into the host membrane, and Tir-intimin interactions trigger formation of A/E lesions, allowing the bacteria to colonize via close association with host intestinal epithelial cells.

There are several other common serotypes that usually have the LEE pathogenicity island, such as O26, O45, O103, O111, O121, and O145 (Mingle et al. 2012). In the US, these six serogroups are the most commonly reported causes of STEC infections (Gould et al. 2013). Thus, STEC of these serotypes have been termed "the big six," and in 2012 were classified as food adulterants by the USDA. However, infection with STEC lacking LEE have also been associated with HUS (Johnson et al. 2006). These isolates are genomically diverse, and their virulence determinants are not fully understood (Franz et al. 2015; Steyert et al. 2012; Haugum et al. 2014; Ju et al. 2013). For example, although these strains are relatively uncommon, some STEC are capable of synthesizing a novel toxin called subtilase cytotoxin, or SubAB (Paton et al. 2004). SubAB was initially discovered in a LEE-negative STEC that caused an HUS outbreak in Australia, but the genes encoding SubAB are spread amongst STEC worldwide. Another striking example of STEC genomic flexibility was the emergence of an STEC strain bearing a hybrid collection of virulence factors, causing a 2011 outbreak in Germany that resulted in over 800 cases of HUS (Rasko et al. 2011). Genomic studies of strains from different sources will yield

insights into the full spectrum of genes important in mediating survival in different environments such as the cattle reservoir, and/or pathogenicity in humans.

After human exposure, STEC must colonize the gastrointestinal tract of its human host where it expresses virulence factors important to its survival in that milieu. The systemic uptake of Stxs from the gastrointestinal tract results in the morbidity and mortality characteristic of STEC infection. Since the organism is non-invasive, Stxs must traverse the intestinal epithelium to be taken up into the host systemic circulation, exerting their cytotoxic effects. Thus, the amount of Stx present in the intestine available for systemic uptake is thought to be important.

It is increasingly evident that pre-existing gastrointestinal tract microbial communities (termed, "microbiota") can influence pathogen virulence in a number of different ways. It is also being appreciated that diet has a role in shaping the intestinal microbiota in a fashion that can be either advantageous or disadvantageous to the host. Recent studies have attempted to assess effects of food, food additives, intestinal microbiota, and probiotics on both cattle colonization and human STEC disease. Ultimately, the goal of these studies is to elucidate interventions that will result in STEC elimination from the cattle reservoir, as well as human disease prevention and/or amelioration.

Treatment of Human STEC Infection with Antimicrobials

In 2000, a prospective cohort study revealed that antibiotic administration during the diarrheal phase of O157:H7 infection was associated with an increased risk of developing HUS (Wong et al. 2000). A subsequent meta-analysis did not support this finding (Safdar et al. 2002). Since then, multiple studies have been done with inconsistent results (Keir et al. 2012). Only one of these studies was a randomized controlled trial; this trial had a small number of patients and showed no effect (Proulx et al. 1992). Debate about the risks of antibiotic treatment continues (Keir et al. 2012; Wurzner et al. 2014; Rahal et al. 2012).

Randomized controlled data are lacking due to the sporadic nature of STEC outbreaks. Even if large randomized controlled trials could be performed, strain diversity (for example, strain-specific differences in effect of antimicrobials on Stx production) could confound the results observed from one outbreak to the next. In order to eliminate strain-specific issues, a 2012 observational, multistate prospective cohort study enrolled over 250 children over approximately 10 years, thus assessing antibiotic use in the setting of infections caused by diverse strains. In this study, children who received antibiotics during the diarrhea phase more frequently developed HUS than those who did not, and this finding was observed across all antibiotic classes (Wong et al. 2012).

Multiple *in vitro* and *in vivo* studies have provided mechanistic insight as to why treatment of STEC infection with antibiotics could result in more severe outcomes. Unlike *Shigella dysenteriae* type 1, in which the *stx* gene is located on the chromosome, *stx* genes are harbored on lambdoid phages in STEC. When phage genes are

repressed, the lambda-like bacteriophage encoding *stx* genes remains integrated into the host chromosome as a prophage. Phage production is linked to Stx production. Upon damage to the bacterial chromosome, the bacterial SOS response induces the lytic cycle, with release of new virions and Stxs from the lysed bacterial host (Waldor and Friedman 2005). Antibiotics can induce the SOS response, particularly those that act via causing DNA damage, such as the fluoroquinolones which target DNA gyrase, and trimethoprim-sulfamethoxazole, which inhibits nucleotide synthesis. In a murine model of STEC infection, administration of subinhibitory concentrations of Ciprofloxacin (a fluoroquinolone) resulted in the production of massive amounts of Stx2 in the feces of O157:H7 infected mice, and caused death (Zhang et al. 2000). In contrast, this effect was not seen with the bacterial cell wall inhibitor, fosfomycin. In a germ-free mouse model, infection with an O157:H7 EDL933 derivative with an uninducible *stx*-encoding prophage could not cause the typical pathological changes seen with the parent strain (Tyler et al. 2013).

Aside from enhancing Stx production from the infecting pathogen, treatments that promote bacteriophage release from the infecting STEC may increase Stx production via effects on other host microflora. Stx-encoding bacteriophages from STEC can infect commensal *E. coli*. Lytic infection of these commensal "bystanders" may result in additional production of Stx by the bystanders as well as the original infecting strain, thus contributing to the burden of toxin in the intestinal tract (Iversen et al. 2015; Gamage et al. 2003, 2006; Cornick et al. 2006). As a result, it has been hypothesized that susceptibility of an individual's intestinal commensal *E. coli* population to transduction by Stx-producing bacteriophage from a given STEC strain may influence whether an individual experiences asymptomatic carriage or illness following exposure (Iversen et al. 2015; Gamage et al. 2003)

Treatment Considerations During the German 2011 *E. coli* O104:H4 Outbreak

Between May 2011 and July 2011, a large epidemic of HUS occurred in Germany; more than 800 patients developed HUS, and over 50 died. The suspected food vehicle was raw fenugreek sprouts. The causative agent was *E. coli* O104:H4, which represents a newly recognized pathotype. This Stx2-producing strain lacks the LEE pathogenicity island, but encodes many virulence factors commonly associated with enteroaggregative *E. coli* (EAEC). It is an extended spectrum beta lactamase (ESBL) producer, but it retains susceptibility to fluoroquinolones. Reflecting this strain's hybrid of virulence factors, one group (Brzuszkiewicz et al. 2011) termed this strain an Entero Aggregative Hemorrhagic E. coli, or EAHEC; another has called it a Shiga toxin-producing EAEC (Rasko et al. 2011). Based on genomic studies, it is hypothesized that at some point, an EAEC strain well-adapted to colonizing humans became transduced with a lambdoid phage encoding Stx2 (Steiner 2014). Prior to this large outbreak, only rare sporadic infections with Stx-producing EAEC had been previously described; reviewed in (Zangari et al. 2013).

There were a few notable features in this outbreak (Jandhyala et al. 2013). The median incubation time following ingestion was 3–4 days longer than with typical O157:H7 infection. The epidemic disproportionately affected adults, whereas in typical O157:H7 infection, a higher risk of HUS is usually seen in children and the elderly. HUS developed in approximately 25% of patients, much higher than the typical HUS rate of 5–10%. This pathogen has been studied in some depth in order to understand why it caused a higher rate of HUS in patients.

Many different treatments were employed, including antimicrobials; reviewed in (Jandhyala et al. 2013; Rahal et al. 2015). The justification for antimicrobial therapy was the fact that unlike O157:H7 infections, which are self-limiting, EAEC infections are typically persistent. Thus, it was speculated that the continued exposure to Stx2 during persistent infection might result in increased HUS severity and/or duration, when compared with O157:H7 infection. However, like O157:H7 strains, subsequent *in vitro* studies with the outbreak organism revealed that low concentrations of Ciprofloxacin still caused increases in *stx2*-encoding phage induction, *stx2* gene transcription, and Stx2 production *in vitro* (Rasko et al. 2011; Bielaszewska et al. 2012; Corogeanu et al. 2012). Also like O157:H7 strains, antibiotics with DNA-neutral mechanisms of action had more favorable effects *in vitro*.

In this particular outbreak, the question of whether antibiotic therapy changed the likelihood of developing HUS, or altered the course of HUS once established, cannot be answered from results of the available studies. This is due to small numbers of study patients, different antibiotic therapies used, co-administration of other therapeutic modalities, and/or other biases (Geerdes-Fenge et al. 2013; Menne et al. 2012; Ullrich et al. 2013). However, antibiotic treatment was associated with lower frequency of long-term pathogen carriage, potentially decreasing the risk of secondary infections (Nitschke et al. 2012; Vonberg et al. 2013).

Effect of Dietary Alterations and Supplements on the Cattle STEC Reservoir

Reduction in STEC shedding from cattle is thought to be an important public health measure for reducing human exposure via food, water, and direct livestock contact. Innumerable studies have revealed wide variations in prevalence, intensity, and duration of STEC shedding within cattle communities. The factors that influence shedding are not entirely understood. However, modeling and epidemiologic studies have revealed that animals who shed O157:H7 at high levels are associated with spread of O157:H7 among cattle communities, so even small reductions in colonization of these high level shedders may have a significant impact on carcass and environmental contamination (Chase-Topping et al. 2007; Matthews et al. 2006a, b; Omisakin et al. 2003; Cobbold et al. 2007). Thus, special mention will be made here of the cattle “supershedder”.

A "supershedder" is defined as an animal that sheds O157:H7 at $>10^4$ cfu per gram of stool at a single sampling time. Cattle can be intermittent supershedders, and/or differ in supershedding duration, reviewed in (Munns et al. 2015). As a result, some have proposed the term, "supershedding event" as more applicable than identifying an animal as a "supershedder" based on a single point in time (Williams et al. 2014). Potential contributions from both host and pathogen may influence the supershedding phenomenon (Munns et al. 2015). Bacterial contributions may include bacterial strain-specific genomic differences in adherence genes, virulence genes, bacteriophage type, growth requirements, and/or biofilm formation.

Supershedder bacterial strains may be genetically distinct from non-supershedder strains. Previous studies have implicated the LEE region in colonization of the rectoanal junction (Naylor et al. 2005; Sheng et al. 2006). However, supershedder O157:H7 strains have demonstrated differences in adherence patterns compared with non-supershedder strains in tissue culture models of rectoanal junction stratified squamous epithelial cells (Cote et al. 2015). Limited mutation analysis suggested that this adherence pattern may be LEE-independent. Thus, further studies are needed to fully elucidate the bacterial gene(s) that may contribute to the supershedder phenotype, and their relative importance.

Differences in cattle intestinal microbiota are postulated to play a role in shedding (Munns et al. 2015). For example, a recent study compared the microbiota of eleven O157 supershedders and 11 non-shedder pen-mates (Xu et al. 2014). Supershedder intestinal microbial communities were more diverse, meaning that there were a greater number of different bacterial strains in the communities within the supershedders, compared to the non-shedders. For some of the bacterial families shared by supershedders and non-shedders, differences in relative abundance of organisms were observed. Further studies will be needed to explore causal relationships. A number of hypotheses have been put forth to explain the effect(s) microbiota may have on supershedding, including the idea that specific bacteria in an animal's microbiota may synthesize quorum-sensing molecules that affect O157:H7 growth and virulence, with subsequent impact on the ability of O157:H7 to colonize. Other interesting hypotheses include the idea there may be differences in microbiota with direct anti-*E. coli* effect(s), or that certain bacteria within the microbiota can create a nutrient environment that lends a survival advantage to O157:H7. Supporting the latter idea, O157:H7 EDL933 strain was shown to preferentially utilize gluconeogenic substrates in a simulated bovine intestinal tract environment, and elimination of this pathway resulted in loss of fitness (Bertin et al. 2014). Thus, as we strive to define the relationship(s) between diet, microbiota and STEC carriage in cattle, specific evaluation of the effect(s) of changing the intestinal metabolome on STEC shedding is also needed. These investigations ultimately may help elucidate feeding and/or probiotic strategies that consistently eliminate the supershedding phenomenon.

A number of different dietary alterations have been studied for their effect on reducing cattle colonization with STEC. These include altering foodstuff composition, adding probiotic organisms to the feed, and adding dietary supplements

such as plant polyphenols, citrus products, vitamin D, or antimicrobial growth enhancers. Three of the best-studied strategies include foodstuff manipulation, addition of direct-fed microbials to cattle diets, and use of the non-therapeutic, growth enhancing antibiotic, monensin.

Examples of foodstuff manipulations in cattle include changing grain proportion and type, grain processing methods, forage proportion and type, and/or introducing distiller's grains into the diet. In some studies, these manipulations altered STEC shedding, although in aggregate, the literature shows contradictory results (Jacob et al. 2009; Callaway et al. 2009; Wells et al. 2014; Smith et al. 2014). Unfortunately, there is no single dietary modification that consistently impacts shedding, and the mechanism(s) underlying effects of foodstuff manipulations are understudied. Possible explanations for inconsistencies across studies include heterogeneity in the colonizing strains, genetic or other variability in cattle populations under study, variability in exact composition of foodstuffs tested, natural variations in shedding patterns amongst cattle, specific environments in which the interventions were tested, and impact of natural colonization vs. deliberate pathogen challenge.

It should also be noted that almost all of these studies focused on reduction of O157:H7, and did not address effects on other serotypes. Thus, even for studies that revealed an impact on O157:H7 shedding, other highly pathogenic STEC of different serotypes may yet effectively colonize and be shed, even if O157:H7 is not. Elimination of a single pathogenic serotype may not be a true proxy for elimination of all HUS-causing organisms.

Direct-fed microbials (DFM) are probiotic strain(s) often used in cattle as growth enhancers. A number of different bacterial strains have been identified as being "probiotic" . The FAO/WHO Expert Committee has defined probiotics as "live microorganisms which, when consumed in an appropriate amount in food, confer a health benefit on the host" (Hill et al. 2014). Some of the mechanism(s) by which probiotics exert these effects include (1) competitive exclusion; (2) immune system modulation; (3) nutrient competition; (4) production of anti-bacterial agents such as bacteriocins, anti-biofilm products, or quorum-sensing molecules; (5) stimulation of host defensins; and (6) fortification of the intestinal epithelial barrier (Papadimitriou et al. 2015). For example, cattle colonization with O157:H7 is influenced by SdiA, a quorum-sensing molecule that responds to substances made by other bacterial species called acyl-homoserine lactones (AHLs). Loss of SdiA is thought to result in defective colonization because the organism cannot appropriately modulate virulence gene expression within the cattle gastrointestinal tract (Hughes et al. 2010; Sharma and Bearson 2013). It has been hypothesized that interruption of the AHL-dependent signaling pathway by "designer" probiotics or dietary additives may prevent colonization (Sperandio 2010).

The use of DFM to reduce shedding of *E. coli* O157 in cattle has been the subject of a recent systematic review and meta-analysis (Wisener et al. 2015). In this review, 16 publications were identified in which pre-harvest stage cattle exposed to the pathogen under natural conditions (not deliberate challenge) were fed probiotics while controls received placebo or no treatment. All but two of the trials

were published in peer-reviewed journals, and most were conducted on research farms located in North America. A number of different probiotic strains were used, primarily *Lactobacillus acidophilus (NP51), Lactobacillus* cristatus (NP45) and *Propionibacterium freudenreichii* (NP24), both alone and in combination. Three trials also included *E. coli* O157:H7 vaccine with probiotics, but had a probiotic-only treatment group. Although limited in power, this meta-analysis supports the conclusion that DFMs decreased shedding prevalence of *E. coli* O157 at the end of the trial period, and throughout the trial period. Whether this strategy will hold up in the setting of a commercial farm is not known. However, a significant impact in preventing human exposure may be realized, even if DFM feeding does not completely eliminate carriage, but is effective in preventing supershedding events.

The exact DFM composition, timing of application, and dose likely impact study results. For example, a novel DFM containing two Lactobacillus species and *Paenibacillus polymyxa* was recently determined to be effective in preventing O157:H7 colonization of cattle, but appeared to have a dosing threshold (Stanford et al. 2014a). It has also been speculated that DFM may be more helpful in preventing initial colonization with STEC, rather than eliminating STEC from the already colonized animal (Stanford et al. 2014b). Additional studies evaluating the effect of individual DFM on cattle microbiota and the ensuing host metabolomes may be helpful in identifying which DFMs have the greatest efficacy on reducing STEC colonization and at what time(s), as well as providing mechanistic insights. Effects of DFM on shedding of non-O157 serotypes are also being studied, which will determine whether this type of intervention will be broadly applicable to other potentially pathogenic STEC (Cernicchiaro et al. 2014; Paddock et al. 2014).

Subtherapeutic concentrations of antibacterials deemed not useful in treatment of human disease are allowed as growth-promoting agents in cattle. One of the best studied is monensin. An ionophore, monensin has minimal activity against Gram-negative bacteria (Aowicki and Huczynski 2013), nor does it appear to induce the SOS response *in vitro* (Kohler et al. 2000). Studies evaluating the effect of monensin on cattle shedding of O157:H7 have shown conflicting results (Paddock et al. 2011). Differences in monensin dosing, timing of application, and diet may be responsible for this disparity. In a recent study assessing risk factors associated with STEC shedding amongst Minnesota dairy herds, use of monensin had a protective effect in the multivariable logistic regression model (Cho et al. 2013). In controls not receiving monensin, the odds ratio for fecal shedding was 4.8, with a confidence interval of 2.5–9.3, compared to monensin-fed animals. It should be noted that this study used shedding of all STEC as an outcome measure, rather than shedding of O157:H7 or LEE-encoding STEC only. Whether monensin will prove to be a useful feed additive, and if so, at what dosing and application timing, requires further study. Similarly, mechanism(s) by which monensin may exert a protective effect require further study, including evaluation of its potential overall effect on cattle microbiota, as it does have activity against a broad range of Gram-positive bacteria (Aowicki and Huczynski 2013).

Effect of Microbiota and Probiotics on Human Susceptibility to STEC Infection and Its Complications

Given the known protective effects of probiotics already discussed, the composition of an individual's intestinal microbiota may impact what occurs following STEC exposure. Following ingestion, pathogens such as O157:H7 must regulate expression of genes important to colonization and virulence, while encountering the resident bacteria, their metabolites and other cell-signaling products such as quorum sensing molecules. These factors may result in modification of STEC gene expression reviewed in (Barnett 2013).

For example, conditioned media from human fecal microbiota inhibited Stx2 synthesis following activation of the antibiotic-mediated SOS response (de Sablet et al. 2009). Further testing revealed that individual bacterial species comprising the fecal microbiota shared this capability. *Bacteroides thetaiotamicron*, in particular, was highly active in inhibiting Stx2 protein expression. The inhibitory factor(s) were retained in filtrates of *B. thetaiotamicron*-conditioned medium subjected to a 3000-MW cutoff. However, this activity could not be related to either autoinducer-3 (AI-3) or SdiA-dependent quorum-sensing mechanisms. Iversen et al. (2015) recently assessed the effect of *B. thetaiotamicron* co-culture and conditioned media on global gene expression of a highly virulent Stx2-producing, LEE-containing STEC associated with an outbreak in Norway. Interestingly, a number of genes comprising the LEE pathogenicity island essential to formation of A/E lesions were upregulated under co-culture conditions, but not by conditioned medium, including the adhesion protein, intimin, and its receptor, Tir. Upregulation of a T3SS gene escU was also observed upon co-culture with the related species *Bacteroides fragilis*, but not with *Clostridium perfringens*. In contrast, both *B. thetaiotamicron* co-culture and conditioned medium resulted in downregulation of *stx2* gene expression and phage-associated genes, as well as decreased Stx2 protein, under phage-inducing conditions. It was hypothesized by Iversen et al. (2015) that the effect of commensals on LEE gene expression may enhance the ability of the pathogen to interact with host cells. It can be further speculated that since this effect was observed with two *Bacteroides species* but not *C. perfringens*, individual gut microbiota composition may have a significant effect on susceptibility to STEC-related disease.

A number of different probiotic bacteria have shown activity against one or more mechanisms thought to be important for STEC virulence in the human host, including probiotic *E. coli*, *Lactobacillus* species, and *Bifidobacterium* species. First described in 1918, the effects of the probiotic *E. coli* Nissle 1917 on STEC colonization resistance and virulence factor expression have been studied. In order to successfully colonize the human gastrointestinal tract, STEC must compete with commensal flora. In a mouse model of competitive exclusion, *E. coli* strains, including *E. coli* Nissle 1917, were evaluated for their relative capacity to prevent subsequent colonization with O157:H7 EDL933. Some strains were ineffective, but *E. coli* Nissle 1917 had a beneficial impact when used alone. When *E. coli* Nissle 1917

was used in combination with two other commensal strains, eventual elimination of EDL933 colonization was achieved (Leatham et al. 2009). Further investigation into this phenomenon implicates nutrient competition as a mechanism (Maltby et al. 2013; Schinner et al. 2015). Other studies have shown a beneficial effect of *E. coli* Nissle 1917 on STEC adhesion, growth, and Stx production (Rund et al. 2013; Reissbrodt et al. 2009; Mohsin et al. 2015).

Beneficial effects of multiple lactic acid-producing bacilli on one or more putative virulence activities of Shiga toxin-producing *E. coli* have been demonstrated; reviewed in (Lievin-Le Moal and Servin 2014). For example, *Lactobacillus rhamnosus* GG has been shown to decrease O157:H7 EDL933 Stx2 mRNA expression via direct contact (Carey et al. 2008). In a mouse model in which challenge with the O157:H7 strain 86-24 is lethal and Stx2-dependent, co-inoculation of animals with O157:H7 86-24 and *L. reuteri* ATCC PTA 6475 resulted in suppression of weight loss, decreased cecal luminal fluid accumulation, and reduced renal tubular necrosis. In this model, the beneficial effect of *L. reuteri* appeared to be distinct from simple outcompetition by the probiotic, and was not observed with *B. thetaiotamicron* (Eaton et al. 2011).

Protection from O157:H7-induced disease with *Bifidobacterium* species has been described in murine models as well (Asahara et al. 2004; Gagnon et al. 2006; Fukuda et al. 2011, 2012). The mechanism appears to involve acetate production, with resultant protective effects on the intestinal epithelium; this mechanism can be reproduced by feeding a prebiotic acetylated starch alone (Fukuda et al. 2011, 2012).

"Designer" *E. coli* probiotics expressing Stx receptor mimics on their surfaces have been developed and tested in animal models (Paton et al. 2000, 2001a, b; Hostetter et al. 2014). Oral administration of live and killed probiotic prevented fatal infection after O157:H7 challenge in mice (Paton et al. 2000, 2001b). In contrast, administration of a probiotic with a Stx2e receptor mimic in a piglet model of HUS decreased intestinal toxin levels, but not clinical disease (Hostetter et al. 2014).

The Effect of Diet, Natural Compounds, and Zinc on Human STEC Infection

Different types of dietary fatty acids have been studied for their effects on STEC growth and pathogenesis, as well as cattle colonization (Harrison et al. 2013). Different fatty acids appear to have different effects. As described above, acetate appears to have a beneficial effect. In contrast, another major intestinal short chain fatty acid synthesized by microbiota, butyrate, has been shown to enhance LEE gene expression and flagella synthesis in O157:H7 (Tobe et al. 2011; Nakanishi et al. 2009), and the mechanism by which this occurs is currently being elucidated (Takao et al. 2014).

Butyrate may also have host effects that contribute to pathogenesis. Cytotoxicity following exposure to Shiga toxins is mediated by interaction with the major cell receptor, globotriaosylceramide (Gb3). It has long been appreciated that butyrate upregulates cell surface expression of Gb3 (Jacewicz et al. 1995). As high fiber diets can alter intestinal butyrate levels, the effect of a high fiber diet on STEC virulence in mice has been studied (Zumbrun et al. 2013). Animals receiving different amounts of fiber in their diet had differences in intestinal microbiota. Specifically, the high dietary fiber-fed animals had reductions in commensal *Escherichia* species. These animals also had an increase in butyrate levels, and increased expression of Gb3 on intestinal tissue and kidney tissue. The mice fed a high fiber diet were more susceptible to colonization, morbidity, and mortality after STEC infection, compared with the low fiber diet controls. Although not conclusive, results of this study revealed that dietary-induced microbiota changes can upregulate Gb3, possibly through a butyrate-dependent mechanism, with ensuing detrimental effects on the infected host.

A number of different natural compounds have also been tested for their ability to modulate STEC-related illness. These compounds have included plant and fruit products, tea components, and spice extracts (Friedman and Rasooly 2013). Some of these compounds inhibited Shiga toxin production or activity. For example, Takemasa et al. (2009) determined that *in vitro*, eugenol from allspice suppressed Stx production, but did not affect O157:H7 growth. Both grape extracts and fresh apple juice had anti-Stx effects (Rasooly et al. 2010; Quinones et al. 2009). Compounds that inhibit production/toxicity of Stx in the intestine could be beneficial by limiting the amount of toxin available for systemic uptake, as well as reducing STEC colonization fitness.

Some data have revealed that Stxs may promote STEC colonization; reviewed in (Mohawk et al. 2010). In a murine model of STEC infection in which animals were challenged with the O157:H7 strain 86-24 vs an isogenic *stx2* deletion mutant, the strain that could not produce Stx2 did not colonize as well as the Stx2-producing strain (Robinson et al. 2006). Oral supplementation with purified Stx2 restored the defect (Mohawk et al. 2010). *In vitro*, the isogenic mutant strain was defective in microcolony formation on HEp-2 cells. However, this activity could be rescued by the addition of purified Stx2. A mechanism involving Stx effects on host cellular nucleolin has been postulated. In light of these findings, dietary supplements that limit Stx production and/or activity in the intestine may be useful in preventing disease.

Zinc is beneficial in prevention and treatment of diarrheal disease in general, hence this micronutrient's effects on STEC and on host responses thought to be important in STEC pathogenesis have also been studied. Zinc decreased production of LEE-encoded proteins, inhibited basal and antibiotic-induced Stx expression *in vitro*, and blocked damage from Stx-producing strains in rabbit ileal loops (Crane et al. 2011). In an intestinal epithelial cell model, zinc protected intestinal epithelial cell monolayers from peroxide-induced damage, preventing Stx translocation. Zinc also blocked the H_2O_2- and ciprofloxacin-induced SOS response (Crane et al. 2014).

Dietary acquisition of a specific sialic acid, alpha-2-3-linked N-glycolylneuraminic acid (Neu5Gc), may affect host susceptibility to certain STEC strains that produce

the toxin SubAB. SubAB is an AB5 toxin, unrelated to the Stxs. SubAB enzymatically cleaves the master regulator of endoplasmic reticulum (ER), BiP, resulting in ER stress (Paton et al. 2006; Wolfson et al. 2008). Highly toxic to eukaryotic cells, studies in animal models suggest that it may have a role in human pathogenesis, causing an HUS-like syndrome when injected into mice (Wang et al. 2007). Interestingly, SubAB preferentially binds to glycans that terminate in Neu5Gc (Lofling et al. 2009). Neu5Gc cannot be synthesized by humans but can be acquired through dietary sources such as red meat or dairy products and incorporated into human cell membranes. It is hypothesized that higher levels of Neu5Gc in human cells may result in increased sensitivity to SubAB, thus diet may influence susceptibility to and/or severity of disease caused by SubAB-producing STEC.

Summary

STEC are a heterogeneous group of microorganisms, with varying potential to cause human disease. The essential virulence factor causing severe disease sequelae, the Stxs, are encoded on lambdoid phages. Antibiotic therapy can trigger the bacteriophage lytic cycle and is thought to increase HUS risk, precluding antibiotic treatment in the diarrheal phase of illness. The mobile nature of these genetic elements allow for dissemination of *stx* genes amongst *E. coli* and other permissive Gram–negative bacteria. As was seen in the German HUS outbreak caused by a Stx2-producing *E. coli* organism with an enteroaggregative phenotype, emergence of pathogens with enhanced disease potential can occur unexpectedly and with disastrous results.

Studies using *in vitro* and *in vivo* models of human disease have revealed that diet, natural compounds, intestinal microbiota and probiotics may influence STEC pathogenicity, but none of these has been tested in human disease. Due to the sporadic nature of human infection and heterogeneity of infecting organisms, it is difficult to assess any intervention aimed at prevention of human colonization, prevention of disease, or prevention of sequelae such as HC and HUS. Due to continued concerns that antibiotic therapy may worsen infection, management of STEC-mediated disease is generally limited to supportive care. As yet, specific medical therapies to prevent HUS following STEC infection are investigational.

A great step forward in preventing human exposure would be identification of measures that mitigate colonization of the animal reservoir. Unfortunately, there are no known animal husbandry techniques that are consistently effective, and insight into the critical determinants of cattle colonization and shedding are needed.

Until we can master our present limitations in the areas outlined above, our armamentarium against this family of pathogens will likely remain inadequate. In conjunction with consumer-initiated food safety practices, important public health interventions such as post-harvest testing, early outbreak recognition with subsequent food recalls, and chlorination of drinking water remain important interventions to prevent STEC-associated HUS.

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The Role of Alternative Sigma Factors in Pathogen Virulence

Mark Roberts, Gary Rowley, Jan Kormanec, and Maria Elisabeth Johanna Zalm

Abstract Alternative sigma factors enable bacteria to change the promoter specificity of the core RNA polymerase to enable the expression of genes that give them advantages in particular situations. The number of alternative sigma factors that bacteria produce varies greatly. Some bacteria, particularly those that reside in the soil have genes for multiple sigma factors. The soil living gram positive bacteria *Sorangium cellulosum* currently holds the record for the number of sigma factor genes at 109. Alternative sigma factors play important roles in the life cycle of many foodborne bacterial pathogens. In this review we will discuss: the structure and function of alternative sigma factors; the different families of alternative sigma factors; their regulation; the role of particular alternative sigma factors and the genes they control in the biology (particularly pathogenesis) of foodborne bacterial pathogens.

Keywords Alternate sigma factor • Pathogen virulence • Foodborne bacterial pathogens • *Campylobacter* • *Yersinia* • *E. coli* • *Salmonella* • *Vibrio* • *Clostridium* • *Staphylococcus* • *Bacillus*

Introduction

Bacteria in their natural habitat are exposed to various stresses. This is also the situation for foodborne pathogens during host infection and during food preparation. All these stresses elicit production of a common group of proteins, the so-called general stress response proteins which have non-specific, essential, protective

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function under stress. In addition, stress-specific proteins are induced that have a protective function against a single stimulus. The responses to these stresses are mediated in many bacteria in part by alternative sigma factors of RNA polymerase, which direct expression of stress genes. The general stress response has been most well studied in two model organisms, *Escherichia coli* and *Bacillus subtilis.* In Gram-negative *E. coli*, general stress response is governed by alternative sigma factor σ^S, which belongs to group 2 sigma factors. Interestingly, in Gram-positive *B. subtilis* and other related bacteria, the general stress response is governed by an alternative group 3 sigma factor σ^B that is sequentially different from *E. coli* σ^S, although both sigma factor regulons contain many homologous genes (Hengge-Aronis 2002; Price et al. 2002; Hecker et al. 2007).

Characterization of Sigma Factors

Transcription, with the RNA polymerase as a principal enzyme, is the first and key step of gene expression and a target of extensive regulation. The process of transcription is divided into three steps: initiation, elongation and termination. During the initiation, RNA polymerase holoenzyme containing a sigma factor recognizes the promoter DNA to form the transcriptionally inactive closed complex which isomerizes to the transcriptionally active open complex with DNA strands separated around the transcription start point (TSP) of the promoter. The holoenzyme of RNA polymerase in this open complex is ready for RNA synthesis (Fig. 1) (Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014).

Sigma factors are key regulatory subunits of RNA polymerase conferring promoter specificity. After binding to the catalytic core RNA polymerase (composed of the β and β´ subunits, two α subunits, and a ω subunit), sigma factors specifically recognize critical promoter elements, which thereby determine promoter specificity of the holoenzyme of RNA polymerase and thus directing expression of a specific set of genes (so-called regulon of the corresponding sigma factor). In addition, sigma factors have another essential function in the transcription initiation process; they initiate strand separation of the double-helical DNA to form a transcription bubble around TSP. Although specific recognition of promoters is mediated by the sigma factor, sigma factors alone are not able to bind promoters. The promoter elements-binding domains are not in the optimal positions in a free sigma factor, they are likely blocked by interactions of its domains forming a compact structural conformation. These domains are optimally spaced by a change of its structural conformation after binding the core RNA polymerase (Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014; Paget 2015).

In principle, the sigma factor dissociates from the holoenzyme of RNA polymerase after the initiation process (Fig. 1). Therefore, the bacterial pool of free core RNA polymerase is available for the competitive binding of various sigma factors, thus reprogramming gene expression for the actual bacterial needs. Due to this competitive step of regulation, the most critical step in almost all sigma factors is the

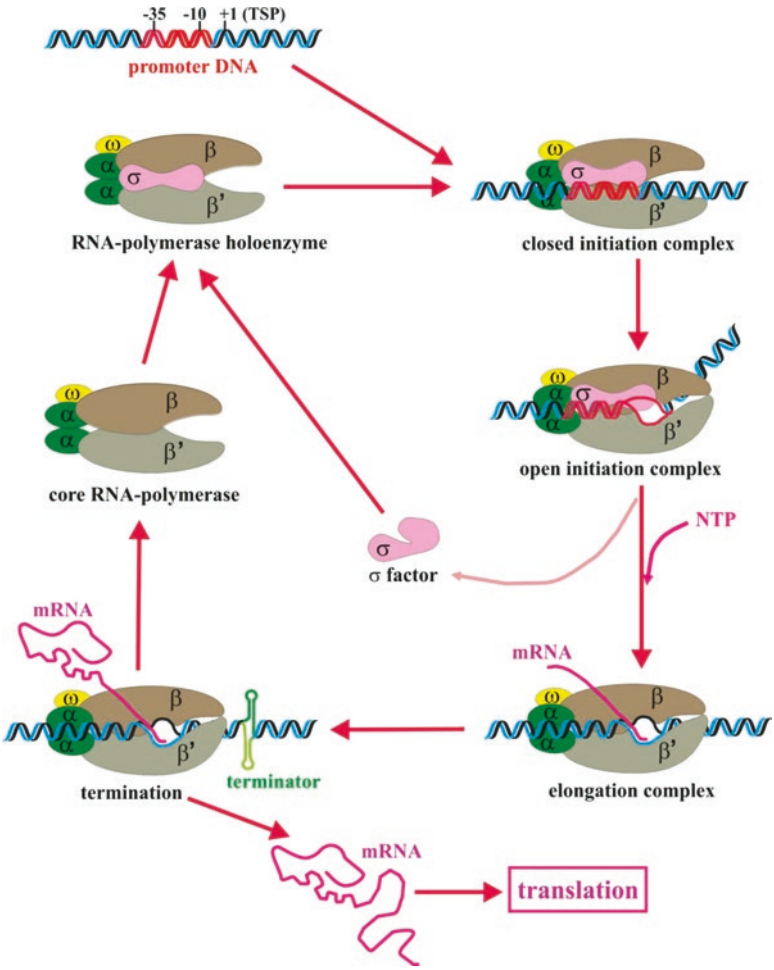

Fig. 1 Cycle of RNA polymerase in the process of transcription

regulation of their levels, which takes place at the transcriptional, translational, and post-translational level. Transcriptional regulation takes place by directing transcription of the given sigma factor genes by promoters that are recognized by different sigma factors, or the sigma factor it encodes. Translation regulation is often accomplished by inhibition or activation of translation of mRNA through the action of specific factors. One frequent mechanism of the posttranslational regulation is the reversible interaction with their cognate negative regulators, anti-sigma factors, which sequestere them from their interaction with the core RNA polymerase. After a variety of signals, this complex is released, making sigma factor free for its association with the core RNA polymerase to direct transcription of a set of cognate genes (Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014; Paget 2015).

All bacteria contain an essential so-called primary sigma factor, directing expression of house-keeping genes and one or more non-essential alternative sigma factors having diverse roles (e.g. responding to stress stimuli, surviving adverse conditions, cell differentiation). Complexity of the cell life corresponds to the number of sigma factors. For instance, the obligate extracellular pathogen *Mycoplasma pneumoniae* has only the primary sigma factor. The genetically-best studied organisms *Escherichia coli* and *Bacillus subtilis* contain 7 and 18 different sigma factors respectively, and the differentiating *Streptomyces coelicolor* contains 65 different sigma factors. Interestingly, some obligate pathogenic bacteria have lost several sigma factor genes after adaption to their host, for instance *Mycobacterium tuberculosis* which can live also outside of its host possesses 13 different sigma factors. Whereas the related but strictly host-dependent *Mycobacterium leprae* has lost many sigma factor genes, and possesses only 4 functional sigma factor genes. Likewise, the Gram-positive pathogen *Staphylococcus aureus*, which is related to the differentiating soil bacterium *B. subtilis*, possesses only 3 functional sigma factors. In general, bacterial species living in a complex habitat (like soil), where they are exposed to a variety of conditions and competing microbial flora (and also often undergo a complex differentiation program), have more sigma factors that those than are obligate pathogens or commensal species (Heimann 2002; Gruber and Gross 2003; Ghosh et al. 2010).

Sigma Factor Families

Sigma factors can be classified into two functionally, structurally, and phylogenetically distinct families, σ^{70} and σ^{54} (based on their representatives in *E. coli*) (Gruber and Gross 2003). The σ^{70} family is dominant and versatile. Sequence alignments of proteins of the σ^{70} family revealed four evolutionary conserved regions, 1 to 4, which can be further subdivided into subregions (Lonetto et al. 1994; Paget and Helmann 2003). Structural data revealed that proteins of this family consist of four-helical domains ($\sigma_{1.1}$, σ_2, σ_3, σ_4) that are connected by highly flexible linkers. Evolutionary conserved regions roughly coincide with the σ structural domains (Fig. 2). All members of this family contain at least two conserved domains, with subdomains $\sigma_{4.2}$ and $\sigma_{2.4}$ directly recognizing -35 and -10 promoter elements respectively, although the consensus sequences and spacing differ for the sigma factors belonging to particular groups(Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014; Paget 2015).

Based on the structural and functional similarities, the σ^{70} family is further subdivided into four groups (Fig. 2). Group 1 contains essential primary sigma factors, closely related to σ^{70} of *E. coli*, which direct expression of most housekeeping genes and which contains four conserved domains ($\sigma_{1.1}$, σ_2, σ_3, σ_4) separated by flexible linkers. The sigma factors of group 2, 3 and 4 are termed alternative sigma factors. Group 2 contains non-essential sequential homologues of primary sigma factors, lacking the $\sigma_{1.1}$ domain. Several of them are involved in responses to a broad range

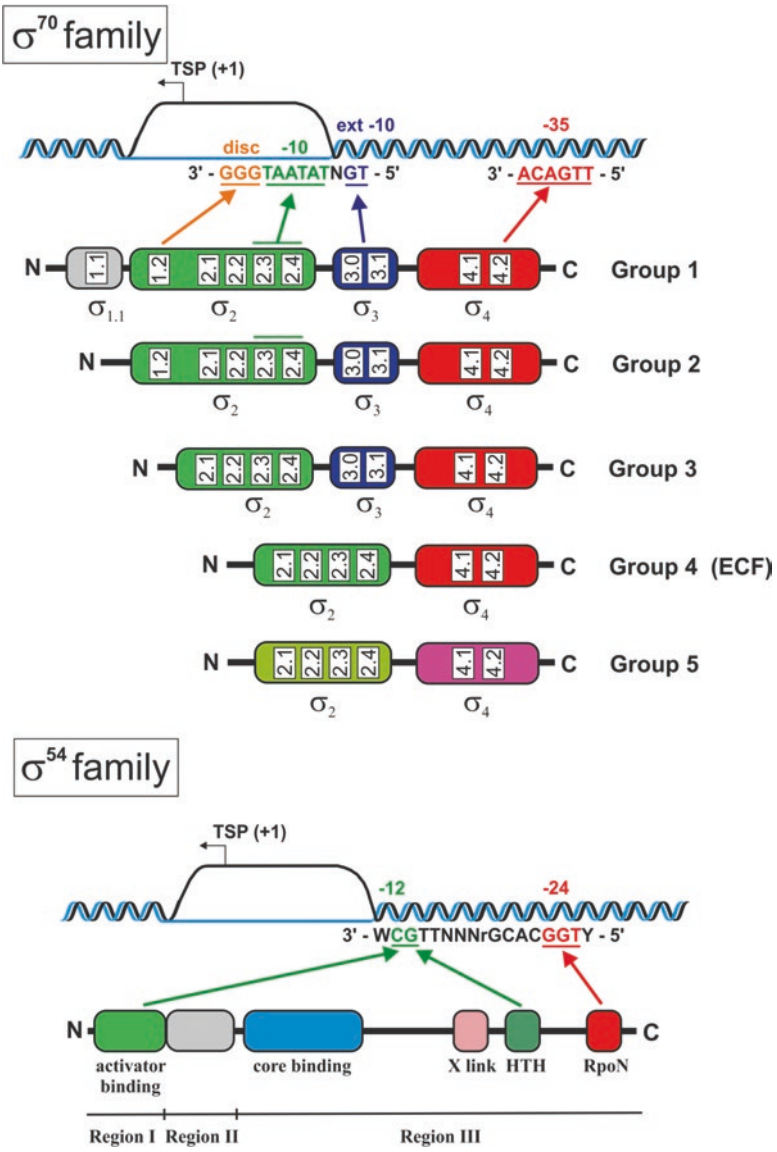

Fig. 2 Domain structure of sigma factors and interactions with the particular promoter elements

of stresses. The best-known representative, *E. coli* σ^S (σ^{38}), is responsible for the general stress response and survival during stationary phase. Group 3 comprises structurally and functionally diverse alternative sigma factors containing only three domains (σ_2, σ_3, σ_4). This group is further subdivided into four phylogenetically-distinct and partially functionally-related subgroups: 1, flagellar sigma factors, (with representative *E. coli* σ^F), 2, heat shock sigma factors (with representative

E. coli σ^H), 3, general stress response sigma factors (with representative *B. subtilis* σ^B) and 4, sporulation sigma factors (with representatives *B. subtilis* σ^F, σ^G, σ^E, σ^K). Group 4 comprises distantly related extracytoplasmic function (ECF) sigma factors and contains only two domains (σ_2 and σ_4). This group is the largest and most diverse at the primary sequence level, consisting of 43 phylogenetically distinct sub-groups. The members of this group are largely involved in the sensing and responding to signals from outside of the cells or in the envelope stress response (Gruber and Gross 2003; Staroń et al. 2009; Feklístov et al. 2014; Paget 2015).

An additional sigma factor specific for the genus *Clostridium*, has been assigned in some reports as group 5. This group is distantly related to the ECF sigma factors, but significantly differs in structure and function from this group 4. This group is represented by four sigma factors, TcdR, TetR, BotR, and UviA from several pathogenic *Clostridium* species. They all are significantly similar to each other, recognize very similar promoters, and are also functionally interchangeable. They also contain only two domains (σ_2, σ_4) and all representatives of this group direct expression of toxin and bacteriocin genes, which are located immediately downstream of the sigma factor genes (Mani and Dupuy 2001; Heimann 2002; Dupuy and Matamouros 2006).

The σ^{54} (or σ^N) family is structurally and functionally distinct from the σ^{70} family.

In contrast to transcription initiation that is mediated by sigma factors of the σ^{70} family, σ^{54}-dependent transcription absolutely requires hydrolysis of ATP, catalyzed by an activator protein bound to an upstream activator sequence (UAS). These characteristics are reminiscent of eukaryotic RNA polymerase II which is activated by transcription factors bound to upstream enhancer elements. Members of this family contain two highly conserved domains (Region I and III), with DNA-binding activities directly recognizing −24 and −12 promoter elements, separated by a flexible linker (Region II), (Fig. 2) (Studholme and Buck 2000; Zhang and Buck 2015). Recent structural analysis of RNA polymerase-σ^{54} holoenzyme revealed that σ^{54} contains four structural domains connected by long coils and loops.

Region I is comprised of two α-helices, region II also contains two α-helices and the core-binding domain (part of region III) consists of two α-helices. This domain is followed by a long loop and extra-long α-helix (called ELH), followed by the HTH domain involved in interaction with the −12 promoter element. The last most conserved domain (RpoN) is comprised of a three helical bundle and it interacts with the −24 promoter element (Yang et al. 2015). Although this family is mainly characteristic of the Gram-negative bacteria, the members of this family were found also in some Gram-positive bacteria (e.g. σ^L in *Bacillus subtilis*), spirochetes, thermophiles, and other bacterial species. While initially identified for their role in nitrogen metabolism (σ^N in *E. coli*), members of this family have been found to control many other physiological processes, like utilization of alternative carbon sources, control of detoxification systems, motility, biofilm formation, and influence the virulence of several bacterial pathogens (Studholme and Buck 2000; Zhang and Buck 2015).

Role of Sigma Factor in Recognition of Promoters During Transcription

Sigma factors play a critical role in transcription initiation, including recognition of promoters, isomerization of the closed complex to the open complex, as well as initial steps in RNA synthesis. However, the process of transcription initiation by RNA polymerase holoenzyme containing sigma factors of the σ^{70} and σ^{54} family is distinct (Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014; Paget 2015).

The RNA polymerase holoenzyme with σ^{70} recognizes promoters that contain promoter consensus sequences at DNA positions −35 and 10 from TSP (at +1). The initial transcriptionally inactive closed complex can spontaneously isomerize to form the transcriptionally active open complex, in which DNA strands are separated around TSP and holoenzyme of RNA polymerase is ready for RNA synthesis (Fig. 1) (Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014; Paget 2015).

Distinct sigma factors of the σ^{70} family, belonging to the individual groups 1–5, recognize distinct bacterial promoters, which direct expression of a regulon of the particular sigma factor. However, each member of the σ^{70} family recognizes and binds to the conserved −10 and −35 promoter element, which are directly recognized by the subdomains $\sigma_{2.4}$ and $\sigma_{4.2}$, respectively (Fig. 2), although the consensus sequences and spacing differ for the sigma factors belonging to particular groups. For instance, *E. coli* polymerase holoenzyme containing primary sigma factor σ^{70} (or σ^{D}) recognizes and binds to the consensus sequences TTGACA at the −35 element and TATAAT at the −10 element, and the spacing 17 ± 1 bp is crucial for transcription initiation. Primary sigma factors from other bacterial species recognize promoters with highly similar consensus sequences (Gruber and Gross 2003; Ghosh et al. 2010; Feklistov 2013; Feklístov et al. 2014; Paget 2015). The *E. coli* alternative sigma factor σ^{S} (belonging to the group 2) recognizes highly similar promoters, due to the extensive sequence similarity of the DNA binding domains with the primary sigma factor σ^{D}. However, there are some slight differences in the promoter recognition between these two sigma factors; the first T residue in the −10 element (TATAAT) is not so strict in the σ^{s}-dependent promoters, and a C residue located just upstream the −10 element is specific for this sigma factor (Landini et al. 2013). The *B. subtilis* alternative general stress response sigma factor σ^{B} (belonging to the group 3) recognizes the consensus sequences GTTTAA – $N_{12–14}$ – GGGTAT (Petersohn et al. 1999). And the *E. coli* alternative ECF sigma factor σ^{E} (belonging to the group 4) recognizes highly conserved consensus sequences GGAACTT at the −35 element and GTCNAA at the −10 element with a strict spacing 15 bp (Rezuchova et al. 2003; Rhodius et al. 2005).

Recent biochemical and structural data revealed the detailed mechanism of promoter recognition and open complex formation mediated by several sigma factors. Bacterial housekeeping promoters, recognized by the *E. coli* σ^{D} primary sigma factor, are characterized by four elements, the −35, extended −10, −10, and

discriminator element, directly recognized by a specific subdomain in the sigma factor. The first stage of transcription initiation is the formation of the closed complex between RNA polymerase holoenzyme and the double stranded promoter DNA (Fig. 1). The −35 element is recognized in a double strand form by helix-turn-helix DNA-binding subdomain $\sigma_{4.2}$. The extended −10 element is recognized also in its double strand form by a long α-helix of the σ_3 domain. Interactions of sigma factors with this extended −10 element stabilizes the initiation complex to such an extent that the otherwise essential −35 element is not required for this type of promoters. This complex undergoes dramatic conformational changes with a bending of DNA, resulting in destabilization of an AT-rich region around the −10 element followed by unwinding 13 bp of the duplex DNA to form open complex that is stabilized by aromatic residues of the σ_2 domain (Fig. 1). The template strand subsequently becomes loaded into the RNA polymerase active site channel ready for RNA synthesis. In contrast to recognition of the −35 element, the −10 and discriminator elements are recognized upon strand separation as a non-template single strand by σ_2 (Fig. 2). Previously it was suggested that subdomain 2.4 in σ_2 is responsible for a direct interaction with the −10 element in its double strand form, and after opening the region around this element, the non-template strand bases are directly recognized by the conserved aromatic residues in the subdomain 2.3. However, later structural and biochemical analysis revealed that the −10 element recognition and initiation of strand separation are coupled. Although alternative sigma factors have not been studied as intensively as the primary sigma factors with regards to the biochemical and structural aspects (only structural analysis of the *E. coli* σ^E with the −35 DNA region has been performed), the overall process of promoter recognition and opening is similar. However, in contrast to housekeeping sigma factors, the group 3 and 4 alternative sigma factors of the σ^{70} family are more sequence specific in the recognition of the −10 and −35 cognate promoter elements with more rigid spacing. Their weakened melting capacity is responsible for this increased specificity, resulting in smaller regulons (Borukhov and Severinov 2002; Murakami and Darst 2003; Feklistov 2013; Feklístov et al. 2014; Paget 2015).

The sigma factors of the σ^{54} family have a distinct mechanism of promoter recognition and activation compared to the sigma factors of the σ^{70} family. RNA polymerase holoenzyme containing σ^{54} binds to a promoter at −24 (GG) and −12 (TGC) consensus sequence upstream TSP (Fig. 2) to form a closed complex, which remains inactive for transcription unless activated by a specific transcription activator protein that belong to the AAA protein family. These activators bind to a DNA site located approximately 150 bp upstream of the promoters (knowns as upstream activating sequences) and use the energy derived from the ATP hydrolysis to isomerize this closed complex to an open complex ready for transcription. Each σ^{54}–specific activator protein is controlled by its own signal transduction pathway, thus allowing tight regulation of σ^{54}-dependent transcriptional response to a wide range of physiological needs (Ghosh et al. 2010; Bush and Dixon 2012; Zhang and Buck 2015). The recent structural data on the holoenzyme of RNA polymerase with σ^{54} revealed the detailed mechanism of promoter recognition. The region I domain plays an inhibitory role and contains sites for interactions with activator proteins. It interacts

with the Region III domain to block entry of the template strand of promoter DNA to the active site of RNA polymerase. Also the first part of the region II (domain RII.1) penetrates deeply into the DNA binding channel to block the transcription initiation process. The interaction of activator protein together with the hydrolysis of ATP is required to unblock these inhibitory interactions and enable the opening of the DNA bubble around the −12 region (Yang et al. 2015).

Alternative Sigma Factors of Gram-Positive Bacteria: Their Regulation and Role in the Pathogenesis of Foodborne Pathogens

Bacillus subtilis σ^B

In contrast to the Gram-negative bacteria in which the general stress response is governed by the alternative group 2 sigma factor σ^S, in the Gram-positive *B. subtilis* and other related bacteria, the general stress response is governed by an alternative group 3 sigma factor σ^B. This sigma factor has been most well characterized in the best-studied representative of Gram-positive bacteria, *B. subtilis*. The gene encoding σ^B (*sigB*) is located in an operon together with other genes (*rsb*) encoding regulators of its activity (Fig. 3). In unstressed cells, the basal expression of the operon is governed by the P_A promoter, which is recognized by RNA polymerase containing the primary sigma factor σ^A. On exposure to stress conditions, the operon is autocatalytically activated by means of the P_B promoter, which is recognized by RNA polymerase containing σ^B (Fig. 3). In *B. subtilis*, the activity of σ^B is tightly controlled by a partner-switching mechanism that utilizes phosphorylation to alter binding specificities of the proteins involved in σ^B activation. These regulatory proteins involve the RsbW anti-sigma factor, the RsbV anti-anti-sigma, and interplay of various protein kinases and phosphatases, which integrate signals from two different stress inputs: energy stress (such as carbon, phosphorus and oxygen starvation), and environmental stress (such as acid, ethanol, heat or salt stresses) (Fig. 3). In unstressed cells, σ^B is held in an inactive state by the anti-sigma factor RsbW, which contains a serine protein kinase HATPase_c domain specific for its antagonist anti-anti-sigma factor RsbV. The phosphorylated RsbV-P cannot interact with RsbW anti-sigma factor. However, after stress activation, one of two PP2C-type phosphatases (RsbU in the case of environmental stress, and RsbP during energy stress) is activated and subsequently dephosphorylates anti-anti sigma factor RsbV-P. Dephosphorylated RsbV can interact with RsbW, thus releasing σ^B from the RsbW/σ^B complex to interact with core RNA polymerase. The resulting RNA polymerase holoenzyme can direct expression of about 200 genes (5% of the genome), whose products confer general stress response to the cell. In the case of energy stress, phosphatase RsbP requires the activator RsbQ, and the induction of the RsbP/RsbQ phosphatase activity coincides with a drop in

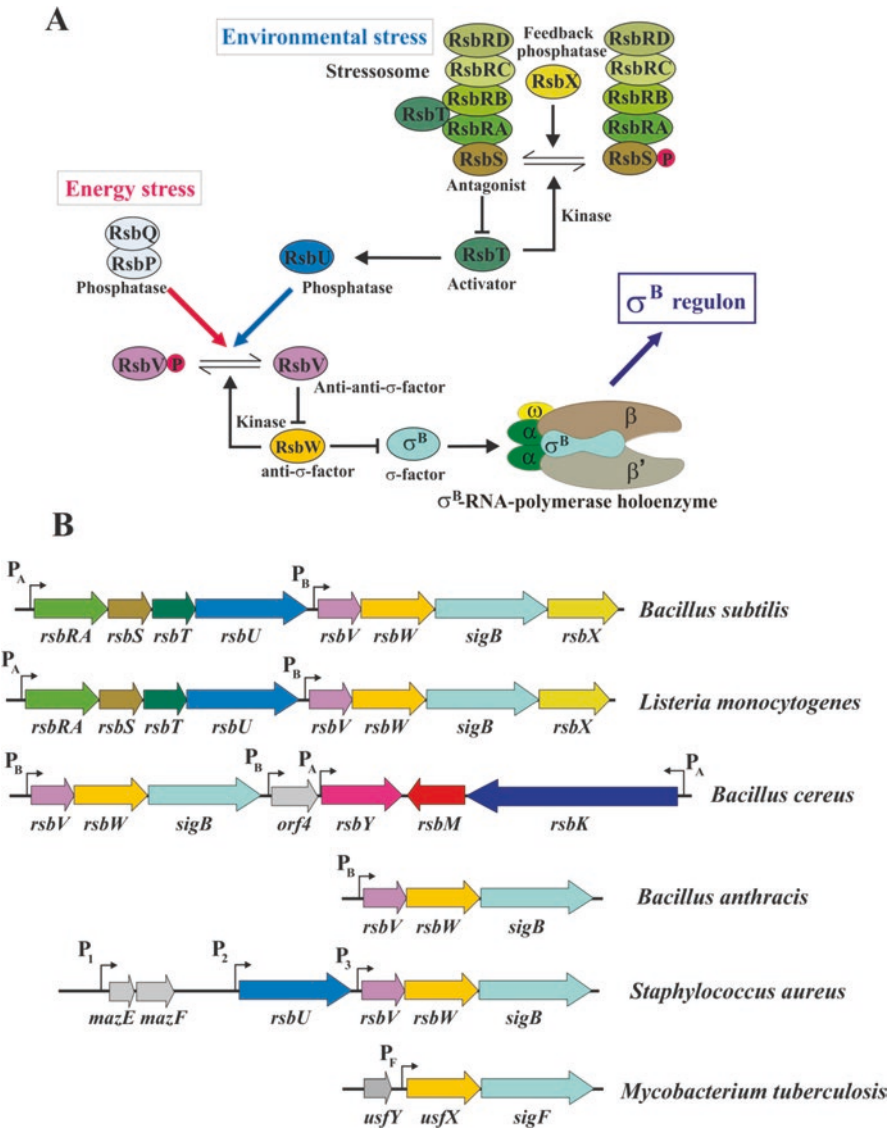

Fig. 3 (**a**) Regulation of *B. subtilis* σ^B by phosphorylation partner-switching mechanism under stress conditions (Price et al. 2002; Hecker et al. 2007; Eymann et al. 2011). The lines ending with an *arrow* or a perpendicular line indicate positive or negative regulation, respectively. See text for further details. (**b**) Genetic organization of the *sigB* cluster in *B. subtilis* and other Gram-positive bacteria. *Thick arrows* denote the positions of genes and *bent arrows* represent the positions of promoters

the cellular ATP level, although the molecular mechanism is unknown. Activation through the environmental stress response pathway is controlled by a large complex, called as the stressosome, which contains multiple copies of the paralogous co-antagonists RsbRA, RsbRB, RsbRC, RsbRD, and the RsbS antagonist, which together bind the RsbT activator kinase. Activation of the stressosome by various

environmental stresses, which is mediated by the different phosphorylation patterns of the RsbR paralogues, leads RsbT kinase activation and the phosphorylation of RsbS. This results in the release of the RsbT activator from the stressosome complex. Subsequently, the released RsbT can activate the RsbU phosphatase to remove phosphate from RsbV-P. Feedback control to ensure reversibility of the process after overcoming the stress conditions is mediated by the RsbX phosphatase, which dephosphorylates RsbS-P (Price et al. 2002; Hecker et al. 2007; Eymann et al. 2011).

More than 150 genes have been identified as belonging to the *B. subtilis* σ^B regulon, most of them encoding proteins with unknown function. However, a significant proportion of the genes encoded proteins involved in oxidative stress resistance, osmotic stress response, heat stress resistance, antibiotic resistance, cold stress resistance, cell envelope function, and proteins from various branches of metabolism. Moreover, phenotypic analysis of mutants in the majority of σ^B-dependent genes with unknown function revealed the have a role in single or multiple stress resistance. These results indicate the main function of σ^B is in nonspecific, multiple, and protective stress resistance (Price et al. 2002; Hecker et al. 2007; Nannapaneni et al. 2011).

σ^B is conserved in diverse Gram-positive bacteria, however, the conservation of its regulators varies, indicating divergence of its function and regulation in the different Gran-positive bacterial species. A *sigB* operon of eight genes is conserved in other close related *Bacillus* strains (*B. licheniformis, B. balodurans, B. clausii*) and also in *Listeria monocytogenes* (Fig. 3) (Hecker et al. 2007). However, a *sigB* operon of the foodborne pathogen *B. cereus* contains genes for only two primary regulators of σ^B activity, RsbV and RsbW, and two other genes, *orf4* and *rsbY*, with the later encoding a predicted regulator of σ^B with a response regulator receiver domain and PP2C phosphatase domain (van Schaik et al. 2004a). Likewise, another pathogenic *Bacillus* strain, *B. anthracis*, possesses a *sigB* operon with only two central regulatory genes, *rsbW* and *rsbW* (Fouet et al. 2000). The *sigB* operon of another related pathogenic strain, *Staphylococcus aureus,* in addition to *sigB* only has the genes for the principal regulators RsbU, RsbV, and RsbW (Senn et al. 2005).

σ^B also occurs in the more distantly related mycobacteria and streptomycetes. In *Mycobacterium tuberculosis* the *sigB*-homologous gene, *sigF*, is co-transcribed with a gene encoding its anti-sigma factor UsfX, homologous to RsbW. However, a gene encoding a homologue of an anti-anti-sigma factor is missing. Like in *B. subtilis*, the operon is autoregulated through the *usfXp* promoter. However, the σ^F activity is regulated by two anti-anti-sigma factors, genes of which are located in different positions of the *M. tuberculosis* genome (Fig. 3) (Beaucher et al. 2002). In contrast to *B. subtilis*, the *sigF* gene has no significant role in stress resistance, but it has a major role in adaptation to stationary phase and virulence of *M. tuberculosis*. The *M. tuberculosis sigF* mutant attenuated in several infection models (Manganelli 2014). Interestingly, in the Gram-positive filamentous soil bacteria of the genus *Streptomyces*, the situation is much more complex. Nine close homologues of σ^B are present in *S. coelicolor*, some of their genes are also in an operon with its anti-sigma factor (homologue of RsbW). However, the genome of *S. coelicolor* contains dispersed genes for 45 homologues of RsbW, 17 homologues of

RsbV, and 44 homologues of PP2C phosphatases RsbU/RsbP. Characterization of these nine σ^B homologues revealed their dominant role in the control of morphological differentiation and osmotic stress response, with several sigma factors having a dual role in these processes. Extensive and complex regulatory interactions determine the activities of these sigma factors (Kormanec et al. 2016). On the contrary, the σ^B-dependent general stress response system does not occur in strictly or facultative anaerobic Gram-positive bacteria (e.g. genera *Clostridium*, *Streptococcus*, and *Lactococcus*) (Hecker et al. 2007).

In addition to the general stress response σ^B, *B. subtilis* contains 7 different ECF sigma factors, σ^M, σ^V, σ^W, σ^X, σ^Y, σ^{Ylac}, and σ^Z, which are mainly involved in cell wall biosynthesis, envelope stress responses and responses to specific environmental stresses. Several of them also contribute to resistance against antibiotics (Souza et al. 2014). *B. subtilis* also contains a homologue of σ^{54} family, σ^L, which has been found to be important for cold shock adaptation (Wiegeshoff et al. 2006).

Bacillus cereus Alternative Sigma Factors

Bacillus cereus is an endospore-forming foodborne pathogen ubiquitous in the environment with a high capacity to adapt to different environmental niches. Its main reservoir is soil, and food can serve as a vehicle to transfer the pathogen to the host. *B. cereus* causes food poisoning resulting in various illnesses due to production of several toxins, such as the heat-stable emetic toxin cereulide that causes vomiting and several heat-labile enterotoxins (e.g. tripartite hemolysin BL) that causes diarrhea. The spores of *B. cereus* can survive many stresses applied during food production, making them difficult to eliminate as contaminants. Surviving spores germinate into vegetative cells, which can also cope with different stressful conditions, such as osmotic stress, heat shock, cold shock, pH stress and oxidative stress (Schoeni and Wong 2005).

The *sigB* operon of *B. cereus* contains only two genes that encode primary regulators of σ^B activity, RsbV and RsbW (Fig. 3). The *B. cereus* genome lacks all other genes involved in the regulation of σ^B in *B. subtilis* (*rsbQ,-R, −S, −T, U, −X*). The *rsbV, rsbW, sigB, orf4* operon is autoregulated by σ^B through the P_B promoter (Fig. 3) and is induced mainly by heat shock and less so by osmotic shock, ethanol stress and entry into the stationary phase. However, an additional σ^B-dependent heat shock inducible promoter was identified upstream the *orf4* gene (van Schaik et al. 2004a). This σ^B-dependent protein is not involved in σ^B regulation in *B. cereus*. However, it functions in response to environmental stress as a Dps-like bacterioferritin and can provide cell protection from oxidative damage through iron sequestration and DNA binding (Wang 2002). Functional analysis revealed that RsbW and RsbV regulate activity of σ^B similarly to as they do in *B. subtilis*. However, instead of utilizing an RsbU-type phosphatase, the activation of σ^B in *B. cereus* requires a different sensing protein, RsbY, containing an N-terminal CheY response regulator receiver domain and C-terminal PP2C serine phosphatase domain. This phosphatase is activated by

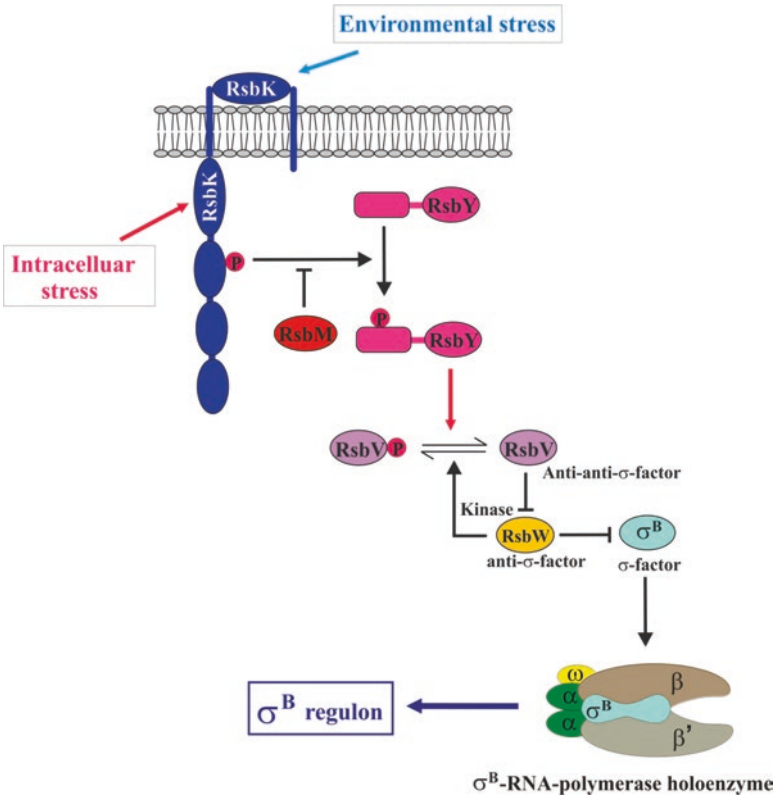

Fig. 4 Regulation of *B. cereus* σ^B by phosphorylation partner-switching mechanism under stress conditions (Chen et al. 2012). The lines ending with an *arrow* or a perpendicular line indicate positive or negative regulation, respectively. See text for further details

phosphorylation by a novel hybrid transmembrane sensor histidine kinase RsbK containing several sensory domains, encoded by a *sigB* operon nearby gene. Therefore, the *B. cereus* σ^B-activation pathway is markedly different (Fig. 4). In contrast to *B. subtilis*, environmental and intracellular stress signalling routes are combined into a single protein RsbK, which phosphorylates the RsbY response regulator receiver domain, thus activating its PP2C phosphatase domain. Subsequently, it dephosphorylates RsbV anti-anti-sigma factor to activate σ^B by sequestering its anti-sigma factor RsbW (van Schaik et al. 2004a, 2005b; de Been et al. 2010). Moreover, the other gene in this cluster, *rsbM*, encodes a specific methyltransferase of RsbK and functions as negative regulator of σ^B in the absence of stress (Chen et al. 2012).

The regulation of σ^B in *B. cereus* indicates involvement of this sigma factor in the stress response in this strain and deletion of the *sigB* gene in *B. cereus* affects heat stress resistance (van Schaik et al. 2004a). Unexpectedly, the mutant was hyper resistant to oxidative stress, this was due to upregulated expression of the *katA* catalase gene (van Schaik et al. 2005b). Moreover, interestingly the *sigB* deletion also

affects spore resistance and germination in this strain (de Vries et al. 2005). However, the activities of several critical virulence factors were not affected in this mutant (van Schaik et al. 2005a).

Two approaches were used to identify σ^B the regulon. In contrast to *B. subtilis*, the σ^B regulon of *B. cereus* is considerably smaller (only 24 heat inducible σ^B-dependent genes) and the overlap with the σ^B regulon of *B. subtilis* is rather small. Only eight genes are common in both strains. Proposed functions of the *sigB*-dependent genes in *B. cereus* indicate a role in metabolic rearrangements in response to stress. Moreover, two identified σ^B-dependent genes have a predicted function in spore germination, thus confirming the phenotype of the *sigB* mutant. As already mentioned, σ^B does not control expression of the main virulence factors in *B. cereus* (van Schaik et al. 2004b, 2007). All of the data indicate that σ^B is likely not directly involved in the pathogenicity of *B. cereus.* In contrast, in the closely related pathogenic species, *B. anthracis* (the etiological agent of anthrax), a s*igB* mutant displayed reduced virulence in a mouse model, indicating the involvement of σ^B in the pathogenicity of this strain (Fouet et al. 2000).

However, the other sigma factor σ^N (of the σ^{54} family) is involved in a large range of cellular processes such as growth at low temperature and anaerobic conditions, motility and biofilm formation and toxin production in *B. cereus*. Production of a non-hemolytic enterotoxin and expression of several other virulence factors, including the virulence regulator PlcR, was significantly down regulated in the *sigN* mutant of *B. cereus* compared to the wild-type strain, thus indicating its role in virulence. Therefore, σ^N is an additional sigma factor essential for adaptation to different environmental niches, ranging from soil, food processing environments and the human host (Hayrapetyan et al. 2015).

Listeria monocytogenes Alternative Sigma Factors

Listeria monocytogenes is an aerobic non-spore-forming Gram-positive, psychrotrophic, foodborne pathogen capable of transitioning from saprophytic survival in the environment to intracellular infection in a wide range of hosts, including humans. It is an etiological agent of uncommon but potentially fatal disease called listeriosis, ranging from mild diarrhea and flu-like symptoms to severe invasive infections. *L. monocytogenes* is wide spread in the environment, from where it can be transferred to the host by a contaminated food. Its notable ability to survive several food preservation stresses (e.g. low temperature, high osmolarity and low pH), makes *L. monocytogenes* of great concern to the food industry. After consuming contaminated food, *L. monocytogenes* can survive gastric passage and colonize the intestinal tract and possibly cause diarrhea. Subsequently, it can invade intestinal epithelia cells and M cells in Peyer's patches. This invasion is mediated by a family of more than six leucine-rich proteins, internalins (e.g. InlA, InlB, InlC), which interact with a host receptor E-cadherin. Following cell invasion, the acidic pH of the phagolysosome activates a bacterial exotoxin, listeriolysin O, and two different

phospholipases C (PlcA and PlcB), initiating release into cell cytosol, where the bacteria replicate and migrate to the eukaryotic cell membrane. This actin-based movement is mediated by another virulence factor, bacterial surface protein ActA. Subsequently, cells can disseminate from the lymph nodes to the spleen and liver (Vazquez-Boland et al. 2001; Gahan and Hill 2005; Chaturongakul et al. 2008; Disson and Lecuit 2013).

Among many factors contributing to stress survival and virulence of *L. monocytogenes* is the general stress-response sigma factor σ^B, which has been extensively studied in this strain (Hecker et al. 2007). The *L. monocytogenes sigB* operon is identical to that of *B. subtilis* (Fig. 3). As in *B. subtilis*, the *sigB* operon in *L. monocytogenes* is autoregulated via a σ^B-dependent P_B promoter (Fig. 3) that is induced by several environmental stresses. However, in contrast to *B. subtilis*, σ^B is also activated by osmotic upshift and temperature downshift, with the highest activation after osmotic stress. This suggests a dominant role of σ^B in the osmotic stress response. A *L. monocytogenes sigB* mutant has substantial defects in its ability to accumulate osmoprotectants (Becker et al. 1998). Interestingly, σ^B also has a role in acid stress response, which might indicate a role in virulence by protecting *L. monocytogenes* from acid stress during stomach passage. However, the *L. monocytogenes sigB* mutant was only weakly impaired in its migration to the liver and spleen in a mouse model (Wiedmann et al. 1998). These and some additional studies indicate that σ^B is critically involved in adaptation of *L. monocytogenes* to osmotic, cold, acid, and oxidative stresses, high hydrostatic pressure and carbon starvation. However, although heat shock and ethanol induce σ^B, stationary-phase resistance to these stresses is likely σ^B-independent. The majority of these stress conditions are typical during food processing and also during infection of the gastrointestinal tract and in the phagosomes of phagocytic cells during intracellular infection (Wiedmann et al. 1998; Becker et al. 2000; Ferreira et al. 2001; Wemekamp-Kamphuis et al. 2004).

Several phenotypic studies have revealed that σ^B is a critical factor of *L. monocytogenes* virulence. Loss of σ^B results in a significantly reduced invasion efficiency of *L. monocytogenes* in two human intestinal epithelial cell lines, partially due to direct effect on internalin *inlA* gene transcription, but not on the virulence transcriptional regulator *prfA* gene transcription. Thus, σ^B plays a critical role in invasion of human host (Bignell et al. 2000; Kim et al. 2004; Garner et al. 2006). In preliminary virulence experiments, the *L. monocytogenes sigB* mutant was only weakly attenuated in the murine model (Wiedmann et al. 1998). Later on it was determined that murine E-cadherin does not interact with internalin, thus the model is not ideal for studying *L. monocytogenes* invasion and virulence. However, guinea pig E-cadherin does interact with internalin, thus the guinea pig is a better model to study certain aspects of *L. monocytogenesis* infection (Lecuit 2001). Accordingly, the virulence of the *L. monocytogenes sigB* mutant was greatly attenuated in intragastrically-inoculated guinea pigs, thus, indicating a critical role of σ^B during the gastrointestinal stage of listeriosis. Interestingly, the *sigB* mutant virulence was not attenuated in intravenously-infected guinea pigs, indicating that σ^B is likely not important for systemic spread of the pathogen (Garner et al. 2006). Moreover, like several other

bacteria, *L. monocytogenes* can form biofilms, and σ^B plays a role in their formation under dual stress conditions (viz., osmotic stress and heat shock) (Lee et al. 2014).

While the structure of the *sigB* operon is identical to that of *B. subtilis*, signal transduction cascades differ in both strains. The *L. monocytogenes* cascade lacks the energy-stress specific RsbQ/RsbP system (Fig. 3). Instead, both energy and environment stress activation of σ^B occurs through a single pathway, which includes RsbT, RsbU, RsbV, and RsbW (Chaturongakul and Boor 2004). However, environmental stresses (acid, antibiotics, cold, ethanol, heat, and osmotic) activates RsbU phosphatase in its upstream pathways through a stressosome complex, as in *B. subtilis* (Fig. 3), and the energy (nutritional) stress enters the network of σ^B activation downstream from RsbU, likely by diminishing the RsbW kinase activity (Shi et al. 2010). Moreover, in addition to activation by environmental and energy stresses by this RsbV-dependent pathway (Fig. 3), σ^B is activated after cold shock by an alternative RsbV-independent pathways in *L. monocytogenes* (Utratna et al. 2014).

Several studies employing different approaches were used to identify the σ^B regulon in *L. monocytogenes* under various stress conditions and strain lineages (Kazmierczak et al. 2003; Abram et al. 2008a, b; Raengpradub et al. 2008; Oliver et al. 2009; Ollinger et al. 2009; Mujahid et al. 2013; Ribeiro et al. 2014). More than 200 genes positively regulated by σ^B were identified, with the majority of them directly regulated by σ^B. The size of the *L. monocytogenes* σ^B regulon is larger than that of *B. subtlis* (about 150 genes). However, there is significant overlap between the general stress response genes of both regulons (Kazmierczak et al. 2003). As in *B. subtilis*, many of the identified σ^B-dependent genes have function in acid stress (e.g. genes of glutamate decarboxylase system), oxidative stress (e.g. a glutathione reductase gene), osmotic stress (e.g. genes for several solutes transport systems), and energy stress responses (e.g. genes of carbohydrate metabolism upregulated during stationary phase). Thus, σ^B-dependent proteins can provide protection against the environmental challenges that *L. monocytogenes* encounters during infection of the gastrointestinal tract (e.g. gastric acid stress or osmotic stress by bile salts).

Consistent with a critical role of σ^B in *L. monocytogenes* virulence, expression of several virulence and virulence-associated genes are under σ^B control. σ^B directly regulates expression of the *bsh* gene for bile salt hydrolase and the *bilE* gene for a bile-exclusion system that are important for virulence of *L. monocytogenes* and (Dussurget et al. 2002; Kazmierczak et al. 2003; Sleator et al. 2004), and σ^B plays a major role in tolerance to bile salts, thus indicating its crucial role in *L. monocytogenes* survival in the bile salts environment of the gastrointestinal tract prior to its colonization, invasion and intracellular propagation (Zhang et al. 2005, 2011). σ^B together with the global virulence gene regulator PrfA regulate the expression of the critical invasion factors the internalins. They contribute differentially in the regulation of eight *inl* genes. Both contribute to *inlA* and *inlB* transcription, PrfA contributes to *inlC* transcription, and σ^B to *inlC2* transcription (McGann et al. 2007).

A tight interaction occurs between σ^B and the main global regulator of virulence genes, PrfA. One of its promoters is under the control of σ^B and some PrfA-dependent genes are also under control of σ^B (Nadon et al. 2002; Kazmierczak et al.

2003; Sleator et al. 2004; Schwab et al. 2005). PrfA is the master regulator of *L. monocytogenes* virulence gene expression directing expression of genes encoding proteins involved in cell entry and intracellular survival (e.g. *hly, mlp, plcA, plcB, actA*). To avoid unnecessary expression in the environment, the PrfA regulon is selectively activated during host cell infection (de las et al. 2011). This interaction indicated a dominant role of PrfA as a direct regulator of virulence genes critical for invasion and intracellular survival, whereby σ^B regulated a wider range of virulence and stress response genes (Ollinger et al. 2008). σ^B, not PrfA, appears to play the major role in human host cell invasion, probably by controlling expression of *inlA* and *inlB*. After cell invasion has completed, PrfA, not σ^B, is the major virulence gene regulator, because the PrfA expression level is strongly induced in intracellular *L. monocytogenes*, whereas the σ^B expression level is not (Kazmierczak et al. 2006). In addition, PrfA, but not σ^B contributes to *L. monocytogenes* hemolytic activity, whereas σ^B, but not PrfA, contributes to intestinal epithelial cell invasion (Ribeiro et al. 2014). Moreover, σ^B downregulates PrfA activity in intracellular *L. monocytogenes*, thus moderating expression of PrfA-dependent virulence genes and thereby reducing host cell damage incurred by these virulence factors (Ollinger et al. 2009). Therefore, this interaction between σ^B and PrfA influences transmission of *L. monocytogenes* during both the gastrointestinal and systemic stages of infection and appears to be critical for appropriate expression of virulence genes, important for the early stages of listerial infection. Confirming these results, a systematic transcriptional profiling of *L. monocytogenes* in different *in vitro* and *in vivo* conditions revealed adaptation mechanisms of this pathogen during transition from the environment to the infected host. σ^B specifically controls expression of genes important for adaptation to the intestinal environment, whereas PrfA is required in survival and replication in blood (Toledo-Arana et al. 2009). In conclusion, the σ^B-dependent general stress response of *L. monocytogenes* provides the pathogen with a multiple, nonspecific stress resistance that is essential for survival in the natural ecosystem, during the gastrointestinal stages of infection, and during food processing. In addition, in contrast to the *B. subtilis* σ^B general stress response, *L. monocytogenes* σ^B is involved in the regulation of critical virulence genes, suggesting that it was adapted to facilitate host-pathogen interaction and host cell invasion.

The genome *of L. monocytogenes* contains genes for three additional alternative sigma factors, σ^H, σ^C and σ^L. The homologue of the *B. subtilis* sporulation and stationary phase specific sigma factor σ^H plays a role in infection in *L. monocytogenes*. Moreover, *L. monocytogenes sigH* mutant has reduces growth in a minimal medium and in alkaline conditions compared to the wild-type strain. The ECF sigma factor σ^C likely plays a role in response to specific stresses. It is activated upon heat shock and a *sigC* mutant is sensitive to heat. No virulence or virulence-associated phenotype have been found for this sigma factor. The σ^{54} family sigma factor σ^L has a role in intracellular replication and stress response. It contributes to osmotolerance and influences susceptibility to antimicrobial peptides. The *L. monocytogenes* σ^L regulon is comprised of genes encoding proteins with diverse cellular function, including protein synthesis, nutrient transport, energy metabolism, cell envelope synthesis and motility (Chaturongakul et al. 2008, 2011; Mattila et al. 2012).

Staphylococcus aureus Alternative Sigma Factors

The Gram-positive bacterium *Staphylococcus aureus* is a versatile human pathogen with the ability to cause a variety of diseases, ranging from gastrointestinal intoxication and superficial cutaneous infections to life-threatening illness including bacteremia, toxic shock syndrome, endocarditis, and osteomyelitis. It colonizes the nares and skin of about one-third of all healthy population. However, this organism, due to its high ability to acquire various resistance determinants, has emerged as a major pathogen for nosocomial- and community-acquired infections. *S. aureus* is not only an extracellular pathogen, but can also invade a wide variety of mammalian cells. The ability of *S. aureus* to cause such diverse infections is linked to its great repertoire of virulence factors, including adhesins, immunomodulatory molecules, and enterotoxins. Its ability to produce a variety of heat-stable enterotoxins makes *S. aureus* a common agent of foodborne diseases, manifested by nausea, vomiting, diarrhea and abdominal pain (Murray 2005; Kadariya et al. 2014; Tong et al. 2015).

Its capacity to cause such a wide spectrum of disease and survive in various unfavorable conditions encountered during the infection process is attributed to a network of global regulatory factors controlling the expression of virulence determinants, enabling it to rapidly sense changes in both the intracellular and extracellular environment, and to respond appropriately. These elements comprise two-component regulatory systems, including the *agr* locus with effector molecule RNAIII, the SarA protein family, and alternative sigma factors, including the general stress response sigma factor σ^B (Cheung et al. 2004).

Compared to its *B. subtilis* counterpart, the *S. aureus sigB* operon is only partially conserved, containing, in addition to *sigB*, genes for its principal regulators *RsbU*, *RsbV*, and *RsbW*. Expression of the operon is governed by three promoters differentially activated during growth *in vitro*; P_1 and P_2 are active mainly in exponential phase, and P_3 mainly in stationary phase. P_1 and P_3 are also induced by heat shock and osmotic stress, and the operon is autoregulated by σ^B through the P_3 promoter (Fig. 3) (Senn et al. 2005). In contrast to other *sigB* operons, in *S. aureus* two upstream genes *mazE* and *mazF* encoding toxin-antitoxin module are co-transcribed with the *sigB* operon (Fig. 3). The P_1 promoter is essential for full σ^B activity and is downregulated by this sigma factor, thus providing a negative-feedback control. In addition, this promoter is also directly activated by a virulence regulator SarA. This P_1 promoter responds to environmental and antibiotic stresses in a way that provide additional levels of *sigB* expression control (Donegan and Cheung 2009). The genome of *S. aureus* lacks considerable part of the σ^B regulatory proteins of *B. subtilis*, including stressosome components RsbR, RsbS, and RsbT, as well as the energy-sensing branch, comprised of RsbP and RsbQ. In concert with this, depletion of the cellular ATP pool and ethanol stress failed to activate σ^B in *S. aureus*. However, salt stress, alkaline shock, and heat shock activated σ^B. Despite these differences, the core of the partner switching mechanism between σ^B, RsbW, and RsbV appears to be functional in *S. aureus* and the activation of σ^B depends upon RsbU in response to nutritional and physical stresses. However, in contrast to *B. subtilis*, σ^B is present also during the exponential phase with a peak at the late

exponential phases and significant decrease thereafter. Also, RsbU is active also in the exponential phase and it is not clear yet how this activity is regulated in *S. aureus*. Moreover, in contrast to *B. subtilis*, it seems that *S. aureus* RsbU does not need a stress stimulus to be activated and some additional unknown regulators are needed for σ^B regulation (Senn et al. 2005; Pané-Farré et al. 2006, 2009).

Several proteomics and transcriptomics analyses were used to identify the σ^B regulon in several strains of *S. aureus* under various conditions. More than 250 genes are positively or negatively regulated by σ^B, with most directly positively regulated by σ^B. The gene products of the regulon are involved in many cellular processes, including cell envelope biosynthesis and turnover, intermediary metabolism, membrane transport, and signalling pathways. Interestingly, the overlap between the *S. aureus* and *B. subtilis* σ^B regulon is rather small and only about 10% of them have orthologues in *B. subtilis*, suggesting that the function of σ^B regulon is different in both bacteria. In addition, expression of several genes for virulence factors are influenced by σ^B, suggesting a role for this sigma factor in *S. aureus* pathogenesis. σ^B positively influences expression of a number of adhesins (e.g *clfA* for fibrinogen-binding clumping factor and *fnbA* for fibronectin-binding protein A) and negatively influences expression of numerous virulence-associated exoenzymes and toxins (e.g *hla* for α-hemolysin, *seb* for enterotoxin B and *spc* for staphylokinase) (Gertz et al. 2000; Bischoff et al. 2004; Homerova et al. 2004; Pané-Farré et al. 2006). However, many virulence factors are indirectly regulated by σ^B through its influence on the expression of major virulence regulators. The level of RNAIII (produced from the *agr* locus) is negatively regulated by σ^B (Gertz et al. 2000; Bischoff et al. 2001, 2004; Homerova et al. 2004). This key virulence regulator is repressed in early exponential phase and activated upon entry into stationary phases (where σ^B is downregulated) and positively regulates expression of a number of extracellular virulence factors (lipases, proteases, toxins) (Gertz et al. 2000; Bischoff et al. 2001, 2004; Homerova et al. 2004; Novick and Geisinger 2008) that are also downregulated by σ^B (Bischoff et al. 2004), thus explaining this indirect regulation. Also, several cell wall-associated adhesion proteins that are positively regulated by σ^B are under the control of another virulence regulator, SarA, whose expression is under the direct positive control of σ^B. Likewise, σ^B positively regulates the virulence regulators SarS and ArlRS (Cheung et al. 2004). σ^B also play a role in regulation of the *tst* gene for toxic shock syndrome toxin 1 that is a major causative agent of this potential fatal illness, toxic shock syndrome. The *tst* gene is strongly repressed by σ^B via at least two distinct global virulence regulators SarA and *agr* (Andrey et al. 2015). The other recently found σ^B-dependent virulence regulator, SpoVG, is required for capsule formation, antibiotic resistance, and secretion of excreted virulence factors, thus explaining similar phenotypes of the *S. aureus sigB* mutant (Schulthess et al. 2009, 2012; Andrey et al. 2015).

Despite the clear impact of σ^B on the regulation of several virulence factors and global virulence regulators, the initial phenotypic and functional analyses of the *S. aureus sigB* mutation revealed no significant impact of this sigma factor on virulence of *S. aureus* in several model systems. Instead, the *sigB* mutation affected heat shock, acid shock, and hydrogen peroxide resistance, but it was not required for

starvation survival, indicating its role in environmental stress response. Moreover, the *S. aureus sigB* mutant showed drastic reduction in methicillin resistance (Wu et al. 1996; Chan et al. 1998; Nicholas et al. 1999; Bakker et al. 2012). However, later, σ^B was found to play a role in virulence in a *S. aureus* murine septic arthritis model (Johnson et al. 1991; Jonsson et al. 2004), thus indicating that the contribution of σ^B to *S. aureus* pathogenesis may depend upon the infection model system. σ^B also plays a role in the virulence of *S. aureus* in a central venous catheter related murine model of multiorgan infection (Lorenz et al. 2008). σ^B also contributes to *S. aureus* binding to fibrinogen and fibronectin *in vitro* and to have a transient effect in the early stages of infection in a rat model of experimental endocarditis, but it was lost in later phases during progression. These results indicated a primary role of σ^B in attachment to host tissue allowing *S. aureus* to persist without harming the host by supressing exotoxin production (Entenza et al. 2005). The other process requiring expression of adhesion molecules is internalization of *S. aureus* by bone-forming osteoblasts. σ^B is also involved in this process (Nair et al. 2003). The other way σ^B can affect the virulence of *S. aureus* is by controlling biofilm formation, thought to be a significant virulence factor. There were several contradictory studies on the role of σ^B in biofilm formation in *S. aureus*, later explained by a detailed study clearly implicating σ^B in biofilm formation(Lauderdale et al. 2009).

In addition to extracellular pathogenesis, *S. aureus* is also able to invade a variety of mammalian phagocytes and survive engulfment by macrophages. Upon phagocytosis by macrophages, *S. aureus* persists intracellularly in vacuoles before escaping into cytoplasm and causing host cell lysis. This survival is dependent upon σ^B, as well as the global regulator Agr, but not SarA, and may serve as a vehicle for the dissemination of infection (Kubica et al. 2008). *S. aureus* infectioncan develop from an acute inflammatory to a chronic persisting stage. Its ability to cause chronic infections has been associated with bacterial host cell invasion and with an altered bacterial phenotype, the so called small-colony variants (SCVs), which are adapted for intracellular long-term persistence. In addition to its role in invasion, σ^B has a critical role also in formation of SCV and in adaptation of *S. aureus* during chronic infections to promote intracellular persistence. Therefore, in the course of the infection process *S. aureus* changes its response. In its acute infection stage, *S. aureus* requires the actions of the Agr and SarA global regulators which positively control expression of secreted virulence factors, such as α-hemolysin, to defend against invading phagocytes by causing inflammation and cytotoxicity and to escape from phagosomes in their host cells to produce an infection of high bacterial density. In contrast, to persist intracellularly *S. aureus* silences *agr* and *sarA* and activates *sigB*. This increased expression of *sigB* is accompanied by upregulation of adhesins and downregulation of toxins. Therefore, σ^B enables bacteria to switch from highly aggressive phenotype during acute infection to a silent SCV-phenotype that allows for long-term intracellular persistence. This specific role in this stage of pathogenesis is consistent with a role of σ^B to cope with the intracellular stress conditions (Mitchell et al. 2013; Tuchscherr et al. 2015).

In contrast to *B. subtilis*, which contains 17 alternative sigma factors, the genome of *S. aureus* contains, in addition to *sigB*, only two additional alternative sigma

factors, σ^H and σ^S. σ^H is a homologue of the *B. subtilis* sporulation and stationary phase specific sigma factor σ^H and is essential for competence of *S. aureus* for DNA transformation, which enable this pathogenic strain to acquire antibiotic resistance genes through horizontal gene transfer, resulting in multiresistant strains, such as MRSA (Morikawa et al. 2012). The ECF sigma factor σ^S is important for *S. aureus* survival during starvation, in the response to specific stresses, in the protection against DNA damage, cell wall disruption, and in interaction with component of the innate immune system. Moreover, this sigma factor plays a role in virulence of *S. aureus*. Using a murine model of septic arthritis, σ^S played a role in systemic infections. Mice infected with a *S. aureus sigC* mutant displayed weight loss, mortality, severity of infection, systemic dissemination, and immune response. Expression of the *sigC* gene is activated upon exposure to a variety of chemical stressors that elicit DNA damage or disrupt cell wall stability. Moreover, its expression is highly induced during growth in serum and upon phagocytosis by macrophages (Shaw et al. 2008; Miller et al. 2012).

Sigma Factors of Relevant Clostridial Foodborne Pathogens

The genus *Clostridium* comprises a large, heterogeneous group of Gram-positive, obligate anaerobic, spore forming bacteria that are widely distributed in various environments. Most of these species are saprophytic, however several are pathogenic to both humans and animals. Selected species of these bacteria cause a variety of diseases, including neuroparalysis, gas gangrene, necrotic enteritis, food poisoning, toxic shock syndrome and pseudomembraneous colitis, which in most cases, result following the production of potent exotoxins. This genus includes also three well-known foodborne pathogens, *C. perfringens*, *C. difficile,* and *C. botulinum*. *C. perfringens* is a common, although often under-reported, cause of food poisoning and causes a spectrum of diseases from self-limited gastroenteritis to an overwhelming destruction of tissues and gas gangrene. This pathogenic potential is attributed primarily to toxins produced by this organism. It also produces the antibacterial protein bacteriocin BCN5. *C. difficile* is a human intestinal pathogen most frequently involved in a spectrum of diarrheal illnesses, ranging from mild diarrhea to severe life-threatening pseudomembranous colitis, that are induced by antibiotic treatment that disrupt the normal gastrointestinal microbiota. Two large enterotoxins, TcdA and TcdB, are considered primary virulence factors responsible for this disease. *C. botulinum* is the etiologic agent of the severe foodborne neuroparalytic disease botulism caused by consumption of contaminated food with botulinum neurotoxin produced by this strain. The botulinum neurotoxin is the most potent substance known, with as little as 30 ng potentially fatal (Carter et al. 2010, 2014).

Toxin production by these bacteria is a very complex process involving quorum sensing regulatory systems, two-component bacterial signal transduction systems, and a new group 5 of alternative sigma factors specific for clostridia (Dupuy et al. 2006; Carter et al. 2014). The first of these sigma factors identified and characterized

in clostridia was TcdR of *C. difficile*. Its gene, *tcdR*, is located in close proximity with toxin genes *tcdA*, *tcdB*, and two other genes, *tcdE* (for a proposed toxin export) and *tcdC* (for a putative anti-sigma factor) in the pathogenicity locus PaLoc (Fig. 5), found only in toxigenic *C. difficile* strains. Sigma factor TcdR directly recognizes promoters directing transcription for toxin genes *tcdA* and *tcdB* and also its own transcription. The expression of *tcdR* is low during the exponential phase of growth and is induced after entry into the stationary phase of growth, and it is also repressed by glucose. It has been suggested that *tcdR* expression is induced by specific environmental signals (Mani and Dupuy 2001; Mani et al. 2002; Mand et al. 2013). In addition, a putative anti-sigma factor TcdC negatively regulates toxin synthesis by interfering with TcdR-containing RNA polymerase recognition of *tcdAp* and *tcdBp* promoters (Matamouros et al. 2007). This was further reinforced by isolation of a natural hypervirulent *C. difficile* epidemic strain with a mutation in the *tcdC* gene that may significantly increase toxin production (Carter et al. 2011).

The other member of this group, BotR, modulates neurotoxin expression in *C. botulinum*. The *botR* gene lies between two operons for the neurotoxin gene and genes encoding the associated nontoxic proteins having a protective effect on botulinum neurotoxin (Fig. 5). The first operon includes neurotoxin gene *bont* and the nontoxic nonhemagglutinin gene *ntnh*. The other operon contains three *ha* genes for nontoxic hemagglutinin proteins. Expression of these operons is tightly regulated and growth phase-dependent (induced after entry into stationary phase), and sigma

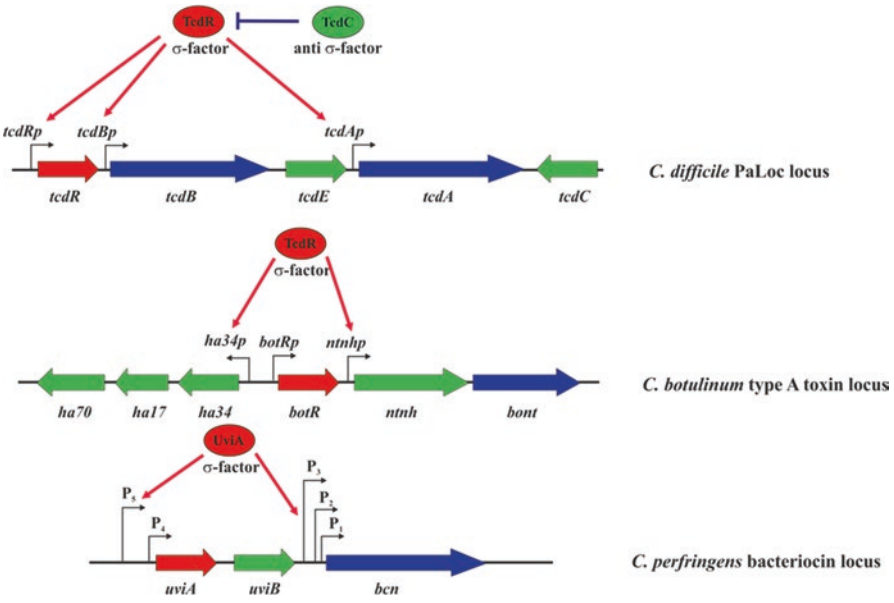

Fig. 5 Genetic organization of the toxin and bacteriocin loci for selected foodborne Clostridium strains (Carter et al. 2014; Dupuy and Matamouros 2006). *Thick arrows* denote the positions of genes and *bent arrows* represent the positions of promoters. The lines ending with an *arrow* or a perpendicular line indicate positive or negative regulation, respectively. See text for further details

factor BotR directly recognizes both promoters for these operons. In addition to this sigma factor, stationary phase global regulator CodY also positively regulates both operons by interacting with both promoters in regions distinct from that recognized by BotR in a GTP-dependent manner. Moreover, six two-component bacterial signal transduction systems have also been involved in this regulation, suggesting control of this expression by various environmental stimuli (Raffestin et al. 2004; Connan and Popoff 2015). No such group V sigma homologue has been identified to control toxin production in *C. perfringens*. Instead, a two-component bacterial signal transduction system VirR/VirS controls expression of the toxin genes (Dupuy and Matamouros 2006). However, a close homologue of this sigma factor group, UviA, controls expression of the UV-inducible gene-encoding antibacterial bacteriocin BCN5. The *uviA* gene is located in close proximity to the *uviB* gene, a proposed immunity protein, and the *bcn* gene which is for the bacteriocin BCN5 (Fig. 5). Transcription of *uviA* is directed by two promoters, of which one is autoregulated by UviA. Both promoters are induced after DNA damage. The *bcn* gene has three promoters directly recognized by the RNA polymerase containing UviA. As a result, bacteriocin BCN5 is induced by treatment with UV light or mitomycin C (Dupuy et al. 2005).

Alternative Sigma Factors of Gram-Negative Bacteria: Their Regulation and Role in the Pathogenesis of Foodborne Pathogens

RpoS in Gram Negative Foodborne Pathogens

In their natural environment, bacteria often encounter stress including changes in nutrition, pH, temperature and osmolarity. Stress response systems, often regulated by sigma factors, allow bacteria to quickly adapt to these challenges. The global stress response sigma factor σ^S (RpoS) was initially described as being upregulated upon entering the stationary phase of growth. However, it has since been determined that RpoS plays an important role in the regulation of a wide range of stresses, including glucose, amino acid, nitrogen and phosphate starvation, biofilm formation and changes in temperature, pH and osmolarity. In pathogens, RpoS plays a role in the regulation of a range of virulence factors, as well as protection against stresses encountered during the infection process (Battesti et al. 2011).

As is common practice for sigma factors, RpoS interacts with the core RNA polymerase to allow transcription of a wide range of genes. In *Escherichia coli*, approximately 10% of the genome is regulated, either directly or indirectly, by RpoS (Weber et al. 2005). The expression and activity of RpoS is strictly regulated at all levels; transcriptional, translational and post-translational, to ensure that sufficient RpoS is present to act instantaneously when encountering stress, while maintaining low RpoS activity during favorable growth conditions (Hengge-Aronis 2002; Battesti et al. 2011).

Transcriptional Regulation of *rpoS*

In most bacterial genomes, *rpoS* is found just downstream of *nlpD*, a gene encoding for a putative outer membrane lipoprotein of unknown function. The main *rpoS* promoter is located internal to *nlpD*, 567 nucleotides upstream from the *rpoS* start codon. This long 5′ untranslated region is critical for the regulation of *rpoS* translation (Lange and Hengge-Aronis 1994; Battesti et al. 2011). Direct promoter binding has been demonstrated for some regulators and alarmones, for other this interaction is hypothesized.

The *E. coli* response regulator ArcA-P negatively regulates *rpoS* transcription by binding directly to the promoter sequence (Mika and Hengge 2005). ArcA is part of the ArcAB two-component system involved in mediating *E. coli's* response to change in respiratory conditions, and it acts as a global regulator of gene expression under micro-aerobic and anaerobic conditions (Loui et al. 2009).

Another protein directly interacting with the *rpoS* promoter sequence, is the nucleoid associated protein Fis, a gene barely detectible at stationary phase, but rising to tens of thousands of dimers upon dilution of a culture into fresh media (Ball et al. 1992). Fis is a DNA binding and bending protein involved in various processes, including: (1) stimulation of excision and integration of Lambda (Ball and Johnson 1991a, b), (2) DNA replication at *oriC*, transposition (Gille et al. 1991) and (3) transcriptional activation and repression of genes (Auner et al. 2003). There are five putative Fis binding regions located directly upstream from *rpoS*. One of these binding sites is located at -50 bp upstream from *rpoS* and is of critical importance for RpoS regulation by the DNA-binding protein Fis. Although the exact mechanism of regulation is unclear, Fis is thought to negatively regulate *rpoS* expression by blocking access to the RNA polymerase binding site (Hirsch and Elliott 2005).

Furthermore, there are some systems thought to be involved in *rpoS* transcriptional regulation, but little is known about the exact mechanisms. The BarA/UvrY system, involved in the induction of siderophore-mediated iron acquisition and colonization of the urinary tract (Zhang and Normark 1996), has been implicated in both positive regulation (BarA) and negative regulation (UvrY) of *rpoS* transcription. It has been suggested that these proteins regulate *rpoS* expression independently of one another (Suzuki et al. 2002; Venturi 2003).

The *rpoS* promoter contains two putative cAMP-CRP binding sites, the first located just upstream from the -35 σ^{70} consensus sequence, the second located just a few nucleotides downstream from the +1 transcription initiation position (Hengge-Aronis 2002). Although cAMP-CRP negatively regulates *rpoS* expression, the mechanisms by which this occurs remain enigmatic (Venturi 2003).

The alarmone guanosine pentaphosphate (ppGpp) is involved in stabilizing RpoS (Bougdour and Gottesman 2007); however, it affects *rpoS* transcription indirectly. Increased levels of ppGpp have a positive influence on the accumulation of inorganic polyphosphate, which is synthesised by polyphosphate kinase and degraded by exopolyphosphatase. Overexpressing the latter does not affect ppGpp levels, but does reduce polyphosphate to barely detectable levels. Under these conditions, *rpoS-lacZ* transcriptions decrease substantially and RpoS levels fail to increase

upon entering stationary phase, suggesting that polyphosphates are important in the positive regulation of *rpoS* transcription (Gentry et al. 1993; Shiba et al. 1997; Venturi 2003).

Translational Regulation of RpoS

Translational regulation of *rpoS* encumbers an inhibitory structure; the long 5′ untranslated region of the *rpoS* mRNA transcript folds into a stem-loop that occludes the ribosome binding site and thus inhibits translation (Brown and Elliott 1997). This structural inhibition can be overcome by *trans*-encoded small RNAs (sRNA), which bind to their leader sequence within the hairpin region and force the hairpin structure open, allowing ribosomes to dock. Using this mechanism, translation of *rpoS* mRNA is positively regulated by at least three different sRNAs (Majdalani et al. 1998, 2002; Mandin and Gottesman 2010; McCullen et al. 2010), each expressed as a result of different types of stress. Translation is positively regulated by DsrA during growth at low temperatures (Repoila and Gottesman 2003), by RprA while responding to envelope stress (Majdalani et al. 2001), and by ArcZ for growth during aerobic-anaerobic stress (Mandin and Gottesman 2010). For interaction with their target mRNA, these sRNAs require interaction with the RNA chaperone protein Hfq, which stabilises the mRNA, in order to regulate *rpoS* mRNA translation. A Hfq-binding sequence present on the target mRNA, $(AAN)_4$, plays a direct and critical role in sRNA-mediated activation, possibly by stabilizing the Hfq-sRNA-mRNA complex (Brown and Elliott 1996; Soper and Woodson 2008; Soper et al. 2010). Hfq binding to the target mRNA also prevents degradation by RNase E, by competition for the target site 2 (Moll et al. 2003).

The sRNA OxyS, expressed in response to oxidative stress (Altuvia et al. 1997), down-regulates *rpoS* expression by a mechanism that is different from RprA, DsrA and ArcZ. Although the exact mechanism is unclear, it is thought to enhance cleavage by RNase E. Observed levels of OxyS cleavage by RNase E, indicate the presence of a cleavage site. Instead of protecting OxyS from cleavage, binding of Hfq to OxyS results in a conformational change which enhances cleavage by RNase E. It has been suggested that OxyS negatively regulates *rpoS* mRNA transcription by forming a ribonucleoprotein complex, consisting of OxyS, *rpoS* mRNA, Hfq and RNase E, and enhances mRNA degradation by recruiting RNase E and promoting cleavage (Henderson et al. 2013).

Various other potential regulators of translation have been suggested. Studies comparing RpoS levels in triple sRNA mutant strains versus *hfq* mutant strains, demonstrated a more attenuated phenotype for the latter, suggesting there may be more Hfq-dependent sRNAs involved in RpoS regulation (Mandin and Gottesman 2010). One of these sRNAs may be GcvB, a sRNA that is upregulated during acid stress that appears to positively regulate cellular RpoS levels by a yet unknown mechanism (Jin et al. 2009). Deletion of *E. coli pst* genes result in constitutive expression of the Pho regulon, which in turn suggests that *pst* encodes a negative regulator of *rpoS* (Ruiz and Silhavy 2003). The transcriptional regulator LrhA is

capable of repressing *rpoS* mRNA transcription in an Hfq-dependent manner, although the exact mechanisms remain enigmatic (Peterson et al. 2004).

RpoS Stability

To ensure a rapid response to environmental changes and cell stress, RpoS is always produced. However, during favorable conditions, RpoS is quickly degraded to prevent unnecessary activation of stress responses (Battesti et al. 2011). This process involves the ATP-dependent protease ClpXP, which does not recognize RpoS directly, but interacts via the adaptor protein RssB. During favorable growth conditions, RssB binds RpoS and targets it for degradation by the ClpXP pathway. When RpoS is delivered, RssB gets recycled, allowing it to interact with multiple RpoS molecules (Zhou et al. 2001). When the cell encounters stress, inhibitor of RssB activity proteins (Ira proteins) are produced. These anti-adaptor proteins bind to RssB, preventing the formation of an RssB-RpoS complex, resulting in rapidly increasing cellular levels of RpoS. There are several known anti-adaptors capable of binding RssB, each produced during different stress stimulants, thereby allowing for stress specific responses. In *E. coli*, the four anti-adaptors currently known to interact with RssB are IraD, IraM, IraP and the more recently discovered IraL (Bougdour and Gottesman 2007; Bougdour et al. 2008; Hryckowian et al. 2014).

RssB is a member of the response regulator family, and like most proteins from this family, it is comprised of a receiver domain and an effector domain (Galperin 2010; Micevski et al. 2015). These domains remain to be biochemically defined in RssB. Response regulator activity is usually regulated with a phosphorylation-mediated switch that regulates the activity of the effector domain (Gao and Stock 2010). Although RssB can be phosphorylated on a highly conserved aspartate residue (Asp58), the function of this phosphorylation remains enigmatic, as strains carrying a mutation in the phosphorylation site only induced a slightly increased level of RpoS stabilization suggesting that phosphorylation enhances RssB function, but is not critically required (Peterson et al. 2004). The mechanisms by which RssB binds RpoS, and delivers it to ClpXP also remain poorly understood (Hengge 2009; Micevski and Dougan 2013).

Thermolysin-mediated digestion of RssB revealed two stable fragments. Further analysis of these fractions revealed that the various Ira proteins interact with RssB in distinct manners, interacting with either or both fragments. Docking of the anti-adaptors is thought to result in a conformational change (Micevski et al. 2015). The *E. coli.*

RssB C-terminal domain contains a predicted coiled-coil region; the *Pseudomonas aeruginosa* full length RssB structure contains a coiled-coil region, which appears to mediate the formation of a homodimer, forcing RssB in an inactive conformation (Protein Data Bank [PDB]: 3EQ2). The coiled-coil region in the *E. coli* RssB is thought to serve a similar purpose. The mode of RssB inhibition by IraD and IraP are thought to involve stabilizing of the RssB homodimer, forcing it to remain in an inactive conformation, therefore stabilising RpoS (Bougdour et al. 2006; Micevski et al. 2015).

The anti-adaptor IraP was first discovered upregulated during phosphate starvation (Bougdour et al. 2006). Transcription of *iraP* happens independently of PhoR/PhoB, but instead its transcription is dependent on increased levels of the alarmone ppGpp, which binds to the *iraP* promoter and stimulates transcription (Bougdour and Gottesman 2007). Its expression is not solely dependent on ppGpp; production of this alarmone can be induced by either RelA or SpoT (Spira et al. 1995). The latter pathway is essential for activation of *iraP* during phosphate stress, whereas the former pathway does not induce *iraP* during phosphate stress. However, during amino acid starvation, another stress that results in ppGpp directed *iraP* expression, the roles are reversed; the RelA pathway is essential for *iraP* expression, whereas the SpoT pathway is not (Bougdour and Gottesman 2007). Cellular ppGpp levels are also elevated during nitrogen starvation and glucose starvation (Villadsen and Michelsen 1977; Metzger et al. 1989). During nitrogen starvation, IraP is involved in stabilizing RpoS, whereas RpoS stabilization occurs independently of IraP, during glucose stress (Peterson et al. 2004; Bougdour et al. 2006). Altogether, this suggests that although ppGpp is essential for *iraP* transcription, more regulatory factors are involved to fine-tune IraP cellular levels in response to different stresses.

The anti-adaptor IraM was identified in a screen for multicopy plasmids that led to mutator phenotypes, and was demonstrated to be an important regulator of RpoS stabilization during Mg^{2+} starvation in *E. coli* (Bougdour et al. 2008). Transcriptional activation of *iraM* requires PhoP, and IraM, is therefore, a connector of the PhoP/PhoQ and RssB/RpoS pathways (Bougdour et al. 2008). Interestingly, whereas *E. coli* utilizes IraM in response to magnesium starvation, *Salmonella* lacks *iraM* and instead utilizes IraP for both the phosphate and the magnesium starvation response (Tu et al. 2006). The *Salmonella* RssC anti-adaptor shares 40% identity with IraM, but is not involved in the magnesium response. The function of this *Salmonella* anti-adaptor remains enigmatic (Bougdour et al. 2008). It should be noted that not all *E. coli* strains carry a copy of *iraM* (Hryckowian et al. 2014). The *iraP* gene contains a second set of upstream promoters capable of responding to PhoP (Bougdour et al. 2008). Although this was previously considered an insignificant promoter, due to the strong ppGpp *iraP* promoter interaction, it is possible that *E. coli* strains lacking *iraM* also depend on PhoP induced IraP during magnesium stress, in a similar manner to *Salmonella*.

The anti-adaptor IraD was identified in the same screen as IraM, and was highly up-regulated in cells treated with hydrogen peroxide, independently of OxyR (Zheng et al. 2001; Bougdour et al. 2008). An independent study also revealed hypersensitivity to DNA damage in *iraD* mutant strains. The predicted mechanism of RpoS stabilization by IraD, is by sequestering RssB away from RpoE. This mechanism was predicted after observation of strong interactions between IraD-RssB and the lack of interaction between IraD-RpoS. However, the inducing mechanisms for *iraD* expression either during oxidative stress or DNA damage remain elusive (Bougdour et al. 2008).

The anti-adaptor IraL was discovered most recently, and needs further research for a better understanding of RpoS function. This anti-adaptor is overexpressed during logarithmic growth in certain uropathogenic *E. coli* (UPEC) strains, resulting in

stable RpoS levels during exponential growth, comparable to the RpoS levels found during stationary phase growth (Culham et al. 2001; Hryckowian et al. 2014). Considering the positive correlation between logarithmic growth RpoS levels and stress resistance in *Shiga*-toxin producing *E. coli* (STEC), the RpoS involvement in gene expression during exponential growth in *E. coli* K-12 and EHEC O157:H7, and the RpoS involvement in the virulence of many pathogenic species, it is likely that the difference in timing and magnitude of RpoS levels helps define the host and niche specificity of pathogenic and non-pathogenic species alike (Dong et al. 2008; Dong and Schellhorn 2009a, b; Dong and Schellhorn 2010; Mand et al. 2013; Hryckowian et al. 2014). BLAST analysis revealed that *iraL* is present in many, but not all, *E. coli* and *Shigella* strains, but not in sequenced strains of other genera. Also, not all EHEC and STEC strains that espress elevated RpoS levels during logarithmic growth, encode *iraL*, suggesting that there are additional mechanisms for elevation of RpoS levels during logarithmic growth. IraL is more frequently found in UPEC strains than in fecal strains, and it has been suggested that the former strains are more adaptable than the latter, possibly as a result of increased RpoS levels upon encountering stress. It is possible that elevated RpoS levels may be favorable for conditions encountered by some pathotypes, but not for others (Andersen et al. 2013; Mand et al. 2013; Hryckowian et al. 2014).

Additional Sigma Factor Regulation

Indirect forms of RpoS regulation include competition. All sigma factors compete for the same pool of core RNA polymerase (RNAP). During favorable circumstances, the vast majority of available RNAP interacts with the general housekeeping sigma factor σ^D (RpoD); however, any process that affects the ability of RpoD to bind to the RNAP, also indirectly affects the ability of RpoS, and other alternative sigma factors, to bind RNAP, and *vice versa*. The affinity of RpoS for RNAP is slightly lower than the affinity of RpoD for RNAP, and cellular RpoD levels are significantly higher than cellular RpoS levels, even during stationary phase growth, thereby preventing disruption of essential housekeeping functions during stress (Tanaka et al. 1993; Jishage et al. 1996). Furthermore, RpoS and RpoD share a preference for the same consensus promoter elements, with RpoS having greater affinity to the −10 region and RpoD having a stronger affinity to the −35 region. This shared affinity for promoter regions, combined with different sensitivity to activator and repressor elements, allows for fine-tuning of gene expression in response to various stimuli (Typas et al. 2007; Battesti et al. 2011).

There is overlap between alternative sigma factors and their inducing stresses. Whereas RpoS is generally associated with starvation and entry of stationary phase growth, RpoE activity is stimulated by envelope stress, and RpoH regulation was firstly associated with heat shock. However, various stresses including hyperosmolarity, heat shock and stationary phase growth induce expression of all three sigma factors (Gruber and Gross 2003; Bang et al. 2005). Bang *et al.* suggest that alternative sigma factor activation can escalate into a cascade of cellular responses,

including activating various other sigma factors. External stress is sensed first by accumulating misfolded proteins in the periplasm, resulting in cleaving the RseA-RpoE complex and activating RpoE. RpoE release stimulates expression of RpoE itself and RpoH. The latter is also activated by the presence of misfolded proteins in the periplasm. RpoH stimulates *hfq* expression, resulting in increased cellular levels of Hfq, which, in turn, is required for the interaction of the RpoS anti-sigma factors with anti-adapters; subsequently releasing the translational inhibition of *rpoS* mRNA and increasing cellular RpoS levels (Bang et al. 2005).

It remains difficult to summarise the stress-specific regulation of, and by, RpoS, as the function of RpoS can differ greatly between different bacterial species, but its function often differs between strains of the same species also. For example, RpoS positively regulates motility and biofilm formation in UPEC UTI89 (Kulesus et al. 2008), however, an *rpoS* mutation in UPEC 536 shows no attenuation in motility or biofilm formation (Beloin et al. 2006). And whereas the growth of UPEC HU734 encoding mutated *rpoS* is attenuated during stress, UPEC CFT073 encoding mutated *rpoS* is not attenuated in colonization of the bladder, kidney and urethra (Culham et al. 2001). Considering the wide range of RpoS alleles and mutations, present within the same species (as well as the differences in the level, timing and magnitude of RpoS regulations between different pathotypes) non-pathogenic isolates and lab strains; it is clear that the activation or inactivation of RpoS can be an advantage as well as a disadvantage depending on the host, niche or environment.

The Role of RpoS in *Salmonella*

Salmonella enterica infections manifest in the form of either salmonellosis or enteric fever, depending on the type of host and the infecting serovar. The serovars causing the more serious enteric fever in humans are *S.* Typhi and serotypes within the *S.* Paratyphi group. The range of serovars capable of causing salmonellosis in humans is much wider. *S.* Typhimurium is generally used as a model organism to study *Salmonella* infections, as it is a causative agent of salmonellosis in humans, but as the name suggests, it is the causative agent of enteric fever in mice (Boyle et al. 2007). After ingestion of *Salmonella*, the bacteria infect the M-cells of the Peyer patches in the distal ileum. Enteric strains migrate to the mesenteric lymph nodes, where they infect macrophages, spreading through the body, infecting the liver, spleen and gall bladder. From the gall bladder *Salmonella* re-enters the intestinal tract, and the cycle starts anew (Everest et al. 2001; Fàbrega and Vila 2013).

Many genes involved in *Salmonella* virulence are encoded *Salmonella* pathogenicity islands SPI-1 and SPI-2. Both SPIs encode a separate T3SS and effector proteins, essential for *Salmonella* virulence. SPI-1 is activated upon contact with the intestinal epithelium; the T3SS encoded on SPI-1 translocates effector proteins across the host cell's plasma membrane. Some of the translocated effector proteins stimulate the host's actin filaments, resulting in localized membrane ruffling, which is required for *Salmonella* invasion (Patel and Galan 2005). Other effector proteins activate the MAPK pathway in the host, thereby activating production of

pro-inflammatory cytokines, resulting in the recruitment of polymorphonuclear leukocytes (Bruno et al. 2009). After *Salmonella* enters the host cell, it remains inside the *Salmonella* containing vacuole (SCV). The T3SS and effector proteins encoded on SPI-2 are required for survival and proliferation inside the SCV and inside phagocytes. During the later stages of SCV formation, RpoS expression is required to regulate the expression of effector proteins that ensure localisation of the SCV in a perinuclear position and stimulate *Salmonella*-induced filaments (Sifs) formation, and elongate SCVs into long tubules, which is required for effective virulence (Beuzon et al. 2000, Abrahams et al. 2006). Other functions of effector proteins secreted by the SPI-2 T3SS are involved with avoidance of SCV-lysosome fusion, the modification of ubiquitin, immune signalling and structure of the host's cytoskeleton (Figueira and Holden 2012). Enteric strains pass through the epithelial cells and exit at the basolateral side where they are ingested by phagocytes. Effector proteins, encoded on SPI-2, allow for survival and proliferation within the phagocyte, while the phagocyte transports them around the body, resulting in infection of the liver, spleen and gall bladder (Darwin and Miller 1999b). While *Salmonella* is contained in the SCV, it encounters a range of stresses, including nutrient starvation, oxidative stress, acidic stress and potential DNA damage and RpoS is thought to mediate prolonged survival by responding to these stresses (Fang et al. 1992) However, RpoS has also been directly implicated by positively regulating SpvR and the *spv* operon. The *spv* genes are located on a virulence plasmid found in a range of *Salmonella* serovars, including, but not restricted to, Typhimurium, Enteriditis, Choleraesuis and Dublin (Fang et al. 1991). The *spvABCD* operon is positively regulated by SpvR, which is capable of binding to an inverted repeat sequence found both upstream of *spvR* and upstream of *spvA* (Grob and Guiney 1996). Transcription at these promoters requires interaction with RpoS (Fang et al. 1992). Eexpression of the *spv* operon is induced by the intracellular environment of the host cell, but the exact triggers remain enigmatic.

The transcriptional regulator SpvR is a member of the LysR family and is expressed at a low, basal level during favorable growth. Expression of *spvR* is upregulated in the SCV, independently of SpvR and RpoS, however, both SpvR and RpoS are required for optimal expression of *spvR*. Other regulators of *spvR* expression remain elusive. It is possible that expression of *spvR* is regulated by various regulatory factors, depending on the encountered growth conditions (Wilson and Gulig 1998).

The function of *spvA* is unclear as it is a predicted outer membrane protein of unknown function (UniProtKB – Q56124) implicated to assist in multidrug resistance (Gebreyes et al. 2009) SpvB has two distinct protein domains. The N-terminal domain belongs to a family of proteins initially characterized in a family of Enterobacteriaceae that infect insects, but the exact function of this widely distributed protein domain remains enigmatic (Lesnick and Guiney 2001). The C-terminal domain of SpvB exhibits ADP-ribosyltransferase activity, capable of modifying the host's G-actin monomers to prevent polymerization into F-actin filaments. SpvB activity results in loss of the F-actin cytoskeleton and apoptosis (Lesnick et al. 2001; Paesold et al. 2002). SpvC is a phosphothreoninelyase,

which irreversibly inactivates host's MAP-kinases by removing phosphates and modifying target threonines (Li et al. 2007). Although the exact role in virulence is unknown, but it has been suggested to play a role in attenuating the intestinal inflammatory response (Haneda et al. 2012). Both SpvB and SpvC are required for the *spv* virulent phenotype (Roudier et al. 1992). SpvD is capable of suppressing the host's pro-inflammatory immune responses by preventing the activation of NF-$_{\kappa}$B-dependent promoters, which play a central role in the host's response to microbial infections. SpvD also interacts with Xpo2, disrupting the nucleus-cytoplasmic importin-α recycling (Rolhion et al. 2016).

Although the *spv* genes are important in *Salmonella* virulence, in the murine model *Salmonella* Typhimurium *rpoS* mutants are twice as lethal as virulence plasmid-cured isogenic derivatives, suggesting RpoS does not only regulate virulence genes located on plasmids, but also regulates chromosomal virulence genes (Fang et al. 1992). Although it is unclear exactly which RpoS-regulated chromosomal genes are involved in this virulence, likely candidates are genes involved in adherence to the host epithelium in the early stages of infection. Using mouse models, Nickerson and Curtiss demonstrated that *Salmonella* Typhimurium *rpoS* mutant strains are defective in their ability to colonize Peyer's patches. These strains are also significantly attenuated in infecting the liver and spleen, with a thousand-fold less colony-forming unit recovery of the *rpoS* mutant after infection of the murine spleen than the wild-type parental strain (Nickerson and Curtiss 1997). Furthermore, *rpoS* mutants are of great interest in vaccine development studies. Various *Salmonella* Typhimurium *rpoS* mutant strains have been used as effective vaccine strains against murine salmonellosis, and *Salmonella* Typhi strains studied for human vaccine development are commonly Ty2 derivatives and thus encode mutated *rpoS* (Coynault et al. 1996; Shi et al. 2010).

The Role of RpoS in *Shigella*

Shigella flexneri is a foodborne pathogen that is capable of invading and colonizing the colon, resulting in bacterial dysentery. They are particularly capable of surviving at low pH throughout the stomach, contributing to the low infectious dose of 10–100 bacteria required for development of shigellosis (Gorden and Small 1993). During infection, *Shigella* invades the highly endocytic M-cells in the follicular associated epithelium via ruffling, and rapidly crosses the cell to be released into the intraepithelial pocket. From here, *Shigella* has access to the basolateral pole of colonic epithelial cells (Mounier et al. 1992; Neutra et al. 1996). Inside the epithelial cells, *Shigella* breaks out of the vacuole and replicates inside the cytoplasm (High et al. 1992), where they activate transcription and secretion of IL-8 to recruit PMN cells. The PMN cells transmigrate through the epithelial lining to reach the luminal bacteria, disrupting the epithelium as they do so, and promoting the local spread of *Shigella* (Perdomo et al. 1994; Beatty and Sansonetti 1997). Infection leads to acute mucosal inflammation and tissue damage, abscesses and ulceration and bloody mucoid stool. Without treatment, the patient risks secondary complications including septicemia, pneumonia and hemolytic uremic syndrome (Bennish 1991).

In *E. coli* RpoS is involved in the expression of the glutamate-dependent acid-resistance (GDAR) pathway. This system, which is induced during growth at mild acidic conditions (pH 5) and in cells growing fermentatively, is comprised of two homologous decarboxylase enzymes, GadA and GadB, and an anti-porter, GadC (Lin et al. 1995; Waterman and Small 2003a). The system removes protons from the cytoplasm through the decarboxylation of glutamate into γ-aminobutyric acid (GABA). The anti-porter exchanges GABA for external glutamate, reversing the cell membrane potential to create an internal positive charge and gradually de-acidifying the extracellular environment (Richard and Foster 2004). RpoS promoter sequences are present upstream of the *gadA* and *gadBC* genes, suggesting RpoS involvement in GDAR regulation (Waterman and Small 2003a). Some studies analyxing *Shigella rpoS* mutants revealed barely detectible *gadB* and *gadC* mRNA during acid stress (Waterman and Small 2003b), whereas others revealed GDAR activity in strains expressing a truncated RpoS (Jennison and Verma 2007), suggesting that the regulation of this acid resistance pathway may be regulated by different response regulators depending on the bacterial environment, growth stage and the stresses encountered.

Regulation of RpoS in *S. flexneri* during infection is dependent on DksA, a protein involved in the regulation of several virulence factors. DksA regulates resistance to environmental stress by ensuring optimal RpoS translation (Webb et al. 1999). Other functions of DksA involve regulation of cell elongation and filamentation as well as assisting in the intercellular spread of *Shigella.* Cell-to-cell spread of *Shigella* usually involves the protein IcsA which localizes the bacteria to a polar location, but *Shigella dksA* mutant strains fail to correctly localize IcsA, resulting in attenuated virulence (Mogull et al. 2001).

The Role RpoS in *E. coli*

Escherichia coli is capable of adapting to and surviving in various niches, both environmental and within a host. Whereas various GI tract colonizing *E. coli* strains exhibit a symbiotic relationship with their host, some strains have diverged into either intestinal or extra-intestinal pathogens (Yan and Polk 2004) One group of extra-intestinal *E. coli* pathogens is commonly referred to as UPEC; uropathogenic *E. coli*. UPEC is an opportunistic pathogen specialized in infecting the intestinal tract of the host, being responsible for approximately 95% of urinary tract infections in humans. Capable of surviving inside the gastrointestinal tract, as well as colonizing the urinary tract, during recurring UTI infections, the UPEC reservoir can often be found in the GI tract of the host (Foxman 2002; Kaper et al. 2004) Most UPEC strains reside in the bladder, colonizing the epithelial cells resulting in cystitis. Some UPEC strains can ascend through the urethra into the kidneys, resulting in pyelonephritis (Bower et al. 2005). To be able to successfully cause a UTI, UPEC must be able to survive stresses including, but not restricted to, nutrient limitation, host immune components and acid stress. Studies in murine models have revealed that UPEC CFT073 strains encoding a mutated *rpoS*, are attenuated in their ability

to colonise the urinary tract of mice, but they present no attenuation during growth in urine, indicating that UPEC CFT073 requires RpoS for successful replication and survival inside the host (Hryckowian and Welch 2013). Furthermore, studies carried out on UPEC UTI89 attenuated in cAMP-CRP production, revealed an increased level of RpoS and remained able to combat acid- and oxidative stress, despite cAMP-CRP being an important regulator when exposed to these stresses. Therefore, RpoS is involved in protection against acid stress and oxidative stress during colonization, especially in the absence of sufficient cAMP-CRP (Donovan et al. 2013).

There are six recognised pathotypes of *E. coli* that are adapted to infecting the GI tract. Two of these: enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC, aka Shiga-Toxin producing *E. coli* or STEC and verotoxin-producing *E. coli* or VTEC) produce a characteristic changes to enterocytes called attaching and effacing (A/E) lesions (Moon et al. 1983). EPEC infections of the small intestine generally manifest as watery diarrhea, whereas EHEC infections of the large intestine result in bloody diarrhea and potential development of hemolytic uremic syndrome (HUS) (Mellies et al. 2007). The latter is capable of secreting Shiga toxin; a protein that targets Gb3-expressing endothelial cells. During EHEC infections, the secreted Shiga toxin is absorbed into the blood stream and dispersed throughout the body. The Gb3-expressing renal glomerular endothelium is particularly vulnerable, resulting in HUS, a syndrome marked by hemolytic anemia, thrombocytopenia and acute renal failure (Sandvig 2001).

As mentioned both EPEC and EHEC cause A/E lesions in the gut. This process involves effacement of the intestinal microvilli by means of reorganization of the cytoskeleton to allow intimate association with bacterial cells (Donnenberg et al. 1997). A/E lesions are one of the common virulence factors of EPEC and EHEC, and the genes required are encoded on the locus of enterocyte effacement (LEE) pathogenicity islands (McDaniel et al. 1995). LEE encodes a T3SS (Lee 1997), the outer membrane protein intimin required for host cell attachment (Jerse and Kaper 1991), several secreted proteins (*espABD*) required for signal transduction pathways required for the formation of A/E lesions, Tir (EPEC) or EspE (STEC) which translocates to the host's cell membrane and acts as a receptor for intimin (Kenny et al. 1997), and the Pas (EHEC) or EscD (EPEC) protein involved in the secretion process (Kresse et al. 1998). Expression of the secreted *esp.* proteins plays a major role in the formation of A/E lesions, and transcription of the *esp.* promoter is dependent on RpoS and primarily occurs during the exponential growth phase (Beltrametti et al. 1999). Furthermore, the first gene on LEE-1 is *ler*, a transcriptional activator essential for the expression of almost all genes encoded on LEE islands (Friedberg et al. 1999). Expression of *ler* is positively regulated by at least two independent pathways; one involving H-NS and GrlR, the other involving RpoS and DsrA (Iyoda and Watanabe 2005; Laaberki et al. 2006).

A common virulence factor for extraintestinal *E. coli* infections is α-hemolysin, which is encoded on the *hlyCABD* operon. The toxin is encoded by *hlyA*, *hlyC* encodes an enzyme required for posttranslational modification, and *hlyB* and *hlyD* encode components of a specific type I secretion system (Welch 2005). Depending on the infecting strain, the *hlyCABD* operon can be located either on a transmissible

plasmid or within chromosomal pathogenicity islands (Muller et al. 1983). Transcription of the *hly* operon is inhibited by H-NS and Hha proteins, which bind and block the promoter region (Juarez et al. 2000). EHEC strains of serotype O157:H7 are responsible for severe intestinal and extraintestinal disease (Kaper et al. 2004). They produce their own type of hemolysin encoded on the non-transmissible O157 plasmid (Schmidt et al. 1994). The EHEC hemolysin is encoded by the *ehxCABD* operon, which shares 60% sequence identity with its α-hemolysin homologue (Schmidt et al. 1995). Similar to α-hemolysin, the *ehx* operon is also inhibited by H-NS. The sRNA DsrA positively influences expression of the *ehx* operon, independently of H-NS, likely by increasing the available levels of RpoS, an essential positive regulator of the *ehx* operon (Li et al. 2008).

RpoS has also been implicated in the regulation of curli expression. Curli, encoded by the curlin subunit gene *csgA*, are thin, coiled and highly aggregative surface fibers involved in the attachment of bacteria to organic and inorganic surfaces (Olsen et al. 1989; Arnqvist et al. 1992). Curli expression is primarily associated with survival outside of the host, as its expression is repressed at temperatures above 30 °C. However, curli- expressing bacteria are capable of binding fibronectin and laminin, which are major components of the eukaryotic extracellular matrix (Olsen et al. 1989, 1993). Transcription of *csgA* is suppressed by H-NS, but this suppression can be reversed by RpoS. It has been suggested that RpoS reverses the H-NS suppression of curli transcription during early infection stages, to increase virulence by promoting the interaction of curli with the eukaryotic extracellular matrix (Olsen et al. 1993).

All *E. coli* K-12 strains carry *csgA*, but only a subset is capable of expressing curli (Olsen et al. 1993). However, many K-12 strains also carry an amber-mutated *rpoS* (Kaasen et al. 1992). Suppressing the amber mutation in K-12 results in previously curli-deficient cells becoming curli-proficient, suggesting that functional RpoS is critically required for curli expression in *E. coli* K-12 (Olsen et al. 1993).

The Role of RpoS in *Yersinia*

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are self-limiting foodborne infectious bacteria that hold their tropism in lymphoid tissue. During infection, *Yersinia* crosses the M-cells in the gut to infect the underlying Peyer's patches, from where they disseminate to mesenteric lymph nodes (Grutzkau et al. 1990). To successfully infect the host, *Yersinia* must be capable of adapting to various stresses, including low pH encountered by stomach acid, and oxidative stress encountered during the host's immune response.

The *Yersinia* RpoS, which shares approximately 90% sequence identity with *E. coli*, is an important factor in the defense against these stresses, as well as a regulator of some known virulence factors (Badger and Miller 1995; Iriarte et al. 1995; Guan et al. 2015). Unlike *E. coli*, the role of RpoS stress protection appears to occur primarily when grown at 37 °C. Experiments carried out comparing *Y. enterocolitica* WT strains and *rpoS* mutant strains revealed that, when

previously grown at 26 °C, both strains recovered equally well when exposed to hydrogen peroxide, osmotic shift, heat and low pH. However, when previously grown at 37 °C, the *rpoS* mutant strains had a two-fold reduction when exposed to hydrogen peroxide and a six-fold reduction when exposed to high osmolarity, high temperatures or low pH. Interestingly, the growth of the *rpoS* mutant was reduced by 13-fold when exposed to starvation stress, following growth at 26 °C. Combined these results indicate that RpoS is important in protection against stress during infection of the host, but it is likely that *Yersinia* expresses different sigma factors to cope with these stresses at lower temperatures (Badger and Miller 1995).

In *Y. pseudotuberculosis*, RpoS regulates the expression of some virulence factors, including genes involved in motility, biofilm formation, expression of the T6SS (type 6 secretion system), and expression of the *Yersinia*-stable-toxin-a (Yst) (Iriarte et al. 1995; Guan et al. 2015). For many Gram-negative bacteria, motility is important for biofilm formation, as well as invasion of the host. In *Yersinia,* RpoS positively regulates expression of the *flhDC* operon, which encodes the flagellum master regulator (Guan et al. 2015). RpoS regulation of motility has also been observed in *Pseudomonas aeruginosa,* in which RpoS positively regulates *fliF* and chemotaxis genes (Schuster et al. 2004), and in *E. coli*, where RpoS suppresses flagellum production (Makinoshima et al. 2003; Dong and Schellhorn 2009a, b). The T6SS is found in many Gram-negative bacteria, and it has often been implicated in virulence, biofilm formation and host cell interactions (Coulthurst 2013). In *Y. pseudotuberculosis,* the T6SS is essential for survival under acidic and osmotic stresses and for resistance against deoxycholate (Gueguen et al. 2013; Zhang et al. 2013) and its expression is positively regulated by RpoS (Guan et al. 2015). As T6SS are involved in the formation of biofilms, RpoS indirectly regulates biofilm formation in *Y. pseudotuberculosis.* However, T6SS also regulates biofilm formation more directly by positively regulating the *hmsHFRS* operon, which is responsible for production of the essential exopolysaccharide Poly-1,6-N-acetyl-D-glucosamine (PGA) (Drace and Darby 2008; Guan et al. 2015).

RpoS is also involved in the regulation of Yst production, an enterotoxin produced by *Y. enterocolitica*. The RpoS-dependent expression of this toxin begins during the late exponential phase of growth and continues throughout the stationary growth phase. Although the exact function of Yst remains enigmatic, the expression of this heat-stable toxin is only found in some *Yersinia* pathotypes, and not in avirulent strains, suggesting it is a virulence-related toxin (Iriarte et al. 1995; Singh and Virdi 2004).

The Role of RpoS in *Vibrio*

Vibrio cholerae is the causative agent of the life-threatening, diarrheal disease cholera. Infections with *V. cholerae* originate from the consumption of contaminated food and water. Although the disease is self-limiting, the severe diarrhea often results in dehydration and even death of young patients (Finkelstein 1996).

V. cholerae major virulence factor is cholera toxin (CT), which is encoded on the bacteriophage CTXφ (Waldor and Mekalanos 1996). CT is a heterogeneous protein which consists of a large subunit that is responsible for its toxicity, and several small subunits, which are responsible for host cell adhesion (Lonnroth and Holmgren 1973; Sattler and Wiegandt 1975). The subunits are assembled in the periplasm prior to being secreted via a type two secretion system (T2SS), which is a piston-like multiprotein complex that allows for the secretion of CT via a pore in the outer membrane (Davis et al. 2000). After infection of the small intestine, secreted CT binds to cells expressing the GM1 ganglioside receptor that are on the surface of epithelial cells lining the GI tract. The uptake of the toxic CT subunit leads to the activation of adenylate cyclase, which in turn leads to increased intracellular cAMP, an imbalance of electrolyte movement and water secretion, resulting in the rice water diarrhea that is typical of cholera (Sanchez and Holmgren 2008).

Unlike *Salmonella*, RpoS is not required during early stages of infection. *Vibrio rpoS* mutants are not attenuated in the suckling mouse model. Instead, RpoS plays an important role during late stage infection. During early infection, *Vibrio cholerae* passes through the stomach, the duodenum and the proximal jejunum before entering the ileum of the small intestine. Here, *Vibrio* swim through the mucosal barrier to colonize the villus epithelium. *V. cholerae* infection is self-limiting, using its host to boost growth before being shed into aqueous environments where further spread may occur. However, once *Vibrio* is ready to abandon its host, the cells require RpoS or escape from the mucus membrane, by regulating genes involved in chemotaxis and motility (Nielsen et al. 2006).

A major difference between RpoS regulation in *E. coli* and *V. cholerae*, is the dependence on Hfq. *E. coli* requires Hfq interaction with the sRNAs DrsA, RprA, ArcZ and OxyS; however, no homologues of these sRNAs are found in the *V. cholerae* genome (Ding et al. 2004). Also, *Vibrio* strains carrying *rpoS* mutations are largely unaffected in their infection model, whereas *Vibrio* strains carrying *hfq* mutations are severely impaired in the suckling mouse model (Yildiz and Schoolnik 1998; Merrell et al. 2000). Although the mechanisms by which RpoS is regulated in *Vibrio* are not fully understood, it is likely that this regulation is independent of Hfq.

Outside of the host, *V. cholerae* is primarily found in aqueous environments. The most abundant biomolecule in these aqueous environments is chitin, originating from the exoskeleton of crustaceans. As a chitinolytic microbe, *Vibrio* is capable of degrading chitin, and using chitin oligosaccharides as signal molecules (Thompson et al. 2004). RpoS is a positive regulator of TfoX, a regulator of chitinase genes, genes regulated in chitin utilization and genes involved in natural competence (Meibom et al. 2005; Yamamoto et al. 2011; Dalia 2016). Natural competence is a process in which bacteria take up DNA from the extracellular environment. If the DNA is homologous to the chromosome, this ingested DNA can be integrated into the chromosome using RecA-dependent homologous recombination (Lorenz and Wackernagel 1994). Competence of *Vibrio* is inhibited in environments rich in carbon catabolytes, suggesting that starvation stress-induced RpoS is required for the process of natural competence.

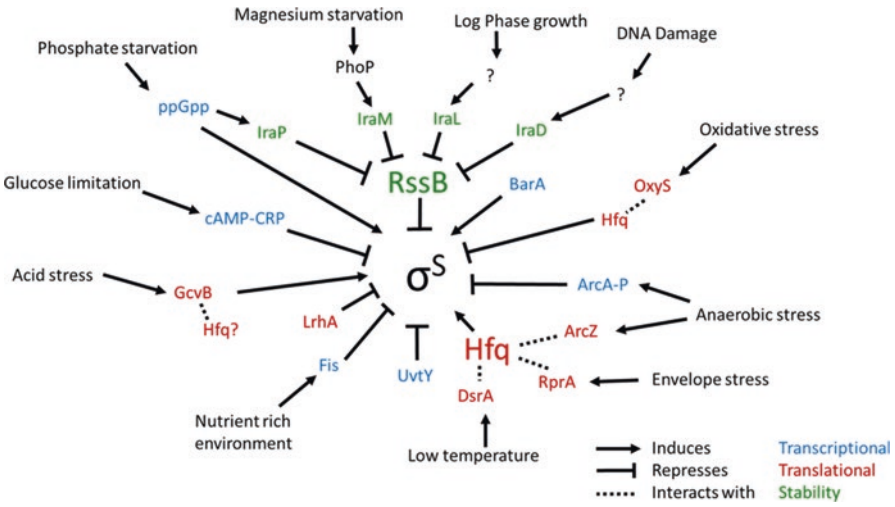

Fig. 6 The expression of rpoS is regulated at different levels. The schematic summarises some of the major stresses that affect the cellular levels of RpoS. Some stresses induce pathways, represented in *blue*, that affect rpoS expression at a transcriptional level. Other stresses encourage the activation of pathways, represented in *red*, that affect rpoS expression at a translational level. Cellular RpoS levels can also be regulated post-translationally, by pathways, represented in *green*, that affect the stability of RpoS, by inhibiting the activity of anti-adaptor RssB and therefore preventing RpoS degradation

The expression of *rpoS* is regulated at different levels. The schematic summarizes some of the major stresses that affect the cellular levels of RpoS. Some stresses induce pathways, represented in blue, that affect *rpoS* expression at a transcriptional level. Other stresses encourage the activation of pathways, represented in red, that affect *rpoS* expression at a translational level. Cellular RpoS levels can also be regulated post-translationally by pathways, represented in green, that affect the stability of RpoS, by inhibiting the activity of anti-adaptor RssB and therefore preventing RpoS degradation (Fig. 6).

RpoE in Gram Negative Foodborne Pathogens

Regulation of RpoE Activation

The stress response regulated by the alternative sigma factor σ^E (RpoE) is one of the most intensively studied stress response systems of *Escherichia coli*. The system is activated in response to envelope stress, both of environmental as well as of cellular origin. Activation of σ^E in *E. coli* results in regulation of more than 100 genes involved in various functions. Among the RpoE-regulated genes are the transcriptional factors *rpoE, rpoH, rpoD* and *rpoN,* regulatory genes, including *rseA, rseB,*

rseC, periplasmic folding factors and proteases such as *skp, surA, fkpA, dsbC, htrA, ecfE*, LPS and phospholipid biogenesis including *lpxD, lpxA, lpxP, psd, htrM*, the sensory proteins *cutC, mdoG, sixA,* and primary metabolic functions such as *fusA* (Rowley et al. 2006). Being involved in the regulation of various essential genes such as *yaeT, yfiO* and *bamC* in *E. coli*, RpoE is a required gene even under nutrient-rich "non-stress" conditions (Dartigalongue et al. 2001). Although RpoE is conserved among most Gram-negative Enterobacteriaceae, its functions within the cell, albeit always involved in envelope maintenance, varies between different bacteria.

During their life cycle, both within and outside of their host, pathogens are exposed to various environmental stresses. To ensure appropriate responses to stress-inducing environments, bacteria have an array of alternative sigma factors at their disposal. To ensure a rapid response to cell stress, alternative sigma factors are expressed at all times, and kept in an inactivate state by anti-sigma factors. RpoE is an alternative sigma factor that responds to damage to the bacterial envelope hence, in the absence of envelope stress, σ^E is inactivated via interaction with its anti-sigma factor RseA. Unlike other alternative sigma factors, which are inhibited in the cytoplasm, inhibition of σ^E involves tethering σ^E to the cytosolic face of the inner membrane, allowing it to react to envelope stress (De Las Penas et al. 1997; Missiakas et al. 1997).

RseA is an inner membrane protein comprised of a single transmembrane segment, an N-terminal cytoplasmic domain ($RseA^{cyto}$) and a C-terminal periplasmic domain ($RseA^{peri}$). During non-stressed conditions, $RseA^{cyto}$ binds tightly to RpoE, thus anchoring it to the inner membrane. Meanwhile, $RseA^{peri}$ is bound to RseB, a periplasmic protein which prevents proteolysis of RseA by proteases (Campbell et al. 2003; Kim et al. 2010). Damage to the bacterial envelope results in the degradation of RseA by DegS and RseP, releasing the σ^E-$RseA^{cyto}$ complex into the cytoplasm, where the complex is further cleaved to finally release σ^E (Flynn et al. 2004).

However, before RseA can be cleaved by DegS and RseP, two things need to happen; the activation of DegS and the release of RseB. DegS is a trimeric periplasmic protein which is anchored to the inner membrane. It consists of a protease domain and a PDZ domain. The trimer forms a funnel-shaped protein, with full access to the active sites. In the absence of a signal, the PDZ domain keeps DegS in an inactive state, during which the active site is not in proper conformation for catalysis (Walsh et al. 2003; Wilken et al. 2004). The C-terminus of outer membrane proteins (OMPs) contain a conserved YxF domain, buried inaccessibly in mature porins and OMPs. However, when OMP folding is disrupted, this conserved peptide is exposed. Upon binding the conserved peptide of denatured OMPs, DegS undergoes a conformational change, allowing the protease domain to become active. As such, damage to the membrane, by means of misfolded porins and OMPs, can be sensed and converted into a signal (Walsh et al. 2003; Wilken et al. 2004).

RseB is a periplasmic chaperone that, in the absence of envelope stress, binds and blocks the cleavage site of RseA, preventing cleavage by DegS and σ^E signalling (Cezairliyan and Sauer 2007). RseB contains a large N-terminal domain and a smaller C-terminal domain connected by a flexible loop, both containing motifs that bind RseA (Kim et al. 2007). Recent studies have revealed that RseB dissociates

from RseA upon interactions with LPS fragments, opening up the RseA cleavage site for interaction with DegS. This allows for sensing envelope stress by means of sensing defects in LPS biogenesis (Lima et al. 2013).

Once RseB has been dissociated from RseA, DegS cleaves the periplasmic site of RseA. The loss of $RseA^{peri}$ opens up a cleavage site in the transmembrane region, which interacts with a second protease: RseP (Alba et al. 2002; Kanehara et al. 2002). The exact mechanism by which RseP cleaves its substrates remains elusive. Once the transmembrane region of RseA has been degraded, the $RseA^{cyto}$/ σ^E complex is released into the cytoplasm. The cytoplasmic adaptor protein SspB recognizes $RseA^{cyto}$ and delivers the complex to ClpXP, which cleaves the remaining of the RseA protein thereby releasing σ^E (Flynn et al. 2004).

RpoE and sRNAs

Although the existence of small RNAs has been acknowledged for decades, with the help of systematic genome wide search, researchers have only recently begun to determine their importance. Small RNAs are plentiful (*E. coli* encodes more than 100 sRNAs) and they contribute in the regulation of various cellular processes, including nutrient uptake, transport, maintenance, homeostasis, biofilm formation and membrane maintenance (Thompson et al. 2007). Regulatory sRNAs can modulate gene expression either positively or negatively. Some sRNAs comprise riboswitches which are part of the same mRNA they regulate; they allow their mRNA to adopt different conformations in response to their specific signals (nutrients, stress, ligand binding) (Breaker 2011). Other mRNAs act by base-pairing to target mRNA, and either modulate transcription or mRNA stability. These sRNAs can both be found encoded within genes and in intergenic regions. To facilitate sRNA-mRNA pairing, many sRNAs are dependent on the RNA chaperone Hfq, but some sRNAs are capable of acting independently of Hfq (Storz et al. 2011; Vogel and Luisi 2011).

sRNAs are often required to allow for fine-tuning of cellular processes, and to allow for high sensitivity to specific signals. As such, their expression is often regulated by signal molecules including specialized sigma factors and two-component systems. sRNAs are often involved in the down-regulation of their activating proteins, and thus their own down-regulation, as feed-back mechanism (Storz et al. 2011).

RpoE is an important alternative sigma factor, responsible for the homeostasis of the bacterial envelope. In response to envelope stress, σ^E regulates expression of some genes directly, other genes are regulated post-translationally by expression of sRNAs. In *E. coli,* and other bacteria, there are a number of σ^E regulated sRNAs (Klein and Raina 2015).

In *E. coli* and *Salmonella,* the sRNA MicA inhibits translation of OmpA mRNA (Rasmussen et al. 2005; Papenfort et al. 2006). OmpA is an abundant membrane protein thatis important in survival in the presence of SDS, cholate, acidity, high salt, or pooled human serum. OmpA deletion strains are more capable of surviving in human tissue, suggesting that OmpA is a target of the host defense system.

By down-regulating OmpA via MicA, σ^E influences virulence, as lower levels of OmpA render bacteria more "invisible" for the host's immune system (Wang 2002).

The *E. coli* sRNA RybB is involved in the regulation of LPS glycoform formation (Klein et al. 2011). LPS is synthesized in different glycoforms, depending on environmental conditions. The core components of LPS are glucose (Glc), heptose (Hep) and 3-deoxy-D-manno-octulosonic acid (Kdo). During nonstress conditions, that is in the absence of induction by RpoE and two-component systems, LPS glycoform I is the most abundant glycoform (three Glc, three Hep, two Kdo, no D glucuronic acid bound to Hep). Activation of σ^E leads to accumulation of glycoform IV, V or VII instead, depending on the inducing conditions. RpoE induction results in transcription of *waaZ, WaaS* and the sRNA RybB. This sRNA mediates translational suppression of WaaR, a glucosyltransferase responsible for attaching a third Glc to LPS, present in glycoform I, but not in form IV, V and VII. WaaZ is a Kdo:Kdo-transferase, responsible for attaching a third Kdo subunit to LPS, which is present in glycoforms IV, V and VII, but not in glycoform I. WaaS is a rhamnose:Kdo-transferase, responsible for adding a rhamnose to the LPS chain, found in glycoform IV, V and VII, but absent in form I. WaaS can attach the rhamnose to either KdoII or KdoIII, depending on the transcriptional levels of EptB. EptB is a phosphoethanolamine transferase, translation of which can be repressed by MgrR and the sRNA ArcZ. However, during σ^E hyperinduction, σ^E induction of *eptB* overrides the repression by MgrR and ArcZ. During EptB repression, WaaS attaches Rha to KdoII, resulting in glycoform IV. When RpoE overrides EptB repression, EptB attaches a phosphoethanolamide to KdoII, and WaaS attachs Rha to KdoIII instead, as is found in glycoform V and VII. Glycoform V is the most abundant glycoform when both *eptB* and *rybB* are expressed, but WaaH is not. WaaH is a HepIII-glucuronic acid glycosyltransferase, which adds a GluUA to HepIII, resulting in LPS glycoform VII, and is expressed during certain stress conditions such as phosphate starvation (Muller-Loennies et al. 2003; Klein et al. 2011, 2013).

The *Salmonella* sRNA RybB is involved in the translational inhibition of multiple major OMPs, including OmpA, OmpC, OmpD and OmpF. OmpD is of particular interest; although it is the most abundant Omp in some *Salmonella* serovars, the gene is absent in *Salmonella* Typhi (Calderon et al. 2011). Ipinza et al. (2014), determined that *Salmonella* Δ*ompD* strains have an increased ability to survive and replicate in target organs of infection. OmpD is a porin that facilitates the uptake of hydrogen peroxide and hypochlorous acid, which are two ROS produced by macrophages during infection. Downregulation of *ompD* translation by RybB, increases *Salmonella*'s ability to survive and proliferate inside macrophages.

E. coli sRNA SlrA negatively regulates σ^E expression and affects translation of *lpp* mRNA (Guo et al. 2014). LpxC, a deacetylase involved in lipid A biosynthesis, and FabZ, a dehydratase involved in phospholipid biosynthesis, share a common substrate, i.e., R-3-hydroxylmyristoyl-ACP. The competition between FabZ and LpxC for this substrate is important for membrane homeostasis (Mohan et al. 1994). When expression levels of LpxC increase, Lipid A outcompetes phospholipids, resulting in a depletion of substrates and a destabilized membrane. To combat an increase in LpxC quickly, σ^E induces SlrA, a sRNA that binds and negatively

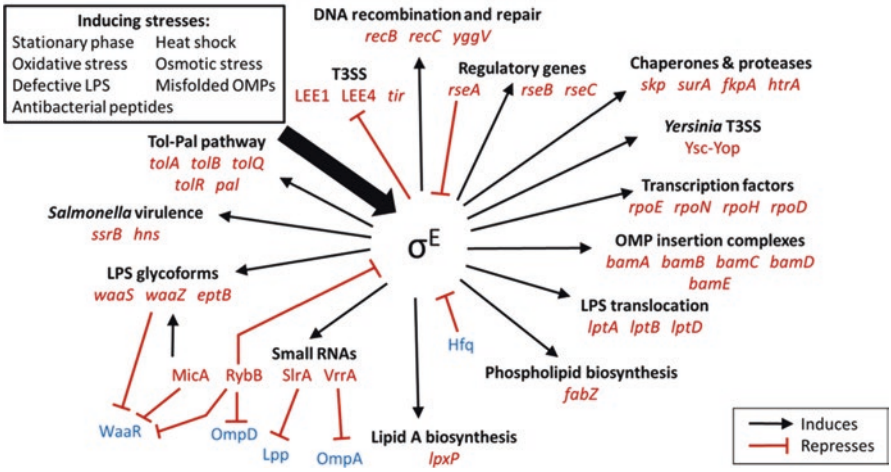

Fig. 7 Overview of σ^E regulation. Processes influencing or influenced by σ^E in *black*; Genes associated with these processes in *red*. Proteins described in *blue* are not directly regulated by σ^E

regulates translation of *lpp* mRNA. Lpp is the most abundant protein in the cell and contains three fatty acid chains. By downregulating Lpp production, the pool of fatty acids increases, which helps restore the LPS::phospholipid balance. Lpp defects themselves are also toxic. With over one million copies per cell, Lpp is the most abundant protein, and is of critical importance to membrane homeostasis. Lpp is embedded in the outer membrane, as well as covalently linked to the peptidoglycan layer. Moreover, Lpp mRNA is unusually stable, so transcriptional repression would be too slow of a response to stress conditions. By SlrA binding to *lpp* mRNA, ribosome binding is blocked, and the mRNA degradation time is significantly reduced, thereby allowing for a rapid response during envelope stress (Guo et al. 2014). An overview of σ^E regulation is given in Fig. 7.

RpoE and *Salmonella enterica*

Unlike *E. coli*, σ^E is not an essential gene for *Salmonella* during normal growth. However, in *Salmonella* σ^E is of critical importance during infection, as Δ*rpoE* mutants are severely attenuated in mouse models. *S.* Typhimurium Δ*rpoE* mutants are defective in both their survival and proliferation in epithelial cells and macrophages alike. Considering these strains display increased sensitivity to reactive oxygen species, their inability to infect is at least partially caused by their inability to defend against the reactive oxygen species created by their host (Humphreys et al. 1999; Testerman et al. 2002). The increased sensitivity to reactive oxygen species (ROS) is at least partially caused by the disrupted regulation of HtrA, which is a σ^E regulated, periplasmic protease involved in the degradation of misfolded OMPs. *Salmonella* Δ*htrA* mutants have an increased sensitivity to ROS (Johnson et al.

1991; Humphreys et al. 1999). Similarly to *E. coli*, σ^E regulates a wide range of genes in *Salmonella*, involving not only defense against ROS, but various different cellular processes. Other σ^E-regulated genes that are involved in virulence include *surA*, *fkpA* and *skp*. SurA and FkpA are both peptidylprolyl-*cis*-*trans*-isomerases, involved in the folding of proteins and OMPs in the periplasm. SurA plays a role in adherence and invasion of eukaryotic cells, and Δ*surA* strains are attenuated both when administered orally and intravenously. *Salmonella* Δ*fkpA* strains are also attenuated in mouse models, although not as severely as Δ*surA* strains (Sydenham et al. 2000; Humphreys et al. 2003; Skovierova et al. 2006). The periplasmic chaperone Skp interacts with native OMPs, as they are translocated through the inner membrane and help with the early folding events of these OMPs. Deletion of *skp* does not affect cell growth significantly in laboratory conditions nor does it affect *Salmonella*'s ability to infect the Peyer's patches. However, *Salmonella* Typhimurium Δ*skp* strains are attenuated in their ability to infect mouse organs, such as the liver and the spleen (Rowley et al. 2011).

RpoE promoter analysis revealed RpoE involvement in the regulation of four genes associated with DNA recombination and repair, i.e., *ptr, recB, recD* and *yggV*. The *E. coli yggV* homologue *rdgB,* is a pyrophosphatase involved in the hydrolyzation of noncanonical purines, removing them from the DNA precursor pool and thus preventing them from being incorporated into the DNA (Chung et al. 2002; Skovierova et al. 2006). The periplasmic metalloprotease Ptr is unlikely to be directly involved in DNA repair, but its promoter appears to overlap with *recB* and *recD*. The genes *recB* and *recD* encode subunits of exonuclease V, an enzyme that prepares DNA breaks for DNA repair during recombination events. Previous studies have revealed that *S.* Typhimurium Δ*recBC* strains are avirulent in mice (Buchmeier et al. 1993; Kabir et al. 2005; Skovierova et al. 2006).

Genes involved in the Tol-Pal pathway are another group of genes that are at least in part regulated by σ^E in *Salmonella (*Skovierova, Rowley et al. 2006*).* The Tol-Pal pathway consists of five proteins, TolABQR and Pal, which form a membrane-spanning complex involved in the translocation of colicins through the envelope (Godlewska et al. 2009). Previous studies have revealed that *S.* Typhimurium Δ*tolA* strains are attenuated in mouse models both in the oral and intravenous route; Δ*tolB* strains are less virulent than their parental strains, and have an increased sensitivity to antimicrobial peptides (Tamayo et al. 2002; Paterson et al. 2009).

RpoE and Pathogenic *Escherichia coli*

One of the main virulence factors of UPEC is the expression of P pili. The distal tip of the P pili, the Cpx-regulated PapG, mediates host adhesion. Although expression of the P pili subunits is not regulated by σ^E, accumulation of misfolded PapG does activate σ^E (Hung et al. 2001; Bower et al. 2005). The assembly of P pili involves HtrA, a σ^E-regulated periplasmic chaperone and protease which is essential for UPEC virulence, possibly because of its involvement in P pili assembly. Another σ^E-regulated gene involved in UPEC virulence is the periplasmic chaperone Skp.

Although Δ*skp* strains are not as severely affected in their capability to infect the bladder as Δ*htrA*, these strains are severely attenuated during infection of the kidneys (Redford and Welch 2006).

RpoE in *Yersinia*

Yersinia enterocolitica and *Yersinia pseudotuberculosis* are self-limiting foodborne infectious bacteriathat hold their tropism in lymphoid tissue. During infection, *Yersinia* crosses the M-cells in the gut to infect the underlying Peyer's patches, from where they disseminate to mesenteric lymph nodes (Grutzkau et al. 1990). To be able to survive and thrive in the lymph tissue, *Yersinia* relies on the Ysc-Yop Type = three secretion system (T3SS). T3SSs are syringe-like structures, comprised of approximately 25 protein components, which span the bacterial envelope and allow secretion of effector proteins directly into eukaryotic host cells (Cornelis et al. 1998). Among other functions, the Yop proteins that are secreted by the Ysc-Yop system inhibit phagocytic activity of macrophages, suppress activity of T- and B-lymphocytes and alter cytokine production of the host tissue (Young and Young 2002). However, the expression of T3SSs is energy-consuming and therefore activation is dependent on environmental factors. The bacterial envelope itself can be considered a data-processing center, with stress response systems acting as sensors and messengers. During infection, host factors can damage the bacterial envelope, resulting in activation of the appropriate stress response systems. At least three different envelope stress response systems are involved in the activity of the Ysc-Yop system; the phage shock protein system (Psp), the Cpx system, and σ^E. The Psp system is involved in alleviating the envelope stress caused by T3SS induction, whereas attenuation of Cpx and σ^E expression results in reduced levels of structural Ysc components present in the bacterial membranes (Darwin and Miller 2001; Carlsson et al. 2007).

The exact mechanism of involvement of σ^E during *Yersinia* infection remains enigmatic. Similarly to *E. coli*, *rpoE* is considered to be an essential gene in *Yersinia.* Studies in both *Yersinia enterocolitica* as well as *Yersinia pseudotuberculosis* failed to produce a viable *rpoE* knock-out. Unlike in *E. coli,* overexpression of RseA did not inhibit cell viability, suggesting that only small amounts of RpoE are required for *Yersinia*'s survival in laboratory conditions.

Studies into the role of *Yersinia* σ^E expression during stress appear contradictory. Heusipp et al. (Heusipp et al. 2003) reported a lack of significant changes in *rpoE* expression in *Yersinia enterocolitica* during either heat-shock or exposure to ethanol, low calcium and high salt, only significantly increasing in *rpoE* expression in the presence of high concentrations (0.49 M) of sugars not fermented by *Yersinia*. In contrast, Palonen *et al.* (Palonen et al. 2013) reported a significant change in expression of *rpoE* in *Yersinia pseudotuberculosis* during exposure to high and low temperatures, acidic and alkaline environments, high osmolality and ethanol. Although the difference in observed results could indicate a different function of σ^E in *Y. enterocolitica* versus *Y. pseudotuberculosis*, it should be noted that not only did

the experimental conditions differ, but also Heusipp *et al.* (Heusipp et al. 2003) measured expression levels using β-galactosidase assays, whereas Palonen *et al.* (Palonen et al. 2013) measured expression levels by QRT-PCR. Considering the latter technique is far more sensitive than the former, there may not be a difference in σ^E function between these strains after all.

RpoE in *Vibrio*

RpoE is required in foodborne *Vibrio* strains; deletion of *rpoE* in *V. cholerae, V. parahaemolyticus and V. vulnificus* demonstrated Δ*rpoE* mutations which could only be created in suppressor mutants (Brown and Gulig 2009; Davis and Waldor 2009; Haines-Menges et al. 2014). The ability of Δ*rpoE* mutants to colonize and kill mice was assessed to determine the influence of σ^E on virulence of *V. cholerae, V. parahaemolyticus and V. vulnificus*. In both *V. cholerae* as well as *V. parahaemolyticus*, the Δ*rpoE* mutants were significantly attenuated in their ability to colonize the GI tract, with a CFU count several logs less than the parental strains. However, despite their reduced ability to infect mice, neither strain displayed reduction in T2SS expression, nor in CT production, suggesting that σ^E is involved in *Vibrio* virulence, independent of cholera toxin production or secretion (Kovacikova and Skorupski 2002; Haines-Menges et al. 2014). Unlike the previously described strains, *V. vulnificus* Δ*rpoE* mutants did not appear to be attenuated in their ability to infect adult mouse models. However, whereas the mice used to study *V. cholerae* and *V. parahaemolyticus* infections were inoculated orally, the mice used to investigate *V. vulnificus* infections were inoculated subcutaneously. This suggests that *vibrio* σ^E may play an important role for survival throughout the gastrointestinal tract (Brown and Gulig 2009).

In both *V. cholerae* and *V. parahaemolyticus, rpoE* has two promoters, i.e., an upstream promoter which binds RpoE and a downstream promotor which binds σ^{70}. The RpoE-binding promoter is stronger than the σ^{70}_binding promoter. This confirms that, similar to *E. coli*, in *Vibrio*, σ^E is capable of self-regulation during stress, as well as regulated by a general house-keeping sigma factor in the absence of envelope stress (Kovacikova and Skorupski 2002; Haines-Menges et al. 2014).

In *Yersinia*, σ^E is involved in virulence as well as envelope stress, and *Vibrio* σ^E also shares this function. Susceptibility assays for Δ*rpoE* mutant strains of *V. cholerae, V. parahaemolyticus and V. vulnificus*, revealed that σ^E responds to envelope stress to different degrees. Whereas all three strains had increased susceptibility to 3% ethanol, only *V. parahaemolyticus and V. vulnificus* Δ*rpoE* mutants were affected by high temperatures. And although neither *V. cholerae* nor *V. parahaemolyticus* Δ*rpoE* mutants were susceptible to SDS or hydrogen peroxide, the *V. vulnificus* Δ*rpoE* mutant was significantly attenuated under these conditions (Kovacikova and Skorupski 2002; Brown and Gulig 2009; Haines-Menges et al. 2014).

Interestingly, σ^E is an essential gene in *Vibrio*, as its deletion is only viable in suppressor mutants. Analysis of these suppressor mutants revealed that most *Vibrio* Δ*rpoE* mutant strains contain suppressor mutations which negatively affect the

transcription of OmpU, a general diffusor porin which constitutes a significant portion of *Vibrio*'s OMPs. Davis & Waldor (2009) noted that OmpU expression was significantly lower in *Vibrio* Δ*rpoE* mutant strains and continued to demonstrate that deletion of OmpU in *Vibrio cholerae* rendered σ^E nonessential. These results reveal there is a clear difference in RpoE function in *Vibrio* and *E. coli as* neither single nor combination of OMP gene deletions has rendered σ^E nonessential in the latter.

RpoE regulates protein expression not only by direct promoter interaction, but also by promoting expression of sRNAs. In *E. coli*, these sRNAs interact with their target mRNA via a RNA chaperone known as Hfq, which modulates gene expression post-transcriptionally. In *Vibrio,* there are no known homologues of the sRNAs regulated by σ^E in *E. coli* (Schu et al. 2015). However, a σ^E-regulated sRNA has been identified in *V. cholerae,* i.e., VrrA. This sRNA is activated by σ^E, and acts independently of Hfq. It modulates expression of *ompA*, which is an important outer membrane porin of *Vibrio*, and encourages the formation of outer membrane vesicles (OMVs). Deletion of *Vibrio vrrA* significantly affects its ability to colonizes eukaryotic cells (Song et al. 2008).

Although *Vibrio* σ^E does not regulate Hfq dependent sRNAs, Ding et al. (2004) determined that Hfq-sRNAs' complexes can suppress *rpoE* mRNA translation. Deletion of Hfq in *Vibrio cholerae* results in overexpression of σ^E, and Δ*hfq* mutants are highly attenuated in suckling mouse models of cholera. However, although Δ*hfq* mutants express elevated levels of σ^E, *rpoE* overexpression does not attenuate mouse infection models, suggesting that Hfq is involved in virulence independently of σ^E.

Interestingly, although σ^E is capable of regulating T2SS (a σ^E- dependent promoter) as well as positively affecting VrrA expression, deletion of σ^E does not affect CT production and excretion. Considering that both VrrA and T2SS are critically important for the translation and secretion of CT, σ^E is likely to be only one of multiple factors capable of regulating the expression of these genes.

Other Alternative Sigma Factors in Gram Negative Foodborne Pathogens

RpoH

RpoH Regulation

The heat shock response (HSR). Placing bacteria at temperatures above the optimum for their growth causes the induction of the production of proteins known as heat shock proteins (HSPs). Many of the HSPs are highly conserved in all living organisms indicating their importance for adaption to elevated temperature and other stresses. However the control mechanisms differ between organisms. In proteobacteria such as *E. coli* and Salmonella the HSR is regulated by the alternative sigma factor σ^H. Despite its name HSR can be induced in *E. coli*, salmonella and

other bacteria by a variety of insults including (ref). What all of these diverse stress lead to and the actual molecular cue that activates the HSR is misfolded proteins in the cytoplasm (VanBogelen et al. 1987; Arsene et al. 2000).

The regulation is complex and operates at the transcriptional, post- transcriptional, translational and post-translational levels. The *rpoH* gene has at least 6 promoters in *E. coli* and salmonella (Janaszak et al. 2009). Promoters P1, P4 and P5 promoters are all recognized by σ^D but are regulated differently (Janaszak et al. 2009). Transcription from P1 during the stationary phase of growth is mediated by σ^S and P3 and P4 are recognized by σ^E and σ^N respectively (Janaszak et al. 2009). At higher incubation temperatures (viz., >42 °C) expression of *rpoH* is directed almost exclusively by the P3 promoter via σ^E (Erickson and Gross 1989). In *S*. Typhimurium, substantial proportion of *rpoH* transcription during stationary phase is dependent on σ^E, but this may be due to the effects of σ^E on σ^S activity (Testerman et al. 2002). A number of other transcription factors also modulate expression of *rpoH* (Ramirez-Santos et al. 2001). *rpoH* is highly expressed by *S*. Typhimurium growing within a macrophage –like cell line (Srikumar et al. 2015) (http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl?query=rpoh;db=salcom_mac_HL).

The *rpoH* mRNA at low temperature adopts a secondary structure that inhibits its translation (Morita et al. 1999). This structure melts when *E. coli* is shifted to a high growth temperature which in a large part accounts for the rapid initial increase in σ^H during heat shock (Morita et al. 1999). In addition, during heat shock there is also an increase in σ^H stability (Guisbert et al. 2008). The stability and activity of σ^H are controlled by cytoplasmic chaperonins (DnaK/DnaJ/GrpE and GroEL/S) and a protease (FtsH) located in the inner membrane (Guisbert et al. 2008; Lim et al. 2013). FtsH degrades free σ^H, while the chaperonins bind free σ^H and make it unavailable to bind core RNA polymerase. Thus, both chaperonins and FtsH negatively regulate σ^H activity. How, then, does sufficient σ^H bind to core RNA polymerase to initiate transcription of genes in the σ^H regulon (the so-called heat shock proteins)? The current model is that elevated temperature (or other stresses) lead to an increase of misfolded proteins within the cytoplasm, which are substrates for FtsH and the chaperonins so will compete for binding of σ^H, hence as the concentration of unfolded proteins increases so will the concentration of free σ^H which binds core RNA polymerase and initiates expression of σ^H-dependent genes. The genes for FtsH and the chaperonins are part of the σ^H regulon and exert homeostasis on the heat shock response. They also modulate σ^H activity so that it is appropriate to the strength of the insult and switch off activity when the stress is removed and the intracellular levels of misfolded proteins decreases. The signal recognition particle (SRP) binds σ^H and delivers it to the SRP receptor, tethering σ^H to the inner membrane which is very important for regulation of σ^H activity (Lim et al. 2013). Interestingly, recent screens for members of the σ^H regulon and inducers of σ^H activity have revealed that inner membrane proteins are over-represented in both categories, suggesting that HSR responds to the protein-folding state of the inner membrane as well as to the cytoplasm (Lim et al. 2013).

The σ^H regulon in *E. coli* contains at least 126 members. Most (88) of these reside and function in the cytoplasm, but a substantial number (27) reside in the inner membrane, and some (8) are periplasmic proteins (Nonaka et al. 2006). In

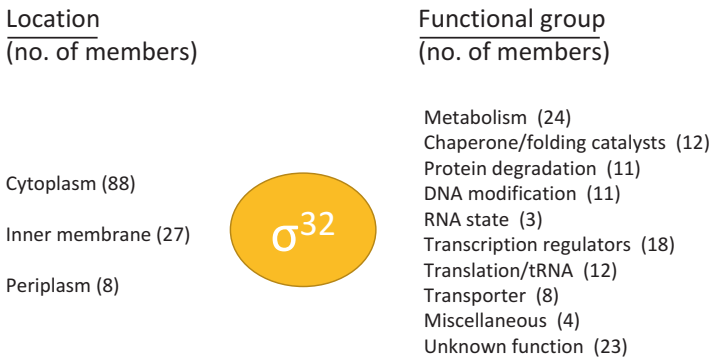

Fig. 8 In terms of numbers, location and functional group of σ^{32} members

terms of function, a substantial portion (22) of the genes encode proteins involved in protein folding or degradation but the largest functional group encode proteins involved in metabolism and 18 of the genes encode transcriptional regulators. A large number of genes (23) have unknown functions. An overview of the number, location and function of σ^H is given in Fig. 8.

RpoH Regulon Members and Pathogenesis

E. coli rpoH mutants are unable to grow above 20 °C and the same is expected of related bacteria. It is therefore not possible to assess the effect of the *rpoH* mutation on pathogenesis and virulence in mammalian and avian model hosts. It is possible to investigate the role of individual HSP (if they are not essential). However the caveat is that many genes controlled by σ^H have multiple promoters so it maybe that under the particular conditions that pertain *in vivo* when the HSP is functioning the expression of its gene may not depend solely or partially on σ^H. In an uropathogenic *E. coli* strain σ^H affects expression of *leuX* (which is associated with pathogenicity island PAI II536) that codes for tRNA5Leu and which is important for the expression of virulence factors (Dobrindt and Hacker 2001).

Vibrio cholerae. *V. cholerae rpoH* appears to be required for growth at all temperatures. An *rpoH* mutant can be isolated if the strain carries a complementing plasmid encoding *rpoH* under the control of an arabinose-inducible promoter. Comparing *in vitro* and *in vivo* (suckling mice inoculated intragastrically) the competition index of the V. *cholerae rpoH* mutant with the *rpoH* plasmid to the WT strain harboring the same plasmid, the mutant was far more attenuated *in vivo* indicating that σ^H-regulated genes are important for the colonization of murine intestines (Slamti et al. 2007).

Conversely, in a separate study, σ^H negatively affected the expression of *toxR* which encodes a positive transcriptional regulator of the genes for cholera toxin and other important virulence genes in *V. cholerae* (Parsot and Mekalanos 1990). The *toxR* gene is adjacent to and divergently transcribed to the *htpG* heat shock gene.

At 37 °C, expression of *toxR* is reduced, compared to at 22 °C. This was attributed to RNAP, with σ^H binding to the *htpG* promoter blocking access to the *toxR* promoter (Parsot and Mekalanos 1990).

Brucella melitensis. *Brucella melitensis* has five alternative sigma factors; including two homologues of σ^H, σ^{H1} and σ^{H2}, two extracytoplasmic function sigma factors, σ^{E1} and σ^{E2} and a σ^N homologue (Delory et al. 2006). Interestingly the *rpoH2* mutant grows normally at 37 °C but is unable to grow at either 21 °C or 42 °C (Delory et al. 2006). The *rpoH2* mutant also exhibits increased sensitivity to hydrogen peroxide. Strains with mutations in the other alternative sigma factor genes do not exhibit any defect in growth at different temperatures or a sensitivity to hydrogen peroxide (Delory et al. 2006). The *rpoH2* mutant was attenuated for growth/survival within macrophages, HeLa cells and mice (Delory et al. 2006). *B. melitensis* strains with mutations in the other alternative sigma factor genes except *rpoN* were also attenuated in mice but not to the same degree as the *rpoH2* mutant. Absence of σ^{H2} significantly reduce expression of two virulence factors in *B. melitensis*, i.e., the flagella and the type IV secretion system (Delory et al. 2006). σ^{H2} controls expression of the *vjbR* gene, which is a positive transcriptional activator of the type IV secretion system genes (Delory et al. 2006).

HSPs are highly immunogenic and antibodies and T cells reactive with HSPs can be detected in humans and animals infected with a variety of microbes. It has been suggested therefore that HSPs make good vaccine candidates. However, immune responses to microbial HSPs have also been implicated in the development of autoimmune disease (Leung and Gershwin 1991; van Eden et al. 2003; Rajaiah and Moudgil 2009).

ClpXP and Type III Secretion Systems (T3SS)

T3SS are major virulence factors of a number of Gram negative pathogens (Galan et al. 2014). They act as molecular syringes to inject effector proteins to alter host cells (Galan et al. 2014) to the advantage of the pathogen. The particular effect on the host cell depends on the specific effector protein(s) that are delivered to the host cell. The flagella apparatus is also an example of a T3SS (Galan et al. 2014). ClpXP protease affects expression of T3SS in a number of Gram-negative foodborne pathogens.

In *S*. Typhimurium ClpXP negatively regulates expression of both flagella and the T3SS encoded by *Salmonella* pathogenicity island 1 (SPI-1). The master transcriptional activator of flagella expression is FlhD4C2 (4 subunits FlhD 2 subunits FlhC). ClpXP degrades FlhD4C2 and this is enhanced by other proteins (Tomoyasu et al. 2002, 2003). The negative effect of ClpXP on T3SS expression is a consequence of the effect on flagella expression. FliZ post-transcriptionally controls the levels of HilD, which is a master regulator of SPI-1 T3SS expression (Kage et al. 2008). Interestingly, *fliZ* is a class 3 flagellar gene with expression is controlled by the alternative sigma factor σ^A (Chilcott and Hughes 2000).

A *S*. Typhimurium *clpP* mutant is attenuated in mice (Yamamoto et al. 2001). Following infection they persist for a long period in the liver and spleens of mice.

However, the mutant was virulent and able to kill mice deficient in interferon–γ or tumor necrosis factor-α and establish a persistent infection (Yamamoto et al. 2001). This suggests that the mutant is defective in survival in activated macrophages and indeed the mutant is attenuated in murine peritoneal macrophages *in vitro* (Yamamoto et al. 2001).

Interestingly, in Enterohemorrhagic *E. coli* (EHEC) O157:H7 Sakai, ClpXP has the opposite effect on T3SS expression. EHEC possess a pathogenicity island called the locus of enterocyte effacement (LEE), which encodes a T3SS. Inactivation of *clpPX* decreases secretion of effector proteins and inhibits transcription from all of the LEE promoters (Tomoyasu et al. 2005). The effect of the *clpPX* mutation can be partially supressed if the strain also harbors an *rpoS* mutation. ClpXP is known to degrade σ^S. ClpXP was concluded to positively control expression and activity of the T3SS by σ^S-dependent and σ^S-independent pathways (Tomoyasu et al. 2005).

In *Yersinia enterocolitica,* ClpP positively affects T3SS activity by degrading LcrG (which functions to block protein secretion by the T3SS) (Falker et al. 2006).

ClpXP is required for *Campylobacter jejuni* to grow at 42 °C which is the body temperature of poultry the major carrier of *C. jejuni* (Cohn et al. 2007). Mutations in *clpX* or *clpP* also reduce motility, autoagglutination and the ability of *C. jejuni* to invade epithelial cells, all of which suggest the virulence of *C. jejuni* maybe impaired (Cohn et al. 2007). Note that *C. jejuni* does not possess σ^H so the regulation of *clp* genes is different than that of the members of the Enterobacteriaceae.

RpoN

Originally identified as a regulator of genes for nitrogen assimilation in *E. coli* (Hunt and Magasanik 1985), σ^N has subsequently been determined to regulate genes with a variety of functions including virulence and transmission (Zhao et al. 1999; Fernando et al. 2007; Riordan et al. 2010; Hwang et al. 2011; Hao et al. 2013; Sana et al. 2013; Liu et al. 2014; Mitra et al. 2014).

In EHEC *E. coli* O157: H7 Sakai σ^N negatively regulates the *gad* genes required for glutamate-dependent acid resistance (Riordan et al. 2010). Conversely, inactivation of *rpoN* reduced expression of the LEE-encoded T3SS genes (Riordan et al. 2010). In *S.* Typhimurium an *rpoN* mutation increased resistance to the antimicrobial peptide polymyxin B. This effect was independent of the two component regulator systems (PhoP/PhoQ and PmrA/PmrB) that play a major role in polymyxin B resistance (Barchiesi et al. 2009).

The Phage Shock Protein (PSP) Response

The PSP response was so named because infection of *E. coli* with the f1 filamentous phage lead to a huge induction of a protein, i.e., phage shock protein A (PspA) (Brissette et al. 1990). Subsequently, a number of stresses have been determined to

induce the PSP response, especially stresses that disrupt the cytoplasmic membrane such as high temperature, ethanol, production of secretins by phages, (T3SS), or protonophores such as CCCP (carbonyl cyanide m-chlorphenylhydrazone) (Joly et al. 2010; Darwin 2013).

The PSP response is also highly induced in *S*. Typhimurium and *Shigella flexneri* by infection of macrophages and also by *S*. Typhimurium infection of epithelial and fibroblast cells (Eriksson et al. 2003; Lucchini et al. 2005; Hautefort et al. 2008; Srikumar et al. 2015). A full list of signals that induce the PSP response in different bacteria can be found in the following reviews (Joly et al. 2010; Darwin 2013).

In *S*. Typhimurium and *E. coli,* the *psp* genes are at two loci. The genes pspA-F are located at one loci and *pspG* at a separate location. *PspA-E* form an operon and *pspF* is located in front of pspA-E (Joly et al. 2010; Darwin 2013). Expression of the pspA-E operon (and *pspG*) is controlled by σ^N (Joly et al. 2010; Darwin 2013). Expression of *pspF* is σ^D dependent and a σ^D promoter also controls expression of *pspE* (Joly et al. 2010; Darwin 2013). The enhancer protein for expression of the *psp* genes by σ^N is PspF (Joly et al. 2010; Darwin 2013).

The major effector protein of the PSP response is PspA, a peripheral membrane protein, which is important for maintenance of proton motive force (PMF) under particular stress conditions (Becker et al. 2005; Joly et al. 2010; Darwin 2013). PspA is also a negative regulator of the PSP response. Under non-stress conditions it binds PspF, preventing it from activating σ^N activity. PspB and PspC are sensory proteins located in the cytoplasmic membrane. They sense particular stresses that require the PSP response and activate the response by binding to PspA and liberating PspF (Joly et al. 2010; Darwin 2013).

PSP Response and Virulence

Yersinia enterocolitica. Recognition of involvement of the PSP response in virulence came first from studies in *Y. enterocolitica*. Expression of the Ysc-Yop T3SS and specifically the YscC secretin protein induces the PSP response and this is required to survive the growth- secretin stress imposed by YscC (Darwin and Miller 1999a; Darwin and Miller 2001). Surprisingly, it was not PspA that was important for protecting *Y. enterocolitica* against T3SS expression but rather PspB and PspC (Horstman and Darwin 2012). A *Y. enterocolitica pspC* mutant was highly attenuated in mice (Darwin and Miller 1999a; Darwin and Miller 2001).

S. Typhimurium. The PSP response when induced in a *S*. Typhimurium *rpoE* mutant helps the survival of this mutant in the stationary phase of growth (Becker et al. 2005). Both the *rpoE* mutant and a *pspA* mutant have increased susceptibility to CCCP, and a *rpoE pspA* double mutant is more susceptible than either single mutant (Becker et al. 2005). The same is true of sensitivity to the antimicrobial peptide BPI. Further evidence indicates that σ^E is required for maintenance of PMF and that the PSP response is induced by a decrease in PMF in a *rpoE* mutant and helps combat the loss of PMF in the absence of *rpoE* (Becker et al. 2005).

Subsequently, it was determined that PspA is required for full virulence of *S*. Typhimurium in Nramp1 (also called Slc11a1)-positive mice, but not Nramp1-negative mice (Karlinsey et al. 2010). The same pattern was observed in survival in macrophages from the respective mice (Karlinsey et al. 2010). Nramp1 is expressed in macrophages and confers resistance to infection against a number of intracellular pathogens that reside within macrophages (Wessling-Resnick 2015). Nramp1 encodes a divalent metal transporter located in the phagosomal membrane. It is thought to deprive microbes within the phagosome of essential divalent cations (Wessling-Resnick 2015). *S*. Typhimurium compensates for this by using divalent transports of its own such MntH, SitABCD and ZupT (Karlinsey et al. 2010). PspA facilitates uptake of manganese by MntH, SitABCD and ZupT, and zinc by ZupT (Karlinsey et al. 2010). Both *mntH* and *zupT S*. Typhimurium mutants have reduced virulence in Nramp1$^+$ mice, but not Nramp1$^-$ mice (Karlinsey et al. 2010).

Studies on the role of individual *psp* genes on *S*. Typhimurium virulence in Nramp1$^+$ and Nramp1$^-$ mice revealed that a strain that lacked *pspABCD* was slightly attenuated in both Nramp1$^+$ and Nramp1$^-$ mice (Wallrodt et al. 2014), and a *pspB* mutant was more attenuated in both Nramp1$^+$ and Nramp1$^-$ mice than the *pspABCD* mutant (Wallrodt et al. 2014). A *pspD* mutant was slightly attenuated (to the same level as the *pspABCD* mutant) in Nramp1$^+$ mice but not Nramp1$^-$ mice, and *pspD* and *pspG* mutations did not affect virulence in either type of mice (Wallrodt et al. 2014).

Unlike *Y. enterocolitica*, *S*. Typhimurium does not require the PSP response to combat secretin stress (Karlinsey et al. 2010). Lack of a PSP response also does not affect secretion of effector proteins by the SPI-1 T3SS (Karlinsey et al. 2010); [unpublished observation] or SPI-2 T3SS [unpublished observation] or production or function of flagella of *S*. Typhimurium (Karlinsey et al. 2010).

PspE is a putative thiosulfate sulfurtransferase. In an *E. coli dsbA* mutant overexpression of PspE helps restore disulphide bond formation in envelope proteins (Chng et al. 2012). Salmonella and *E. coli* possess another thiosulfate sulfurtransferase, i.e. GlpE. A *S*. Typhimurium mutant lacking both PspE and GlpE but not either enzyme alone was slightly attenuated for systemic infection of mice (Wallrodt et al. 2013).

FliA

Flagella are organelles of motility that consist of large multimeric structures that in Gram negative bacteria are assembled via a T3SS (Aldridge and Hughes 2002). They have been attributed many roles in bacterial pathogenesis. These include: movement towards host cells and tissues; adhesion to host tissues; biofilm formation, the secretion of virulence factors and promoting bacterial invasion (Duan et al. 2013). They also act as pathogen associated molecular pattern molecules that are sensed by host innate microbe recognition systems (such as TLR-5) leading to the production of various cytokines and other signalling molecules that promote an inflammatory response (von Moltke et al. 2013).

In *Salmonella* and *E. coli* at least 50 genes are required for flagella assembly and function (Aldridge and Hughes 2002). The genes for flagella assembly are grouped into three classes according to the temporal order that they are transcribed (Aldridge and Hughes 2002). The alternative sigma factor σ^A (FliA) is responsible for expression of the late (class III) genes (Aldridge and Hughes 2002). *fliA* is a class II gene but FliA does not associate with core RNAP to enable class III gene expression until the flagella hook basal body (HBB) has been assembled. This is due to the action of the anti-sigma factor FlgM (Karlinsey et al. 2000). FlgM binds FliA and prevents it from binding core RNAP. When the HBB has been formed it transports FlgM to the exterior of the bacterial cell freeing FliA to bind core RNAP and initiate class III gene expression (Karlinsey et al. 2000).

Campylobacter

In *Campylobacter* flagella are critical virulence factors (Guerry 2007; van Putten et al. 2009; Lertsethtakarn et al. 2011; Bolton 2015). Flagella are required for colonization of poultry, infection of human volunteers and invasion of human intestinal epithelial cells (Black et al. 1988; Wassenaar et al. 1991, 1993; Grant et al. 1993; Nachamkin et al. 1993; Yao, Burr et al. 1994; Hendrixson and DiRita 2004). Many of the genes involved in flagella assembly in *E. coli* and *Salmonella* have homologues in *C. jejuni* and the genes are also classified in terms of temporal expression as class I-III. However, the control of flagella gene expression is different. A full description of flagella assembly is beyond the scope of this review but interested readers should consult the excellent recent review (Lertsethtakarn et al. 2011).

Both σ^N and σ^A regulate *C. jejuni* flagella gene expression. Most rod and hook genes have σ^N-dependent promoters whereas the major flagellin gene and other filament genes have σ^A-dependent promoters (Hendrixson and DiRita 2003; Carrillo et al. 2004; Wosten et al. 2010).

As well as transporting the flagella assembly proteins, the *C. jejuni* T3SS secretes a number of other proteins some of which (called *Campylobacter* invasion antigens, Cias) have been associated with interaction with host cells (Konkel et al. 1999, 2004; Song et al. 2004; Poly et al. 2007). *C. jejuni* mutants lacking CiaB, CiaC or CiaI are defective in invasion and/or survival within eukaryotic cells (Konkel et al. 1999; Christensen et al. 2009; Buelow et al. 2011).

As well as flagella genes, σ^A is required for expression of *ciaI* and four other genes termed feds (flagellar co-expressed determinants). *C. jejuni* mutants lacking CiaI, FedA, FedB, FedC or FedD have reduced ability to colonize the intestinal tract of chickens and the *fedA* mutant also has a reduced ability to invade a human intestinal epithelial cell line (Barrero-Tobon and Hendrixson 2012).

The effect of mutations in *rpoN, fliA* and *flgK* on production of flagella, secretion of proteins and virulence phenotypes was compared (Fernando et al. 2007). In *S.* Typhimurium *flgK* mutants retain the hook capping protein FlgD and cannot form a flagellum filament (Muramoto et al. 1999). All *C. jejuni* mutants had severely reduced motility. The *fliA* mutant was not affected in its ability to adhere to HeLa

cells, but the *rpoN* and *flgK* mutants were affected, albeit slightly (Fernando et al. 2007). All the mutants were significantly less invasive for HeLa cells than the WT parent. The *rpoN* and the *flgK* (but not the *fliA*) mutations abolished the ability of *C. jejuni* to secrete proteins. All three mutations compromised the ability of *C. jejuni* to colonise the cecum of chickens (Fernando et al. 2007). However, the attenuation of the *fliA* and *rpoN* mutants was much greater than that of the *flgK* mutant (Fernando et al. 2007).

Conclusions

Alternative sigma factors play critical roles in the biology of all the major bacterial foodborne pathogens. They regulate the expression of genes that allow the pathogen to adapt to environments inside and outside of the host. Their strict recognition of promoter sequences allows them to regulate the expression of large set of genes (regulons) in global way. Many alternative sigma factors are constitutively produced but are held in an inactive state (for example by binding an anti-sigma factor or phosphorylation state). This allows for rapid activation of the sigma factor and the regulon it controls enabling the bacteria to adapt swiftly. This is critical if the sigma factor is essential for responding to threats that are capable of liking the bacteria.

A great deal is now known about alternative sigma factors but there is much to learn particularly about their regulation and the function of specific regulon members during different stages in the pathogenesis of infection and in environments outside of the host including during food processing. For example what are the particular cues and pathways that activate different alternative sigma factors during infection? Are the same cues responsible for activating the homologous alternative sigma factors in bacteria with different life-styles, e.g. intracellular vs intracellular pathogens?

The roles of many genes that are members of regulons controlled by alternative sigma factors are still to be determined. Traditionally they would be investigated by mutating the gene to inactivate it or so that it produces a mutant protein with altered properties. There may be homologues of the particular gene or genes that encode a protein with a similar biochemical activity to the product of the gene of interest. If this is the case then analysis of strains with multiple mutations maybe necessary to completely elucidate the role of all alternative sigma factor regulon members *in vivo* and *in vitro* (Humphreys et al. 2003; Farn and Roberts 2004).

There is a great deal of interesting in the role microbiome in different clinical conditions in both humans and animals. The microbiome is known to be important in providing natural resistance against infection with a number of foodborne pathogens. Whole genome sequencing of microbiome populations has identified many new species of bacteria and thousands of new genes. It is almost certain that many of these genes will be regulated by alternative sigma factors. It will be very interesting to see if these alternative sigma factors and the genes they control are involved in any of the pathogenic or protective functions associated with the microbiome.

Finally, there is an urgent need for new antimicrobial agents, particularly against Gram negative pathogens. Given the importance of alternative sigma factors to bacterial pathogens *in vivo* they are potentially attractive targets for developing novel antimicrobial agents. An inhibitor of σ^E activity has already been identified (El-Mowafi et al. 2015).

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The Effects of Environmental Conditions and External Treatments on Virulence of Foodborne Pathogens

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Abstract Pathogenic microorganisms engage an array of virulence factors to successfully infect their host. The expression of such virulence factors is modulated by changes in the biosphere, and chemical cues through receptor-mediated communications. Recent research has identified several sensory mechanisms and compounds that facilitate chemical cross-talk between microbes or between microbes and the external environment, thereby facilitating pathogens to efficiently utilize their virulence attributes. This chapter highlights the various virulence mechanisms applied by microbes, especially foodborne pathogens, and the effects of external environmental conditions and physico-chemical treatments on the expression of major virulence factors, with an aim to develop effective intervention strategies for enhancing the safety of foods.

Keywords Foodborne pathogens • Virulence • Temperature • Desiccation • Irradiation • Pressure • Packaging • pH • Cross protection

Introduction

The development of agriculture around 12,000 years ago was one of the greatest scientific advancements of humans that transformed the human society from a traditional hunter-gatherer lifestyle to a more developed and organized culture with a reliable food supply. This led to permanent settlements around fertile lands and laid the foundation of the modern age. The industrial revolution followed and the world human population increased from around 5 million people to 7.3 billion today (http://www.census.gov/popclock/). The world population is further expected to grow over a third, reaching ca. 9.1 billion people by 2050 (FAO 2009). Greater

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demand for food and a dwindling farming community present twenty-first century agriculture with a formidable challenge. Today, ca. 2.1 billion tons of cereals are produced to meet the demand for both human and animal food (FAO 2009). In the last 50 years, meat production has nearly quadrupled from ca. 78 million tons in the 1960s to 314 million tons in 2014 to meet the unprecedented growth in demand for animal protein (Tilman et al. 2011; FAO 2014). The latest scientific projections suggest that feeding a population of ca. 9.1 billion people will require raising overall food production by 70% between 2009 and 2050 (FAO 2009).

To meet the rapidly increasing demand for food and maintain cost-efficient production, the agriculture industry has adopted highly intensive and vertically integrated crop and livestock production systems. However, the ecology of these production systems coupled with the stress they put on plants, agricultural soil and food animals, facilitates the survival and transmission of pathogenic foodborne microorganisms. Today, foodborne illnesses are one of the leading causes of morbidity and mortality in humans. In the United States, foodborne diseases results in ca. 48 million annual illnesses, 128,000 hospitalizations and 3000 deaths, which cost the U.S. economy ca. $77 billion in healthcare expenses (Scallan et al. 2011; Scharff 2012). When extrapolated to a global scale, this equates to ca. ≥1 billion cases of foodborne illnesses/year (Choffnes et al. 2012). The major food commodities implicated in these infections include produce (46% of illnesses, 23% of deaths), meat and poultry (22% illnesses, 29% deaths), dairy and eggs (20% illnesses, 15% deaths) and fish and shellfish (6.15% illnesses, 6.4% deaths) (Painter et al. 2013). Moreover, with expanding international trade, a breach in food safety procedure at one location affects consumers globally. Antibiotics have been used in animal agriculture to prevent disease and promote growth. Currently, the global usage of antibiotics in food animals is nearly double of that used in humans (Aarestrup 2012). In the United States, this accounts for nearly 80% of national annual antimicrobial consumption (FDA 2010). However, with increasing antimicrobial resistance in microbes and concerns over the toxicity of synthetic chemicals, maintaining a microbiologically safe food supply is becoming more difficult. These health concerns are further heightened due to slow development of novel pharmaceutical antimicrobials. This situation warrants development of effective intervention strategies for controlling pathogens in food processing environments and foods for reducing the risk of human infections.

Hurdle technology is one approach with potential to provide a microbiologically safe and economical supply of food to consumers. Hurdle technology employs a combination of preservation factors or hurdles that harmful microorganisms find difficult to survive (Leistner 1995). Significant advancements in the knowledge of the major factors involved in food preservation (e.g., temperature, pH, water activity) have facilitated rapid development of this technology. Since its genesis more than 60 hurdles have been identified (Leistner 2000, 2007; IFT/FDA 2003).

Although hurdle technology is effective at significantly reducing pathogen load in food products, the dynamic nature of the microbial ecology at pre-harvest, harvest and post-harvest stages makes it difficult to control all potential sources of contamination. Considerable research is currently being conducted to better understand

microbial interactions with the environment, with an aim to develop improved intervention strategies and enhance food safety. Recently, a novel concept for developing control strategies based on anti-virulence strategies, has emerged that takes into account the effects of food preservation methods on the physiological and virulence attributes of microbes. This approach targets the virulence of pathogens that might survive the hurdles, while applying less selection pressure on the development of resistance, as compared to traditional strategies that primarily aim to kill microbes, but potentially select for resistant pathogens (Rasko and Sperandio 2010).

This chapter highlights major virulence mechanisms in foodborne pathogens and effects of various environmental changes, chemical cues and external treatments on expression of bacterial virulence attributes.

Virulence in Microbes: Major Factors and Mechanism(s) of Action

Traditionally, the term virulence is used to describe the inherent ability of a microbe to cause infection in its host (Casadevall and Pirofski 1999). It is derived from the Latin word *virulentus* meaning full of poison (Pepper 1949; Wain 1952). With a deeper understanding of microbial sensory mechanisms, and cell-to-cell communications (i.e. quorum sensing), the concept has now broadened to include host-microbe interactions that promote disease progression, and takes into account the role of host's internal environment, physiology and immune status. Therefore, a key point in this chapter is that virulence is a phenomenon that is heavily dependent on the interaction of the pathogen with its immediate environment rather than being an independent and constitutively expressed characteristic (Poulin and Combes 1999; Casadevall and Pirofski 2003).

Virulence Factors and Pathogenicity Islands

Microbial virulence is mediated by an array of virulence factors that facilitate pathogen survival in the environment and infection in the host (Falkow 1991; Finlay and Falkow 1997). These factors include (1) motility organelles that promote movement to favorable microbiological niches, adherence to external surfaces and colonization of host organs (Josenhans and Suerbaum 2002; Haiko and Westerlund-Wikström 2013), (2) surface receptors and membrane proteins critical for adhesion and invasion of host tissues (Foster et al. 2014; Ribet and Cossart 2015), (3) biofilms and polysaccharide capsules that facilitate persistence of pathogens in the processing environment and confer protection from phagocytosis in the host (Bridier et al. 2015; Cappitelli et al. 2014; Chmielewski and Frank 2003; Parsek and Singh 2003), and (4) secretory proteins, especially toxins that destroy host tissue (Taylor and Roberts 2005; Wu et al. 2008). These factors are either concentrated

on the cell surface, released into the external environment, or are directly injected into target tissue via specialized secretory systems to mediate pathophysiology in the host (China and Goffaux 1999; Tomich et al. 2007).

The genes for most virulence factors are located on distinct genetic elements called pathogenicity islands on the chromosomes of microbes (or sometimes plasmids). Although pathogenicity islands exhibit significant inter-genus or inter-species variability in their structure and function, some key aspects are common among many pathogens (reviewed in Gal-Mor and Finlay 2006). These common pathogenicity islands occupy relatively large regions of the microbial chromosome that can range from ca. 10 to 100 kb. In addition to virulence genes, pathogenicity islands harbor genes coding for genetic mobility such as integrases, transposases and origins of replications, which are often flanked by tRNA genes for efficient protein synthesis (Gal-Mor and Finlay 2006). The 3′ end of tRNA genes also act as preferred targets for integration of certain plasmids and phages due to sequence similarity with the natural attachment sites of phages (Reiter et al. 1989). Some pathogenicity islands are also flanked by direct repeat sequences (ca.16–20 base pairs [bp]) that are homologous to phage attachment sites and act as recognition sequences for excision enzymes, contributing to mobility of the island through horizontal gene transfer to other microorganisms (Hacker et al. 1997; Jain et al. 2002). Pathogenicity islands have been identified in majority of pathogenic bacteria, including but not limited to *Escherichia coli* (Middendorf et al. 2001; Oelschlaeger et al. 2002), *Salmonella* spp. (Hansen-Wester and Hensel 2001; Hapfelmeier et al. 2005), *Shigella* spp. (Moss et al. 1999; Ingersoll et al. 2002; Schroeder and Hilbi 2008), *Vibrio cholerae* (Karaolis et al. 1998; Faruque and Mekalanos 2003; Rajanna et al. 2003), *Listeria monocytogenes* (Vazquez-Boland et al. 2001), *Staphylococcus aureus* (Mir-Sanchis et al. 2012), and *Clostridium difficile* (Cohen et al. 2000).

Virulence Regulation and Role of Stress Signals

Environmental conditions and chemical treatments that perturb the physiological balance of microbes constitute a stress. In the case of foodborne microbes, these include various treatments used in processing foods and the environmental changes encountered in the host. Microbes have well-developed stress response systems contributing to the survival of pathogens. The two most extensively studied stress response systems are the SigB (alternative stress sigma factor) in Gram-positive microbes and the RpoS (alternative sigma subunit of RNA polymerase S) in Gram-negative bacteria (Abee and Wouters 1999; Wesche et al. 2009). These systems control the expression of more than 50 genes involved in stress response, including temperature fluctuations, pH, osmotic changes, nutrient starvation and oxidative stress (Volker et al. 1992; Loewen et al. 1998; Abee and Wouters 1999; Hengge-Aronis 2002; Rowbury 2003). It is becoming increasingly evident from recent research that stress regulation and virulence expression are interconnected (Garner et al. 2006; Chaturongakul et al. 2008; Wesche et al. 2009; Begley and Hill 2015).

The role and mechanism of action of transcriptional regulators have been thoroughly discussed in previous reviews (Kazmierczak et al. 2005; Chaturongakul et al. 2008; Dong and Schellhorn 2010); however, the relation between external environmental factors and virulence response of pathogens has received relatively little attention. The subsequent sections of this chapter summarize the effects of various stressors on foodborne pathogen virulence attributes and potential mechanism(s) of action.

Effects of Environmental Conditions and External Treatment on Pathogen Virulence

Since microbial virulence is governed by its interactions with the surrounding physico-chemical environment, it is possible to modulate the virulence attributes of a pathogen by judiciously changing the external environment. The following discussion highlights studies revealing the effects of changes in environmental conditions or chemical cues on pathogen virulence.

Temperature

Environmental temperature is a critical signal that triggers the expression of important survival and virulence traits in many microbial species. Pathogenic microbes have acquired a plethora of molecular mechanisms that utilize nearly all macromolecules, including proteins, lipids and nucleic acids as thermosensors to detect changes in environmental temperatures and respond rapidly (Johansson et al. 2002; Shapiro and Cowen 2012). In the food industry, thermal processing is one of the most commonly employed intervention strategies to control foodborne pathogens and provide a commercially sterile and/or shelf-stable product. Commercial sterility/shelf stability refers to product conditions that render it free of microorganisms capable of reproducing under normal non-refrigerated conditions of storage or distribution (Awuah et al. 2007). The choice of a thermal process depends on many factors, including (1) the physical characteristic of food, (2) the thermal resistance of microorganism, and (3) the pH, water activity and salt content of food (Fellows 2009). Over the years, the technology has been improved from "in-container sterilization" to encompass several processing and packaging techniques that minimize the impact of thermal treatments on nutritional and organoleptic properties of food. These include agitation retorts, thin profile processing, aseptic thermal treatments, and electromagnetic heating (reviewed in Awuah et al. 2007).

Since thermal inactivation of microorganisms follows first-order semi-logarithmic kinetics, it is theoretically impossible to obtain a product completely devoid of microorganisms irrespective of the length of processing time (Fellows 2009). Moreover, an exposure to sublethal heat shock, or change in environmental temperature (e.g., temperature abuse of food product) can trigger a transition from

a saprophytic to a pathogenic lifestyle (Freitag et al. 2009), changes in membrane composition (Annous et al. 1997; Edgcomb et al. 2000), and expression of factors critical for persistence in food processing environments such as biofilm formation (Bonaventura et al. 2008; Pan et al. 2010; Lee et al. 2013; Upadhyay et al. 2013a, b). Therefore, recent research has investigated the effects of various temperature treatments on virulence attributes of foodborne pathogens.

The foodborne pathogen *L. monocytogenes* is known for its ability to grow at a wide range of temperatures from 4 to 45 °C. Mechanistic studies have revealed that temperature-dependent expression of virulence genes in *L. monocytogenes* is controlled by a transcriptional activator, PrfA (Leimeister-Wächter et al. 1990, 1992; Mengaud et al. 1991; Renzoni et al. 1997), and an RNA thermosensor (Johansson et al. 2002). PrfA binds to a 14-bp palindromic sequence present at PrfA-regulated promotors (Freitag et al. 1993; Bockmann et al. 1996; Sheehan et al. 1996) and results in the expression of virulence genes. The protein fails to bind to the promoter a temperatures below 30 °C resulting in reduced expression of the virulence genes, although the *prfA* gene is transcribed (Leimeister-Wächter et al. 1992; Renzoni et al. 1997). Duodu, et al. (2010) investigated the effects of storage temperature (0, 4, 20 °C) on the expression of virulence genes in *L. monocytogenes* grown in a salmon matrix. Of the four virulence genes tested (*hlyA, actA, inlA* and *prfA*), the expression of *inlA* coding for invasion protein Internalin A was significantly upregulated at higher temperature, indicating that exposure to temperature abuse might influence the virulence potential of *L. monocytogenes.* Follow-up Caco-2 invasion assay and mouse infection experiments revealed that the strains, maintained at lower temperature (0, 4 °C), had reduced invasion and virulence potential as compared to strains maintained at 20 °C. In another study, the global transcriptome response of *L. monocytogenes* was studied after heat shock using microarray analysis (van der Veen et al. 2007). Transcription levels were measured after exposure to 48 °C and compared to a control (37 °C). The genes that form part of the heat shock response had increased expression. In addition, PrfA-regulated genes coding for invasion (*inlA, inlB*) and intracellular survival (*plcA*) were upregulated. A change in environmental temperature (cold stress of 10 °C for 24 h) also increases resistance in *L. monocytogenes* to antibiotics, including streptomycin, gentamycin, ampicillin, penicillin, tetracycline and doxycycline (Al-Nabulsi et al. 2015).

Similar to *L. monocytogenes*, *Salmonella* has a well-developed genetic thermosensing and regulatory mechanism (Hurme et al. 1997). Yang et al. (2014) investigated the effects of growth temperatures (10, 25, 37, 42 °C) on *Salmonella* Enteritidis heat/acid resistance, membrane composition and virulence-related gene expression. These researchers observed an elevated expression of virulence-related genes-*spvR, hilA, avrA* at 42 °C as compared to lower temperatures (10, 25, 37 °C). Incubation at 42 °C increased heat resistance of *Salmonella,* whereas acid resistance was maximum at human body temperature (37 °C), indicating appropriate virulence attributes were selected based on environmental cues in the pathogen. An increase in heat resistance was also accompanied by an increase in saturated fatty acids in the membrane to maintain optimal fluidity and function of lipid bilayer. Similar results were obtained by Hinthong et al. (2015) who investigated the effects of temperature

(29–40 °C) on the expression of fimbrial gene expression, adherence and biofilm formation in Enteroaggregative *E. coli*. An increase in temperature resulted in an increased expression of fimbrial gene *aafA;* however, bacterial adherence to Hep-2 cells was not significantly affected. Interestingly, biofilm formation was higher at 29 °C than at 38 °C. Since biofilm formation is a survival strategy in the environment, increased biofilm formation at ambient temperature as compared to a human body temperature indicates the presence of a well-developed thermosensory mechanism in the pathogen.

Irradiation

Commercial application of ionizing radiation to improve food safety and shelf life was initiated in the 1960s (Olson 1998), but due to consumer concerns, its usage in foods has been limited (WHO 1999). However, with advancement in technology, safety and social awareness, the perception towards irradiated food products is changing. The irradiation process involves applying ionizing (electromagnetic waves, X-rays, or electron beams) or non-ionizing (ultraviolet light) radiation to foods at doses of 2–10 kGy and 400 J/m^2, respectively (FDA 2015a, b). The technology has been very effective in reducing pathogen loads, with minimal deleterious effects on the organoleptic qualities of food (Yip and Konasewich 1972; Abu-Tarboush et al. 1996; Sherry et al. 2004). Rajkowski and Thayer (2000) determined that gamma radiation significantly reduced *E. coli* O157:H7 and *Salmonella* spp. on naturally contaminated sprouts with a radiation D-value in the range of 0.3–0.5 kGy. Similar results were obtained on alfalfa seeds (Thayer et al. 2003), and chicken meat (Thayer and Boyd 1993; Thayer and Boyd 1991).

The primary target for irradiation is the bacterial chromosome. Ionizing irradiation inactivates bacterial cells by either acting directly on the target molecules (nucleic acid proteins) or indirectly, e.g. through the production of reactive chemical species such as the hydroxyl radical leading to DNA disruption, impaired cellular metabolism and cell death (Sharp 1939; Hill 1970). The sensitivity of microbes to irradiation depends on membrane composition (Patterson and Loaharanu 2000), DNA repairing mechanisms (Moseley 1989; Hanawalt 2012), and composition of the treatment atmosphere (van Gerwen et al. 1999). Sublethal injury has also been detected in irradiated cells that can lead to modifications in microbial DNA and development of tolerance or resistance in the organism (Ayari et al. 2012). Radiation resistance has been observed in foodborne pathogens as a result of cross-adaptation by other stresses. Mendonca et al. (2004) determined that starvation stress increased the survival of *L. monocytogenes* in ground pork as compared to a control. In addition, no reduction in the virulence attributes of starvation-adapted, irradiation-treated cells was observed. Similarly, acid stress induced radiation resistance in *E. coli* O157:H7 (Buchanan et al. 2004). Other researchers reported similar findings with salt stress (Sommer et al. 2003), temperature and desiccation (van Gerwen et al. 1999). Although very few studies have investigated effects of radiation on

virulence attributes of microbes it is likely to occur due to induced random mutations by irradiation, presence of protein moonlighting (Henderson and Martin 2011, 2013) and transcriptional factors controlling multiple protective mechanisms in bacteria. For example, DNA repair in most bacterial species is mediated by the RecABCD, AddAB and AdnAB systems (reviewed in Dillingham and Kowalczykowski 2008; Wigley 2013). Interestingly, RecA also modulates adhesion and invasion of *L. monocytogenes* while imparting acid and bile resistance in the organism (van der Veen and Abee 2011). An in-frame deletion in *recA* reduces adhesion-invasion efficacy and reduces survival of the pathogen at low pH and in bile. Therefore, a mutation in the DNA repair system would potentially affect certain virulence factors. In a recent study, low-dose irradiation modulated virulence attributes, including biofilm formation in the oral pathogens *Streptococcus salivarius* and *Klebsiella oxytoca* (Vanhoecke et al. 2015). In another study, inactivation of staphylococcal virulence factors was observed in response to photodynamic therapy, consisting of laser (665 nm wavelength) and light-activated methylene blue (Tubby et al. 2009). Major virulence factors affected include protease, hemolysin and sphingomyelinase activity.

Desiccation and Osmotic Stress

Desiccation or removal of water from cells is widely used for food preservation. This is usually achieved by air-drying or the addition of large amounts of salt or sugar during food processing, thereby reducing water availability, or water activity (a_w; $a_w < 0.87$) for bacterial metabolism and growth. Bacterial communities undergo a change in surface area, shape, and texture in response to desiccation (Mattick et al. 2001, 2003; McMahon et al. 2007). At the cellular level, changes include membrane damage, increased viscosity, reduced fluidity and modifications in physiological processes such as the viable but nonculturable state (VBNC) (Rahman et al. 1994; Stern et al. 1994; Pommepuy et al. 1996; Asakura et al. 2002; Potts et al. 2005). At the molecular level, DNA damage is observed due to dehydration, chemical metabolism (Maillard, Fenton reactions), and the generation of reactive oxygen species (Potts 1999, 2001). Osmotic stress, a physiological consequence of the process of desiccation, is observed at an early stage of the process and consists of both physiological and genetic changes (Potts 1994; Hill et al. 2002). Ion uptake is stimulated through the Kdp/Trk system along with changes in the cytoplasmic osmolarity by storing osmolytes such as glycine, betaine and carnitine (Wood et al. 2001; Sleator and Hill 2002). Osmolyte transporter (e.g., BetL, Gbu, OpuC) and synthesis proteins (e.g., ProAB, involved in proline generation) play a critical role in this osmoadaptation (Wood et al. 2001; Sleator and Hill 2002; Wemekamp-Kamphuis et al. 2004) and are upregulated during osmotic stress and the desiccation process (Aspedon et al. 2006; Weber et al. 2006).

Although an environment of low-a_w foods (e.g., cereals, dried meat, milk powder, peanut butter) poses a significant challenge to microbial growth, foodborne

pathogens can survive in low-a_w foods for long periods of time (Beuchat et al. 2013). The major foodborne pathogens occurring in low-a_w foods, include *Bacillus cereus* (Farakos and Frank 2014), *Cronobacter* species (Iversen and Forsythe 2004; Restaino et al. 2006; Baumgartner et al. 2009; Norberg et al. 2012), *L. monocytogenes* (Blessington et al. 2012), pathogenic *E. coli* (Deng et al. 1998; CDC 2011; Beutin and Martin 2012), *Salmonella* (Barrile and Cone 1970; Archer et al. 1998; Beuchat and Mann 2010), *S. aureus* (ICMS 1996; Stewart et al. 2002) and *Clostridium spp*. (Barash et al. 2005; Silva and Gibbs 2010). In the desiccated state, vegetative cells and spores can remain viable for several months or even years (Silva and Gibbs 2010; Beuchat et al. 2013). Desiccation stress also confers cross protection against thermal inactivation (Shen et al. 2011; Chen et al. 2013) and other stresses, including oxidative damage, and irradiation (Gruzdev et al. 2011).

Among the foodborne pathogens, *Listeria* species can tolerate high osmotic stress (ca.10% salt). Exposure to salt stress results in increased production of carboxypeptidases (Bergholz et al. 2012), salt shock proteins, and transcriptional activator Sigma B that facilitates the development of osmo-tolerance in *Listeria* (Gandhi and Chikindas 2007). For *Salmonella*, most invasion factors are influenced by the osmolarity of the surrounding environment. OmpR, one of the important virulence regulators in *S.* Typhimurium, is activated by hyperosmolarity (high water activity) (Dorman et al. 1989). In *E. coli*, osmotic stress induces Pex and heat shock proteins while simultaneously inhibiting DNA replication, cell growth and nutrient uptake (Chung et al. 2006). In addition, accumulation of trehalose (Abee and Wouters 1999), betaine and proline via activating specific transporters occurs to increase cytoplasmic osmolarity and prevent damage to the cell (Csonka and Epstein 1996). Bile salts serve a major signal for osmotic stress and virulence in pathogenic *E. coli*. Research reveals that bile salts induce the topical antibiotic polymyxin resistance and the production of cationic antimicrobial peptides in enterohemorrhagic *E. coli* (Kus et al. 2011) and significantly increase the adhesion of enteropathogenic *E. coli* to host cells (de Jesus et al. 2005). A similar increase in adhesion, invasion and intracellular infection due to salt stress was recently observed in *Burkholderia pseudomallei* (Pumirat et al. 2014).

Pressure

High-pressure processing (HPP), also known as high hydrostatic pressure (HHP) or ultrahigh pressure processing (UHPP), is a novel approach for reducing the load of foodborne pathogens and extending shelf life of foods without significantly deteriorating the sensory or nutritional value of food products (San Martin et al. 2002). In this technique, the food (liquid or solid) is subjected to pressures ranging from 100 to 800 MPa (58,015–116,030 psi). Process temperatures vary from 0 to 100 °C and the treatment time from milliseconds to over 20 min (although a time this high would preclude its use in industrial food applications) (Paterson 2005; FDA 2015). This technology has been effective in inactivating foodborne pathogens such as

S. aureus, *Streptococcus agalactiae, S.* Enteritidis*, E. coli* O157:H7, *and L. monocytogenes* in food products, including milk (Vachon et al. 2002; Koseki et al. 2008; Viazis et al. 2008), juice (Nienaber and Shellhammer 2001; Whitney et al. 2008) and pork (Koseki et al. 2007; Marcos et al. 2008; Porto-Fett et al. 2010). The main site of action for high-pressure processing is the bacterial cell membrane. The lipid bilayer of the membrane undergoes phase transitions while under exorbitant pressure, which causes a disruption of hydrocarbon chains leading to leakage of intracellular substances from the cytoplasm of microorganisms (Kato and Hayashi 1999). Because of its action on membranes, the antimicrobial efficacy of high-hydrostatic pressure varies with cell membrane structure (Hoover et al. 1989), the shape of microbial cells (Ludwig and Schreck 1997), the bacterial physiological state (Ludwig et al. 1996; Mc Clements et al. 2001), and the medium in which organisms are placed (Patterson 2005).

The class three-stress gene repressor (CtsR) negatively regulates the expression of heat shock genes (*clpP*, *clpE, clpC*) and contributes to pressure tolerance in *L. monocytogenes* (Karatzas et al. 2003). Liu et al. (2011) used microarray technology to study effects of *ctsR* deletion on the expression profile of *L. monocytogenes.* Compared to the wild-type strain, the entire *clpC* operon contributing to pressure tolerance was up-regulated in the *ctsR* mutant. Additionally, there was reduced expression of flagellar synthesis and virulence in the *ctsR* mutant indicating that the repressor protein potentially contributes to both pressure tolerance and virulence in the pathogen. Van Boeijen et al. (2013) reported similar findings, observing that *L. monocytogenes* strains surviving HPP treatments had attenuated virulence in a mouse infection model. In another study, 30 strains of *L. monocytogenes* were screened for pressure tolerance at 400 MPa for 2 min at 21 °C and compared based on nucleotide sequence analysis of the *ctsR* region (Chen et al. 2009). The strains had significant differences in pressure tolerance; the reductions ranged from ca. 1.9 log CFU/ml in resistant strains to ca. 7 log CFU/ml in sensitive strains. However, the sequences 200 bp upstream of the *ctsR* start codon were virtually identical among the strains, indicating that differences in the pressure tolerance phenotype observed in the strains could be based on yet unidentified transcriptional factors and/or post translation modifications of the protein. HPP modulates the master transcriptional regulator of virulence (PrfA) in *L. monocytogenes* (Bowman et al. 2008), where it was observed that high-pressure treatments (400–600 MPa) for 5 min resulted in strong suppression of the SigB (alternative sigma factor controlling stress response) and PrfA regulons, along with a reduction in energy production and carbohydrate metabolism. Expression of genes associated with DNA repair, peptidoglycan synthesis and flagellar assembly was also upregulated.

High-hydrostatic pressure modulates virulence expression in Gram-negative foodborne pathogens as well. Malone et al. (2006) used DNA microarray analysis to identify genes involved in pressure resistance of *E. coli* O157:H7. A sublethal pressure treatment (100 MPa, 15 min, 23 °C) sufficient to induce a pressure stress response was used and the expression of genes was studied. The results revealed that expression of genes varied significantly between pressure-tolerant (EC-88) and pressure-sensitive (ATCC 35150) strains. The pressure treatment down-regulated

the UspA (universal stress protein A) family of stress response genes; furthermore the DNA-binding protein gene (*dps*) was critical for protecting DNA against reactive oxygen species. The expression of Fe-S cluster assembly genes was upregulated. Since Fe-S cluster proteins store intracellular Fe, an upregulation of these genes could lead to microbial Fe toxicity and reduced resistance to pressure. The role of sigma factor (RpoE), thiol-disulfide redox system, and trehalose synthesis in modulating tolerance to pressure was also identified. Aertsen et al. (2004) determined that high pressure induces an SOS response in *E. coli* characterized by RecA and LexA-dependent expression of *uvrA, recA,* and *sulA*. The proteins, RecA and LexA, arecritical for DNA repair mechanisms in the pathogen.

Although effective, inactivation of microbial spores still remains a challenge for high-pressure technology. Spores can survive at a high pressure of 1200 MPa. Moreover, low and sublethal pressure treatments induce spore germination due to electrostriction (increase in ionization of spore contents) (Gould and Jones 1989). However, combination treatments of pressure, heat and irradiation are more effective for inactivating spores (Crawford et al. 1996; Lee et al. 2006; Silva et al. 2012). Taken together, the above studies reveal that HPP modulates stress response mechanisms in microbes, including virulence expression.

pH

Preservatives are used in the food industry to prevent spoilage, extend shelflife and improve safety. Commonly used chemical preservatives in the food industry include organic acids (e.g., acetic, lactic, benzoic and sorbic acid), chelators (e.g., citric acid and EDTA), hydrogen peroxide (Brul 1999) and salt (Taylor and Henry 2010). Most of these chemicals exert their antimicrobial action by changing pH and/or osmolarity of the environment surrounding the microbes. Many foodborne pathogens are able to tolerate changes in environmental pH through acid tolerance responses that include (1) production of acid shock proteins responsible for protein and DNA repair, (2) enzymes resulting in intracellular homeostasis through efflux pumps and acid decarboxylase systems, and (3) membrane modifications (Ryan et al. 2008; Taylor and Henry 2010; Alvarez-Ordonez et al. 2011, 2012). The acid-induced adaptive proteins, in turn, protect pathogens from death due to subsequent acid stress. Acid tolerance responses have been observed in major foodborne pathogens, including *S.* Typhimurium, *E. coli* O157:H7, *L. monocytogenes,* and *S. aureus* (Foster and Hall 1991; O'Driscoll et al. 1996; Garren et al. 1998; Samelis et al. 2004; Bore et al. 2007; Melo et al. 2013), and confer cross protection to preservatives such as sodium lactate and sodium chloride (Garren et al. 1998) or to refrigerated storage (4 °C) and refrigerated abuse temperatures (ca.10–15 °C) (Samelis et al. 2004).

Several studies revealed the effects of acid stress on foodborne pathogen virulence factors. O'Driscoll et al. (1996) determined that under low pH conditions, *L. monocytogenes* acquired enhanced acid tolerance and virulence. Acid-adapted

strains produced increased lethality in mice, relative to the wild-type strains, when inoculated intraperitoneally and had greater colonization in the spleen. In another study, exposure to low pH (ca. 5) increased the invasiveness of *L. monocytogenes* to Caco-2 cells and its ability to survive in macrophages (Conte et al. 2000). Werbrouck et al. (2009) obtained similar findings with invasiveness of *L. monocytogenes* in human intestinal cells. These researchers also determined that exposure to an acidic environment altered the expression of virulence genes (*inlA, opuC, sigB*) in the pathogen. Acid stress induces the master transcriptional regulator of virulence (PrfA) in *L. monocytogenes* (Neuhaus et al. 2013). Additionally, epithelial cell invasion and the ability to infect *Caenorhabditis elegans* was enhanced. Increased tolerance to acid stress in *L. monocytogenes* is mediated by the glutamate decarboxylase (GAD) system (Cotter et al. 2001; Gandhi and Chikindas 2007) and a two-component regulatory system, LisR-LisK (Arenas et al. 2013). The glutamate decarboxylase system facilitates intracellular pH homeostasis by neutralizing protons in a decarboxylation reaction that generates gamma-aminobutyrate from glutamate (Feehily and Karatzas 2012). A similar potentiation of virulence, following exposure to acid stress, has been observed with other foodborne pathogens, including *S.* Typhimurium (Riesenberg-Wilmes et al. 1996), *S.* Enteritidis (Humphrey et al. 1996), and *E. coli* O157:H7 (Barnett Foster 2013). Enterohemorrhagic *E. coli* has three distinct systems that confer acid tolerance, including sigma factor, cAMP receptor protein, and a glutamate/arginine-dependent system controlled by decarboxylase and antiproters (Barnett Foster 2004). House et al. (2009) observed profound variations in virulence properties of acid-stressed *E. coli* O157:H7. The acid-stress impacted major virulence attributes which include bacterial motility, adhesion to host epithelial cells, and induction of host-cell apoptosis, along with significant changes in the expression of motility, adhesion and type III secretion system genes. In another study, Yin et al. (2012) observed similar findings on the effects of acid treatment on *E. coli* O157:H7 adherence *in vitro* and in ligated pig intestine; expression of *gadE* (a gene involved in quorum sensing) and the global transcriptional regulators *cyaA, fis, himA* were significantly upregulated. Likewise, Humphrey et al. (1996) determined that acid-adapted *S.* Enteritidis exhibited enhanced virulence in mice and invasiveness in laying hens.

Biopreservatives

In addition to synthetic chemical preservatives, several natural compounds have been tested as preservatives due to an increased demand for natural foods and concerns over the potential toxicity of synthetic chemicals. These include plant extracts, essential oils and probiotic metabolites such as bacteriocins and diacetyl (Holzapfel et al. 1995; Ross et al. 2002; Gálvez et al. 2007). Recent research has revealed that plant-derived compounds, in addition to their efficacy in reducing foodborne pathogens in food animals (Kollanoor-Johny et al. 2012a; Upadhyaya et al. 2015a, b) and food products (Mattson et al. 2011; Upadhyaya et al. 2013a, 2015c; Upadhyay et al.

2013b, 2014b, 2015a), can modulate microbial virulence in Gram-positive (Qiu et al. 2010, 2011; Azizkhani et al. 2013), Gram-negative (Upadhyaya et al. 2013b; Baskaran and Venkitanarayanan 2014) and fungal pathogens (Yin et al. 2015). Upadhyay et al. (2012) determined the efficacy of trans-cinnamaldehyde, carvacrol, and thymol in reducing the virulence of *L. monocytogenes in vitro.* Sublethal concentrations of these plant compounds reduced the adhesion and invasion of *Listeria* on Caco-2 cells, inhibited listeriolysin and phospholipase activity, which are critical for intracellular survival, and downregulated the expression of major virulence genes, including *inlA, inlB, hly, actA*, and *prfA*. Follow-up *in vivo* studies in the larva of invertebrate host *Galleria mellonella* (Greater wax moth) revealed that these phytochemicals increased the survival of the host challenged with lethal doses of the pathogen (Upadhyay 2014). Mooyottu et al. 2014 determined that *trans*-cinnamaldehyde and carvacrol inhibited *C. difficile* toxin production and toxin-mediated cell cytotoxicity *in vitro*. Gene expression analysis and mechanistic studies with mutant strains revealed that these compounds downregulated the expression of toxin production genes *(tcdA, tcdB)* with the potential mechanism of inhibition of CodY, the global transcriptional regulator of virulence in *C. difficile.* Similar anti-virulence has been observed with other foodborne pathogens, including *S.* Enteritidis, *C. sakazakii*, *E. coli* O157:H7, and *V. cholera,* where the plant compounds reduced bacterial motility (Kollanoor-Johny et al. 2012b), biofilm formation (Amalaradjou and Venkitanarayanan 2011; Upadhyay et al. 2013b), attachment and colonization (Baskaran and Venkitanarayanan 2014) and toxin production (Bhattaram et al. 2013). A detailed discussion on the effects of phytochemicals on microbial virulence attributes can found in published reviews (Negi 2012; Savoia 2012; Upadhyay et al. 2014a, b, 2015b).

Food Packaging

Food packaging is a technology that safeguards food products against nutrient losses, quality deterioration, and pathogen contamination. Food packaging design is governed by various physical-chemical characteristics of food products (e.g., pH, water activity) and storage conditions (e.g., temperature, duration). The common types of packaging systems include vacuum packaging, modified atmosphere packaging (MAP), and active packaging (Zhou et al. 2010; Singh et al. 2011). The packaging systems have been effective at enhancing microbiological safety (Ming et al. 1997; Gadang et al. 2008; Jin et al. 2009) and preserving the organoleptic quality of food products (Sante et al. 1994; Seydim et al. 2006; Devatkal and Naveena 2010; Siripatrawan and Harte 2010). Only a few studies have determined the effects of different packaging systems on bacterial virulence and are summarized here. Bodnaruk and Draughon (1998) investigated the effects of packaging atmosphere (vacuum and CO_2) and pH on the virulence of *Yersinia enterocolitica* on pork stored at refrigeration temperature. Following 30 days of refrigerated storage, no significant change in the stability of its virulence plasmid was observed due to the

modified atmosphere or pH. Sharma et al. (2011) determined the effects of modified atmosphere packaging in combination with temperature abuse (4 and 15 °C) on the expression of genes (*stx, eae, ehxA, iha* and *efbE*) critical for adherence and toxin production in *E. coli* O157:H7 present on iceberg lettuce. The expression of virulence genes was maximum at 15 °C and near-ambient air packaging temperatures. Modified atmosphere packaging with nitrogen or carbon dioxide reduced the expression of virulence genes, indicating that a change in packaging atmosphere potentially affects bacterial virulence. Lopez-Velasco et al. (2010) reported similar findings and observed that temperature abuse (10 °C) during storage of packaged spinach resulted in the modulation of growth and virulence in *E. coli* O157:H7.

Cross Protection by Stresses

Many foodborne pathogens are often exposed to several stresses simultaneously during food processing steps. They possess a repertoire of proteins that are induced by more than one environmental/chemical signal and perform one or more unique functions beyond the principal action of the protein. This phenomenon is known as protein moonlighting (Henderson and Martin 2011) and contributes to the development of cross protection against stresses (Liu et al. 2014). As a result of this cross-protection phenomenon, bacterial cells adapt to a particular physiological or environmental condition, which in turn protects them from stresses to which they have not been previously exposed (Juneja and Novak 2003). For example, starvation and acid stress induce thermotolerance in *L. monocytogenes*, *Salmonella,* and *E. coli* (Leyer and Johnson 1993; Lou and Yousef 1997; Rowe and Kirk 2000; Leenanon and Drake 2001). Similarly, increased tolerance against nisin and lacticin 3147 was observed in *L. monocytogenes* exposed to acid stress (Van Schaik et al. 1999). In other studies, acid adaptation of *E. coli* O157:H7 enhanced its resistance to thermal treatments and survival in apple juice (Mazzotta 2001), other refrigerated fruit juices (Hsin-Yi and Chou 2001) and fermented meat sausage (Lund and Baird-Parker 2000). Leyer et al. (1995) determined that acid-adapted enterohemorrhagic *E. coli* strains survived better during fermentation of sausage and exhibited increased resistance to lactic acid than the nonadapted cells. In addition, acid resistance enhanced survival of the pathogen in shredded dry salami (pH 5.0). Acid adaptation also confers protection against the activated lactoperoxidase system in microorganisms (Leyer and Johnson 1993; Ravishankar et al. 2000). Likewise, cross-protective effects have been observed with other stressors, including heat (Lou and Yousef 1997; Wang and Doyle 1998; Walker et al. 1999), cold (van Gerwen et al. 1999; Bollman et al. 2001; García et al. 2001), and desiccation (Browne and Dowds 2002; Skandamis et al. 2008). In contrast, potentially negative interactions between stresses leading to increased susceptibility of the pathogen can occur. For example, increased sensitivity of *Listeria* and *Vibrio* to heat was observed when the pathogens were preexposed to cold temperature (Bayles et al. 2000; Lin and Chou 2004). Molecular studies have revealed that cross-protective mechanisms in Gram-negative

and Gram-positive bacteria are regulated by RpoS and SigB transcriptional regulators (Hengge-Aronis 1996; Hecker et al. 2007) that result in the expression of several stress adaptive genes, protein folding, and DNA repair, thereby conferring an overall protective effect to the microbes in various environmental niches and in the host (Abee and Wouters 1999; Rosen and Ron 2002; Griffith 2005).

Conclusion and Future Directions

Foodborne pathogens encounter an array of environmental signals and chemical cues during their movement from farm to food products, and eventually to the human gastrointestinal tract. Research suggests that pathogens have well-developed sensory mechanisms that trigger stresses and virulence pathways that potentially adapt them for a more efficient and pathogenic existence in the host. With the availability of next generation sequencing and "omics" technologies, research investigating the effects of various physico-chemical interventions on bacterial virulence from a whole-genome perspective has gained attention in recent years. However, the challenge would be to develop strategies to incorporate the new knowledge **into practical concepts and protocols that can be implemented in industrial processes** to further increase food safety.

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The Rise of Genomics and the Promise of Whole Genome Sequencing for Understanding Microbial Foodborne Pathogens

Eric W. Brown, Narjol Gonzalez-Escalona, Robert Stones, Ruth Timme, and Marc W. Allard

Abstract Next generation sequencing of pathogens is revolutionizing the science behind clinical diagnostics, epidemiology and the field of microbiology in general. Never before have scientists had access to, and been able to analyze and compare thousands of complete genomic sequences of bacteria, parasites and viruses. This vast amount of genomic data permits a better understanding of virulence traits, adaptability to food manufacturing environments, geographical traceability and transmission to humans, along with a host of other valuable information important to food scientists and regulators. Prior to the very recent (2011) introduction of bench-top, next generation sequencers, sequencing and annotation of complete pathogen genomes took months and was too expensive to be practical on the large scale. Today, through the introduction of a few novel technologies, it is now practical for any facility to sequence a pathogen in 1–2 days at a cost of $100 or less. The widespread availability of small, easy to use, next generation sequencers is resulting in a paradigm shift in the way in which scientists approach the identification and traceability of pathogens in the environment and clinic. A longstanding problem associated with foodborne disease outbreaks is the ability to rapidly identify the food and source of the contamination. Despite the best efforts of food safety experts, the tools available for tracking and tracing foodborne outbreaks are simply too slow and insufficiently resolved to effectively pinpoint the source of the outbreak, resulting in weeks of effort, and in many cases never identifying the source of the contamination. To this end, FDA, in 2012, created an integrated pilot network of state and federal laboratories to use whole genome sequencing to enhance traceback of foodborne pathogens. Known as GenomeTrakr, the network is creating a publically available, global database containing the genetic makeup of thousands of foodborne disease-causing bacteria including *Salmonella*. At present, WGS impacts food microbiology and regulatory food science in several ways

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including: (i) support of traceability efforts during foodborne contamination events; (ii) enhanced microbiological workflow; and (iii) quality assurance of food microbiological sampling programs. Taken together, early applications of WGS deployments underscore its extraordinary utility in food safety as well as the potential for complete characterization of bacterial pathogens as they emerge in the food supply.

Keywords Whole genome sequencing • Next generation sequencing • Microbial foodborne pathogens • Adaptability • Traceability • Tracking

Introduction

Next-generation sequencing (NGS) or whole-genome sequencing (WGS) refers to highly parallel robotic genomic sequencers that are now providing the full-length genetic code or whole genome sequence (WGS) of a bacterial pathogen in a matter of hours. When coupled with effective analytical bioinformatic pipelines, accurate and stable genetic changes can be identified that can distinguish foodborne outbreak strains to the isolate level. Numerous recently published examples illustrate the ability of WGS to discern high-resolution genetic relatedness of otherwise indistinguishable isolates. At its essence, next generation sequencing of microbial pathogens is revolutionizing the science behind clinical diagnostics, epidemiology and the field of microbiology in general. Never before have microbiologists had access to, and been able to analyze and compare thousands of complete genomic sequences of bacteria, parasites and viruses. This vast amount of genomic data permits a better understanding of virulence traits, adaptability to food manufacturing environments, geographical traceability and transmission to humans, along with considerably more valuable information important to food scientists, public health specialists, and regulators.

Prior to the 2011 introduction of bench-top, next-generation sequencers, sequencing and annotation of complete pathogen genomes took months and was too expensive to be practical on a large scale. Today, through the introduction of a few novel technologies, it is now practical for any facility to sequence a pathogen in 1–2 days at a cost of $100 or less. The widespread availability of small, easy-to-use, next-generation sequencers is resulting in a paradigm shift in the way in which scientists approach the identification and traceability of pathogens in the environment and clinic.

Whole Genome Sequencing: A New Way Forward to Understanding Foodborne Pathogens and Associated Contamination Events

Metagenomic data are impacting food safety in ways not previously foreseen. Several recent applications of WGS now include enhancing surveillance of foodborne contamination events, more accurate and effective development of pathogen detection

diagnostics, and understanding the unique adaptations that now give rise to pathogen persistence in food growing and processing environments. In the US alone, associated economic burdens of foodborne illnesses are estimated collectively at $152 billion dollars annually (Scallan et al. 2011). Mitigating foodborne illness, at times, seems an intractable challenge; however, WGS is now providing timely and innovative solutions. For example, WGS is revolutionizing the science behind many aspects of food safety. The ability to compare and analyze thousands of complete genomic sequences of bacterial pathogens is a great stride forward for understanding pathogen traceability and persistence. This vast amount of genomic data even now permits a better understanding of virulence traits, adaptability to food manufacturing environments, along with other valuable information important to food scientists (Lienau et al. 2011; Allard et al. 2012, 2013; Hoffmann et al. 2014).

Perhaps most notable has been application of NGS in conjunction with phylogenetic analysis as a molecular epidemiologic tool for providing directional leads to epidemiologists in distinguishing lineages of foodborne pathogens (e.g., *Salmonella*) and revealing potential associations between isolates collected from food or environmental sources and isolates collected from clinical sources. The topic of phylogenetic methods has been well-reviewed elsewhere (Hillis et al. 1996; Kitching et al. 1998; Yang and Rannala 2012); however, the specific application of phylogenomic methods to molecular epidemiology is fairly new and thus has not been validated or extensively reviewed in the literature. In this chapter, we attempt to cover various topics around the implementation of WGS for foodborne pathogen traceability and other characterizations. First we will address the recent explosion of *Salmonella, E. coli,* and *Listeria monocytogenes* genome availability through the GenomeTrakr network and database. Second, we will review potential issues associated with skipping the classical steps of sequencing analysis, and, alternatively, moving directly from raw reads from whole genome sequences to phylogeny. Finally, we will explore methods for revealing nucleotide substitutions in their evolutionary context and what this potentially means for understanding adaptation of foodborne pathogens in food growing and processing environments.

The GenomeTrakr: An Engine for *Foodborne Pathogen* Genome Collection

Phylogenetic analysis methods are at the forefront of large-scale whole bacterial comparative genomics analysis. Harnessing the capability of the latest next-generation sequencing platforms is growing, both in government and academic research facilities. Phylogenetics is at the forefront in allowing subsequent downstream analysis, and is giving research, clinical and regulatory scientists the ability to not only use these tools to trace the source of foodborne outbreaks, but to also gain a better understanding of pathogenicity, survival mechanisms in diverse environments, and antibiotic resistance. These tools offer a great benefit and capability in both protecting human health and in reducing the damaging impact of foodborne outbreaks through more thorough understanding of how these outbreaks occur.

GenomeTrakr, managed by the US Food and Drug Administration, is an expansive, publically available genomic database for foodborne pathogens. The US section of this database for *Salmonella* can be accessed here: http://www.ncbi.nlm.nih.gov/bioproject/183844; where over 19,000 draft genomes are available for download at the time of this writing. The GenomeTrakr database and vast network of public health labs that feed it demonstrates the utility of distributed data acquisition collected in a consistent format [i.e. NGS platform, metadata fields, and quality control (QC) standards]. This database was implemented to create a pathogen detection network in which state and federal public health agencies can contribute data towards a publicly available and transparent reference database. The database is hosted by National Center for Biotechnology Information (NCBI), which is a member of the International Nucleotide Sequence Database Collaboration (INSDC). As of winter 2015, the number of labs contributing to the GenomeTrakr database exceeded 30. The GenomeTrakr members are mostly public health laboratories, with a few academic and private partners contributing as well. Details of the GenomeTrakr network and database can be found at http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS.

The hardware and software implemented by NCBI enables the comparison and clustering of draft genomes for tens of thousands of taxa and at the same time producing daily phylogenetic trees for source tracking of food and environmental isolates (results available via FTP here: http://www.ncbi.nlm.nih.gov/projects/pathogens). By using an open strategy and comparative genomics, comparative analysis of *Salmonella* and other foodborne pathogen genomes enhanced molecular epidemiologic insights are gained, which were previously deemed indistinguishable by conventional subtyping methodologies. These results reveal the important investigative role NGS tools have within a regulatory environment, while highlighting the novel additional insights provided to epidemiologic investigations through comparison to a reference database. Numerous interests can mine this large public database to gain additional molecular evolution and systematics insights into the nature of foodborne pathogens (Fig. 1).

The Application of Phylogenetics in Molecular Epidemiology (Suggest Providing a Glossary for Terms Such as Phylogenetics)

Molecular phylogenetics is a relatively mature field, with three solid decades of peer-reviewed evolutionary theory manuscripts, validating traditional methods of phylogenetics and more recently genome-scale phylogenetics, or phylogenomics. A recent paradigm shift in NGS has drastically reduced the cost of DNA sequencing, making it rapid and inexpensive enough to collect a draft *Salmonella* genome for less than fifty US dollars (i.e. NextSeq, Illumina). The shift to NGS has also had a major impact in identifying variable genomic regions on which evolutionary inference is based. Traditionally, phylogenomic analyses have used multilocus sequence

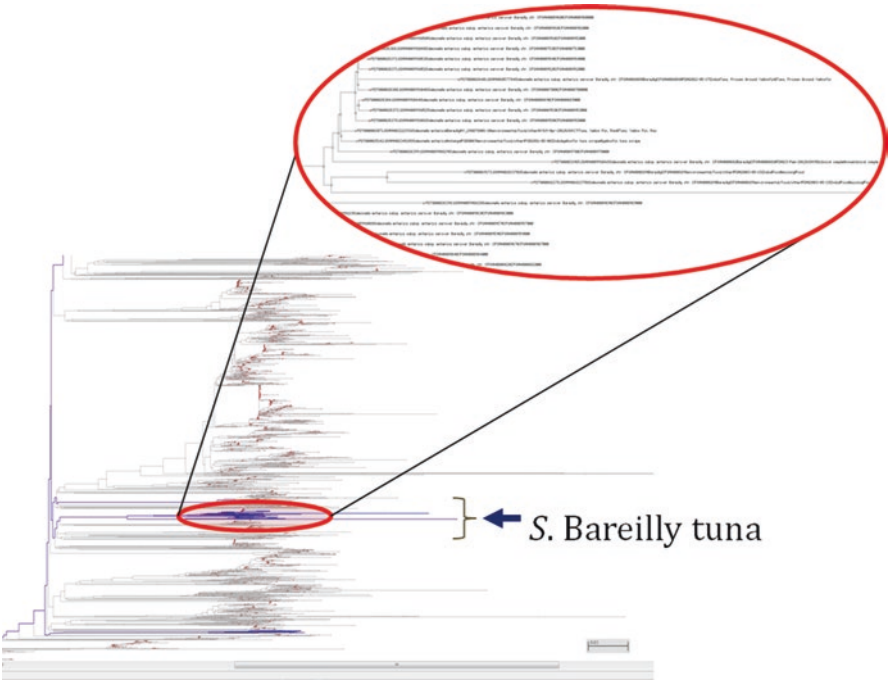

Fig. 1 Open-source phylogenetic tree of *Salmonella enterica* genomes generated by NCBI as part of the GenomeTrakr whole genome sequencing network. The tree shown highlights how specific serovars of *Salmonella* (*e.g.*, *S.* Bareilly associated with tuna) can be traced (tree leaves in *blue*) in the context of surrounding *Salmonella* genomes (tree leaves in *black*). The ovular inset in the *upper right* depicts a magnification of the bracketed *S.* Bareilly clade where more detailed relatedness and associated metadata can be observed. The tree shown is a K-mer- based tree and reflects 8244 leaves or individual whole genome sequences of different *Salmonella* isolates and strains. The tree is updated at regular intervals to include additional foodborne pathogen genomes as they are generated and reported to the database

alignments, in which orthologous housekeeping genes were determined across the taxa of interest, and then aligned orthologs were concatenated for downstream analyses. This approach bridged the 2005 NGS technology shift, starting first with PCR and Sanger sequencing, then scaling up to thousands of orthologous loci with NGS data (A. R. Lemmon and Lemmon 2012; Townsend et al. 2008; Bertozzi et al. 2012; Timme et al. 2012; Smith et al. 2014; den Bakker et al. 2011; Gonzalez-Escalona et al. 2014). In turn, the phylogenetic inference methods followed these data types with built-in assumptions that data matrixes contained contiguous discrete segments of DNA sequence.

However, this approach leaves a few areas of uncertainty when applied at the genome scale. First, ortholog determination is a hypothesis of shared ancestry, which can result in systematic errors if incorrect. Second, multigene alignments often have large amounts of missing data (i.e., gene presence/absence is highly

variable). Finally, multigene alignments can be very long with few variable characters (e.g., 20 SNPs across a 4.8 MB genome alignment for *Salmonella*), requiring vast computational resources for analysis and result interpretation.

Due to these issues, a different approach was devised where just the variable characters (SNPs) are extracted and included in the nucleotide character matrix, from which a phylogenetic tree is inferred. These are nominally called SNP-based phylogenies. There are several approaches to identifying the SNPs in a given set of taxa. Some require a reference genome as in the FDA CFSAN SNP pipeline (Pettengill et al. 2014; Davis et al. 2015), and others are reference-free, as in kSNP (Gardner and Hall 2013). The approaches are quite different from the traditional method described earlier in that these methods attempt to go from the raw reads as input files to a SNP matrix all in one pipeline, with kSNP extending the pipeline all the way to the phylogenetic tree. This eliminates the time involved in assembly, annotation and ortholog determination, greatly reducing the complexity of the character matrix to the variable-only sites. These benefits enabled SNP-based phylogenomic methods to emerge as the method of choice when analyzing real-time pathogen sequence data, such as GenomeTrakr. Unlike other rapid methods, such as Kmer (Sims and Kim 2011) and wgMLST (Applied Maths, Inc., Ridom Seqsphere+), SNP-based methods utilize the actual nucleotide changes as the core data. Identifying a physical nucleotide change aids in a regulatory framework, and having nucleotides as the core data also enables stronger statistical phylogenetic analyses. SNP-based methods have been applied across a broad evolutionary scale: the largest *Salmonella enterica* phylogeny to date was reconstructed using the kSNP method (Timme et al. 2013), whereas a much shallower *Salmonella* outbreak was investigated using the FDA CFSAN SNP pipeline (Hoffmann et al. 2015).

Even though there has been great success with SNP-based methods, many parameters need extensive validation before they can be widely utilized in the regulatory context for molecular epidemiology. Depending on the parameter, the values might have minimal or far-reaching effects. For example, when tree topology is used to identify the contamination source of foodborne outbreaks, only one split (or branch) on the tree has to be correct, i.e., the one separating the outbreak strains from non-outbreak strains. It is noteworthy that the amount of sequence coverage is important for the confidence of SNPs recovered. Typically the community collects 20–50X average coverage of Illumina MiSeq reads per genome. If the coverage is below 1X, a significant degradation in results is likely. Before phylogenomics becomes fully implemented within the regulatory framework, each of the above parameters, and probably others, will need to be fully validated. A set of publically available benchmark datasets (both simulated and real) are needed to rigorously test currently available and future phylogenomic pipelines. Several research teams are working towards this much-needed goal.

Genomics Predicts Bacterial Pathogen Phenotypes

Several additional applications and benefits of WGS technology include: (1) readily available antimicrobial resistance (AMR) profiling to 98% accuracy; (2) serotyping characterization without a need for classical antibody testing for STEC and

Salmonella (virulencefinder- https://cge.cbs.dtu.dk/services/VirulenceFinder/, and seqsero- http://www.denglab.info/SeqSero); and (3) virulence pathogenicity assessment for emerging bacterial pathogens.

Antimicrobial Resistance Typing by Whole Genome Sequencing

Genetics and WGS have been used for predicting serotypes, drug resistance and virulence of bacterial pathogens for a few years. Numerous software tools are available both free of charge, as well as fee for service (see webliography at end of chapter for a few examples). The best approach for building accurate prediction software is to combine isolates that include both the genotype with the phenotype for the same sample (Hendriksen et al. 2008; Hoffmann et al. 2014, 2015). Combining accurate phenotypic information into the metadata fields of genetic databases such as NCBI will be needed to build the knowledge database required for more accurate predictions (Pecora et al. 2015). When investigators have both the full genotype along with the antimicrobial resistance (AMR) phenotype, then they can often determine which gene goes with each AMR phenotype and can occasionally discover novel mechanisms of resistance (Chen et al. 2013). High quality WGS data coupled with molecular biology of these pathogens is uncovering new mechanisms and interactions among the AMR genes and metabolic pathways (Cao et al. 2015; Carraro et al. 2015). As the genes and mechanisms of AMR are discovered and entered into a database, it becomes relatively easy to query the database to see if the genes are present. Recent tests of the predicting power of phenotype from genotype have been conducted, and results revealed that approximately 95–100% of AMR can be accurately predicted based on the genes present in a draft genome of a bacterial pathogen (Zankari et al. 2013; Stoesser et al. 2013; Gordon et al. 2014; Zhao et al. 2015), with some differences in accuracy due to different genes in different species of the bacterial pathogen. Many investigators are working on this problem, as the increase in antibiotic resistance is a global public health concern that requires immediate action. These growing databases and software tools (McArthur et al. 2013; Carattoli et al. 2014; Saputra et al. 2015) enhance the surveillance, determine the distribution, and predict the emergence of new AMR pathogens by public health agencies.

Virulence Profiling and Other Important Phenotypic Predictions Using WGS Data

Virulence and pathogenicity markers for Shiga toxin-carrying *E. coli* (STECs) have been known for some time. Numerous genes are known and regularly tested for virulence. STEC produces one or more Shiga toxins Stx1 and/or Stx2, which are encoded by *stx*1 and *stx*2 genes, respectively, which are major virulence factors responsible

for disease. The *stx*1 gene has three subtypes (*stx*1a, *stx*1c and *stx*1d), whereas *stx*2 includes seven subtypes: *stx*2a, *stx*2b, *stx*2c, *stx*2d, *stx*2e, *stx*2f and *stx*2g (Karmali et al. 2010). Other virulence factors include intimin (*eae*), enterohemolysin (*hlyA*) serine proteases (*i.e. espP*), type II secretion effectors (*i.e. etpD*) among many others (Chapman et al. 2006; Bugarel et al. 2011; Joensen et al. 2014; Rump et al. 2015). Many of the Shiga toxin tests are PCR based, leading to numerous publications characterizing STECs from various environments (Paton and Paton 1998; Zhang et al. 2000; Durso et al. 2005; Jenkins et al. 2012; Sasaki et al. 2012; Buvens et al. 2011; Wang et al. 2012; Mellor et al. 2012), though alternative subtyping technologies also are available (Shen et al. 2012; Norman et al. 2015). Due to their pathogenicity and the danger of these pathogenetic bacteria in foods, several tests are commercially available to the food testing industry (see r-biopharm and dupont web urls). While *Salmonella* was the first outbreak foodborne pathogen tested using WGS and phylogenetics (Lienau et al. 2011), phylogenomic analysis of STECs rapidly followed, including early data sharing of German outbreak WGS draft genomes in real-time (Rhode et al. 2011 and see crowdsourced/BGI-data url). As more investigators use whole genome sequenced STECs, the databases grew, providing information on new variants and full sequences for many of these virulence factors (Fricke et al. 2008; Slanec et al. 2009; Rump et al. 2011, 2012; Mellmann et al. 2011; Bono et al. 2012; Ju et al. 2012; Ribeiro et al. 2012; Underwood et al. 2013; Cao et al. 2013; Toro et al. 2014; Lindsey et al. 2014; Pettengill et al. 2015). Investigators have been building data repositories for genes that cause pathogenicity (http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Escherichia), and a few open source genomic tools have been created to predict virulence from draft genomes (https://cge.cbs.dtu.dk/services/VirulenceFinder/). Real-time GenomeTrakr monitoring of pathogenic foodborne bacteria started in 2012 and WGSs are now available for STEC, *E. coli*, *Salmonella, Listeria* and *Campylobacter* (http://www.ncbi.nlm.nih.gov/pathogens/), which includes input from local departments of health (den Bakker et al. 2014; Taylor et al. 2015) and global collaborations from large government reference centers (Dallman et al. 2015).

The Evolutionary Context of Nucleotide Substitutions Within the Pathogen Genome

Focusing on the broader context of microbial evolution, we see that researchers are beginning to develop a better understanding of the evolutionary selective pressures that can influence the phylogeny, and therefore identify biological groupings of specific serovars or strains of bacterial pathogens implicated in foodborne illnesses. Nucleotide evolution (SNPs, indels, rearrangements, etc) across bacterial populations result in lineage differentiation, which might also contribute to phenotypic changes in the bacterium. As a result, it can be informative to map these nucleotide changes (SNPs in this case) onto the phylogenic tree (*i.e.,* the tree of strain relatedness). One method is outlined here to do this using Maximum Parsimony optimization.

One annotated genome for at least one of the samples being compared is essential. Most often bacterial genomes are annotated using NCBI's Prokaryotic Genome Automatic Annotation Pipeline (http://www.ncbi.nlm.nih.gov/genome/annotation_prok). Optimizing the characters on the branches of the strain tree to know when and where all of the nucleotide substitution changes have occurred is essential to understanding the genomic adaptive changes within a population of strains.

Once the strain lineage of interest along with the nucleotides that changed along that branch are determined, these characters can be mapped back to an annotated reference genome for further understanding of whether the changes were synonymous (did not result in an amino acid change) or nonsynonymous (resulted in an amino acid change) substitutions. Examples of genes and amino acids that are evolving among pathogens and potentially causing outbreaks and or illness are regularly published (Lienau et al. 2011; Allard et al. 2012). Interestingly, if a gene has less nonsynonymous substitutions compared to synonymous substitutions, this can be implied to be of functional significance because it is a characteristic of sequences that are being constrained to code for proteins. Nonsynonymous substitutions can give rise to nonsense mutations in which a codon is changed to a premature stop codon. This can result in truncation of the translated protein and the protein becoming nonfunctional. The result of accumulation of multiple mutations or insertions and deletions of nucleotides within a gene also provides an indication that this could be a pseudogene, whose product is no longer required for the survival of a bacterial pathogen in a particular environment.

Synonymous substitutions that alter the amino acid sequence of a protein can be explored in greater detail to determine at how this may or may not alter the structural properties of the protein. Depending on the amino acid substitution, the substituted amino acid may have similar physicochemical properties (chemical or structural), that does not have structural significance on the biological function of the protein. For example, a small aliphatic amino acid such as glycine could be replaced by alanine from the SNP mutation in the codon G<u>G</u>G to G<u>C</u>G and may have little biological significance or an acidic amino acid such as aspartic acid replaced by glutamic acid through a SNP mutation in the codon GA<u>C</u> to GA<u>G</u> and will not change the structural properties of the protein. However, the replacement of a small aliphatic amino acid leucine with the basic amino acid arginine from SNP mutations in the codon C<u>T</u>T to C<u>G</u>G potentially may have significance on the biological function of the protein. This is illustrated in the 3D graphical amino acid SNP viewer in Fig. 2 from an example of an *E. coli* protein pseudouridine synthase, in which the genetic code for the amino acid is mutated near the enzyme's active site. This mutation being detected originally as one of a number of DNA SNP mutations from one of the clades carried determined in a phylogenetic analysis.

Therefore, from a foodborne illness outbreak analysis, gene mutations identified from phylogenetic analysis and associated with a bacterial clade of interest can be translated into their corresponding protein sequences. These can be further identified as not only amino acid mutations on the primary protein sequence alignment, but can be shown to potentially alter biological function from mapping amino acids

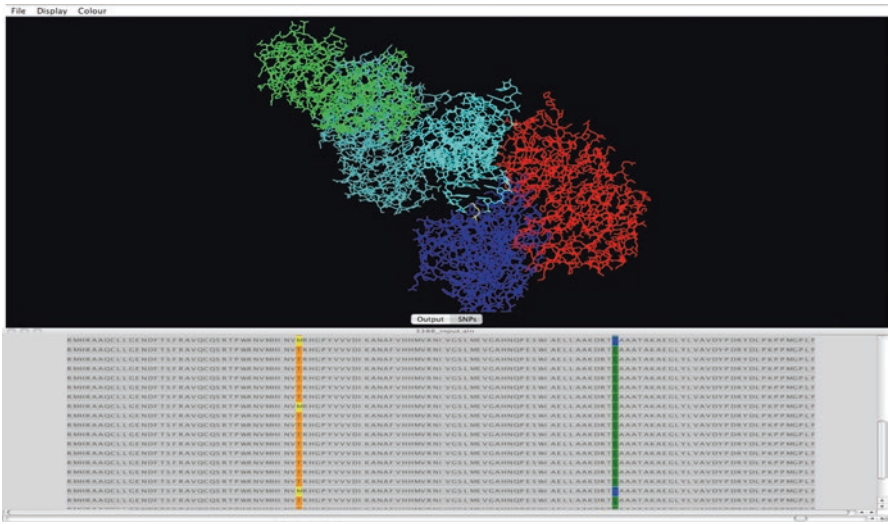

Fig. 2 Schematic overlay of primary sequence onto 3-dimensional protein structure. Comparisons of this nature reflect the merger of genomics and proteomics in predicting amino acid function and critical positions in the gene with respect to phenotype. Programs of this sort also permit real-time *in silico* evaluation of the effects of nonsynonymous nucleotide change on final protein structure

on the three-dimensional protein homology structure (Fig. 2). Using the process of annotation, assembling the variant calls, building the phylogenetic tree and optimizing the characters on the phylogenetic hypothesis, predictions can be made regarding which genes may be important to the lineages, leading to an outbreak or contamination event (Allard et al. 2012, 2013; Lienau et al. 2011, 2013).

SNPs that are not in protein-coding regions may still affect biological function of numerous noncoding RNAs, e.g., transcription factor binding and messenger RNA degradation. Noncoding RNA genes include highly abundant and important RNAs such as transfer RNAs (tRNAs) and ribosomal RNAs (rRNAs) and noncoding bacterial small RNAs (sRNAs), which belong to several groups involved in many cellular processes. These sRNAs can, for example, bind to protein targets and modify its function, or bind to mRNA targets and regulate gene expression. Virulence genes in some bacteria can be regulated by sRNAs. For example, the *invR* RNA in *Salmonella* represses synthesis of the outer membrane protein OmpD, and *sgrS* sRNA regulates the expression of the secreted effector protein SopD.

Determining cause and effect of a specific phenotype is always difficult and may require significant molecular biology experimentation. Many investigators are working to sequence genomes that have been phenotypically characterized so that one can build smart databases that include both genotype and phenotype. The promise of well-curated databases, including a detailed listing of the phenotypic metadata that accompanies each sample, will help improve these genetic predictions in absence of functional molecular biology.

Researchers are beginning to understand, from phylogenetic studies, more about the significance of DNA mutations that can be associated with a clade of interest. DNA mutations, both in coding and noncoding regions, may play a pivotal role in biological function, and may give rise to a selective advantage with regard to foodborne pathogens having the ability to survive harsh environmental conditions, or increase their degree of virulence and pathogenicity through evolutionary selective pressure.

Because of increasing DNA sequencing read lengths from next generation sequencing platforms, researchers now have the ability to completely sequence bacterial chromosomes and associated plasmids and phages. Hence, phylogenetic analysis can not only be determined from building data matrices based on SNP DNA mutations, but also from the presence and absence of genes. This enables the comparison of the loss and gain of genes between more closely related or between completely unrelated bacteria. This is because gene gain and loss can occur in two different ways, either through vertical transmission of inherited changes, or through horizontal acquisition of mobile elements that are present in a shared environment. This horizontal loss and gain is referred to as horizontal gene transfer (HGT) and plays an important role in maintenance and transmission of virulence. It is thought that acquisition of a mobile element with a cassette of genes may enable foodborne pathogens to survive in what was once unfavorable environments and also provide an increased resistance to antibiotics. In a similar manner, if the environment changes and those traits are no longer needed, then the bacteria can lose that element and not maintain it as part of their genome. The gain and loss of genes can be identified and associated with clades from the phylogenetic analysis, and can be further investigated as to the biological significance of the loss and gain of genes. For examples of published closed pathogen genomes and the characterization of mobile elements such as plasmids and phages, see Chen et al. (2013) and Hoffmann et al. (2014) who studied the genetic basis of antimicrobial resistance and the organization of genes on mobile elements.

Furthermore, genes can be compared to metabolic pathways databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG), which is a collection of manually curated databases of genomes, biological pathways and diseases. KEGG can be utilized to search against a set of genes to identify important metabolic pathways. An example pathway and the genes identified from next generation sequencing and phylogenetic analysis from a *Listeria* study searched against the KEGG metabolic pathway database is shown in Fig. 3. It could be anticipated that HGT of a complete operon might pass on a function or portion of a metabolic pathway to take advantage of a local resource in response to environmental changes and the pathogen's needs.

Whole Genome Sequencing Benefits Several Sectors of the Food Industry

Expansion and outreach of WGS technology to the various sectors of the food industry will be critical to its full implementation and success in mitigating foodborne illnesses from farm to fork. The commercial food production and processing industry,

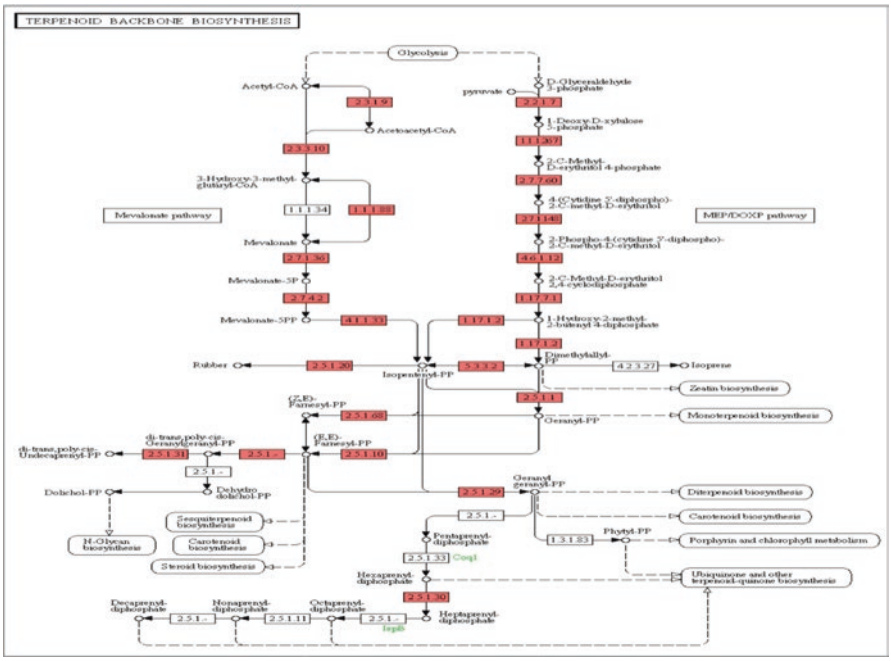

Fig. 3 Example of a functional pathway from the Kyoto Encyclopedia of Genes and Genomes (KEGG) – a collection of highly detailed and manually curated databases addressing biological/physiological pathways. KEGG can be applied to better understand the role of an operon or a disparately positioned set of genes in a metabolic or physiological pathway. The schematic specifically illustrates an example pathway and associated genes identified from next generation sequencing and phylogenetic analysis of a *Listeria monocytogenes* strain searched against the KEGG database

as a whole, performs most food safety monitoring compared to the public sector. Genomics is a new field that some industry leaders are beginning to explore. There are many applications in the area of food quality and standardization that would immediately benefit from the use of genomic technologies. Food manufacturers could use highly discriminatory data provided by WGS to track the source of pathogen contaminations to a specific ingredient or to a specific niche in the manufacturing environment. Moreover, the data could be used to enable manufacturers to efficiently detect and correct problems, early in the process. The degree to which the food industry adopts this new technology will depend, in part, on the cost of acquiring and implementing the technology. Food industry outreach and education will be key to this process, and will need to engage industry in both wet laboratory and bioinformatics training. Agriculture extension services will also be essential for outreach directly to growers through university affiliates. In addition to producers and processors, the food testing and quality sectors also represent important targets for outreach. However, the potential utility of the technology goes beyond the food production and manufacturing sectors. Diagnostics and test development companies could benefit

from WGS in extraordinary ways. Having the complete genomic maps of thousands of diverse *E. coli*, *Salmonella*, and *Listeria monocytogenes* strains from a free and open source has enabled diagnostic assay development and design to be far more comprehensive and specific than ever before. Methods including but not limited to qPCR, X-map liquid hybridization technology, and LAMP, are all technologies that can benefit from an unlimited suite of genomic maps of foodborne pathogen strains, including shiga-toxigenic and enterohemorrhagic *E. coli*. When considering potential applications of the GenomeTrakr database, it is difficult to envision all the future directions for which this database might provide a developmental foundation.

International Considerations and the Global Rise of Food Safety Genomics

Global outreach is also important and will include coordination with organizations such as the World Health Organization (WHO), International Standards Organization (ISO), and The Wellcome Trust. This includes efforts in training as well as validation of methods and integration across the global community. Such a genomic system will enable enhanced and more detailed pathogen risk assessments and preventive control strategies for various sectors of the food supply. Indeed, economic, scientific, and public health drivers exist for continued development and deployment of such "omic" technologies as WGS and, at least in the case of pathogen detection, characterization, and traceability, the framework for the operation and regulation of such a system, has recently emerged (Aarestrup et al. 2012). However, how the data from such systems will be shared among global stakeholders, including developing countries, remains an important question to be answered.

Perhaps most importantly, global deployment of such a database provides a standardized approach to frontline stakeholders such as the food testing and food safety/quality assurance communities. This should make harmonization for testing much more achievable between countries, because much of the information learned about a particular contaminant will be derived from a common shared global database with a common, transferrable, and portable data-input platform (e.g., desktop sequencers). Although partially encompassed already under the umbrella of food safety, additional drivers from academia will likely contribute to the advancement and deployment of a globally distributed next generation WGS system. That is, from an academic perspective, a database comprised of a comprehensive, sharable, and standardized type of data (i.e., WGS data) provides a single data mining vehicle along with a cornucopia of biomarkers and other diagnostic targets for international scientists to engage in more effective molecular and biochemical methods development for food safety testing and pathogen detection. Moreover, in order to operate most effectively, a genome sequence database of this nature should include a vast number of environmental strains as well as human isolates. Thus, at an even more basic level, a single data platform for microbes should encourage the unification or merging of a number of academic microbiological subdisciplines, including

environmental, clinical microbiology, and food and industrial microbiology (GMI Consensus Rep., 2011, http://www.globalmicrobialidentifier.org/News-and-Events/Previous-meetings/1st-Meeting-on-GMI). Additionally, public health organizations in both developed and developing countries would greatly benefit from an "omic-based" food safety system, in part, by providing more accurate risk assessments.

Summary and Conclusions

WGS technology platforms, similar to all methods utilized in food testing, are moving rapidly toward formal validation and certification for use in food, animal, and associated clinical settings (Allard et al. 2012; Pightling et al. 2014). Validation will be required for global acceptance of the results obtained by these genomic techniques. Validation of these platforms will also help establish the general principles for certification and legalization of these emergent molecular reference methods (Wilson et al. 2013). Whole genome sequencing is ripe for formal validation and international harmonization. It is a powerful technique that is now being used for routine analysis for the identification, serotyping, and high resolution subtyping of foodborne bacterial pathogens, including *Salmonella*, *Listeria*, and *Campylobacter*. Its use in delimiting the scope of foodborne outbreaks has been demonstrated repeatedly over the past 7 years. Moreover, open-source genome sequence databases for pathogens are now being populated heavily for food safety and other applications.

The ability of WGS technology to sort bacterial strains based on their geographic identity, host reservoir, and genetic relatedness is well known. The deployment of WGS technology will enable the sequencing and categorization of many important isolates of *Salmonella*, STEC, *Listeria monocytogenes*, and other enteric pathogenic species. These data, when submitted with the relevant meta-data, including geographic locale, food or environmental source, host, date of isolation, and serotype/pathotype, will greatly expand our knowledge of potential pathogen reservoirs as well as reveal specific geographic regions that may harbor unique WGS strain types. WGS has ushered in a new day for microbiological testing of foods for foodborne pathogens, and there is likely even more unforeseeable and proficient ways this technology will augment a safe food supply.

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Web Tools and Databases

AMR prediction software tools and databases, Ridom and SeqSphere. http://www.ridom.com/company/

BAX system for testing STECs. http://www.dupont.com/products-and-services/food-protection/dupont-food-diagnostics/brands/bax/products/bax-assays/bax-product-details/bax-ecoli-stec-rt.html

Center for Genomic Epidemiology DTU. https://cge.cbs.dtu.dk/services/VirulenceFinder/

CFSAN-snp-pipeline. https://github.com/CFSAN-Biostatistics/snp-pipeline

Database of Virulence factors in Pathogenic Bacteria. http://www.mgc.ac.cn/cgi-bin/VFs/genus.cgi?Genus=Escherichia

E Coli 0104:H4 genome analysis crowdsourcing. Sequences. https://github.com/ehec-outbreak-crowdsourced/BGI-data-analysis/wiki

FDA-CFSAN's GenomeTrakr Program. http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWG/

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PlasmidFinder, ResFinder, VirulenceFinder. http://www.genomicepidemiology.org/

Reads2Type rapid bacterial identification tool. http://www.cbs.dtu.dk/~dhany/reads2type.html

SeqSero Salmonella Serotyping by Whole genome sequencing. http://www.denglab.info/SeqSero

The Comprehensive Antibiotic Resistance Database. http://arpcard.mcmaster.ca/

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Part II
Foodborne Pathogens, Host Susceptibility, and Infectious Dose

Host Innate Immune Factors Influencing Enterohemorrhagic *Escherichia coli* Pathogenicity

Ying Zhang, Jaclyn S. Pearson, and Elizabeth L. Hartland

Abstract Enterohemorrhagic *Escherichia coli* (EHEC) is a significant foodborne attaching and effacing (A/E) pathogen that causes diarrhea, hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) in humans. EHEC is closely related to enteropathogenic *E. coli* (EPEC) and both induce characteristic A/E lesions on the gut mucosal surface. During EHEC and EPEC infection, host innate immune responses, such as inflammation and cell death are rapidly activated, upon the detection of bacterial components and virulence factor activity. To promote A/E lesion formation and dissemination of the pathogen in the body, EHEC and EPEC deliver a repertoire of effector proteins, including Tir, NleA/EspI and NleB to -H, to the host cell cytosol via a type III secretion system (T3SS). These interfere with a range of host cell processes, including host defense mechanisms. Several T3SS effector proteins specifically modify or cleave host proteins involved in inflammation and cell death, thereby inactivating these pathways. The identification of the host targets and the characterization of the biochemical function of T3SS effectors have greatly contributed to understanding the pathogenesis of EHEC and EPEC infections.

Keywords Enterohemorrhagic *Escherichia coli* (EHEC) • Type III secretion system (T3SS) • Inflammation • Apoptosis

Abbreviations

A/E pathogen	Attaching/effacing pathogen
AP-1	Activation protein-1
BI-1	Bax inhibitor-1
CLR	C-type lection receptor
CRL	Cullin Ring E3 ligases
DC	Dendritic cell

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DD	Death domains
DISC	Death-inducing signaling complex
EHEC	Enterohemorrhagic *Escherichia coli*
FA	Focal adhesion
GlcNAc	N-acetylglucosamine
HUS	Hemolytic uremic syndrome
IE	Integrative element
ILK	Integrin-linked kinase
ITIM	Immunoreceptor tyrosine-based inhibitory motifs
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MTS	Mitochondrial targeting sequence
NF-κB	Nuclear-factor κB
NLR	NOD-like receptor
NZF	Npl4 Zinc figure
OI	O-island
PAMPs	Pathogen-associated molecular patterns
PKC	Protein kinase C
PRRs	Pattern recognition receptors
RLR	RIG-I-like receptor
SAM	S-adenosyl-L-methionine
Stx	Shiga toxins
T3SS	Type III secretion system
TAB2 and TAB3	TAK1-binding proteins 2 and 3
TAD	Transcription activation domain
TIM17b	Translocase of inner mitochondrial membrane 17b
Tir	Translocated intimin receptor
TLR	Toll-like receptor
TNFR1	Tumor necrosis factor receptor 1
TRAIL	TNF-associated apoptosis-inducing ligand

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) is a major threat to human health in both low-income and high-income countries. EHEC was first recognized as a human pathogen in 1983 during two outbreaks of hemorrhagic colitis in the USA which were caused by contaminated undercooked hamburger meat (Riley et al. 1983). Early studies revealed, approximately 75% of O157:H7 EHEC outbreaks in the US were attributed to the consumption of contaminated beef products (Callaway et al. 2009), although agricultural plants, pork products, freshly consumed fruits and vegetables and water, have also been reported as a source of EHEC infection due to

contamination with animal feces (From the Centers for Disease Control and Prevention 1995; Feng and Reddy 2013). Apart from food contamination, other identified routes of EHEC transmission include person-to-person contact, animal-to-person contact and airborne transmission (Durso et al. 2005; Spika et al. 1986; Varma et al. 2003).

Among EHEC strains, serotype O157:H7 is the most prominent clinical serotype and is responsible for a large number of cases linked to food and waterborne outbreaks worldwide. EHEC O157:H7 has a very low infectious dose, with as few as 10–100 microorganisms able to cause disease in humans (Boyce et al. 1995). Upon ingestion of O157:H7, diarrhea begins after 1–8 days (average onset 3–4 days) and rapidly progresses into bloody diarrhea in more than 70% of EHEC-infected people (Mead and Griffin 1998). After ca. 7 days of diarrhea, approximately 5–10% of patients develop hemolytic uremic syndrome (HUS), with children and the elderly particularly at risk of this life-threatening complication (Manning et al. 2008; Karmali et al. 2010).

A feature of the interaction of EHEC with the intestinal epithelium is the ability of the bacteria to form attaching and effacing (A/E) lesions and to produce Shiga toxins (Stx). A/E lesions are histopathological changes on the intestinal mucosa characterized by extracellular attachment of the bacteria to the cell surface, the formation of pedestal-like structures underneath the adherent bacteria and the local effacement of microvilli (Frankel et al. 1998). A/E lesion formation by EHEC is dependent on a pathogenicity island known as the locus of enterocyte effacement (LEE), which is conserved across all A/E pathogens, including closely related enteropathogenic *E. coli* (EPEC). The LEE encodes more than 40 genes, including regulators, the adhesin, intimin, translocators, chaperones, a type III secretion system (T3SS) and translocated effector proteins that play an essential role in EHEC pathogenesis (McDaniel et al. 1995; Elliott et al. 2000). For example, the LEE-encoded T3SS effector, termed the translocated intimin receptor (Tir), binds the bacterial outer membrane adhesin, intimin, and promotes actin polymerization at the site of bacterial attachment by recruiting host proteins associated with cytoskeletal actin dynamics (Wong et al. 2011). Although EPEC and EHEC share a similar mechanism of host cell attachment, Stx additionally influences the interaction of EHEC with the intestinal mucosa. Bacteriophage-encoded Stx comprise two subgroups, Stx1 and Stx2, and individual EHEC isolates may carry one or both Stx1 and Stx2 (Kaper et al. 2004; Muthing et al. 2009). Stx is toxic for human cells and mediates the inhibition of protein synthesis resulting in large-scale cell death through apoptosis and the severe pathologies of hemorrhagic colitis and HUS (Endo et al. 1988; Karmali et al. 1983).

Aside from A/E lesion formation, EHEC and EPEC employ T3SS effectors, including LEE-encoded and non-LEE-encoded effectors, to promote bacterial colonization and survival within the host. In recent years, T3SS effectors from EHEC and other A/E pathogens have emerged as novel enzymes that specifically modify or directly degrade target host proteins, thereby interfering with fundamental biological processes such as cytoskeleton organization, inflammatory responses and apoptosis signaling pathways (Wong et al. 2011). The inhibition of pro-inflammatory

responses and apoptosis that are involved in eliminating the pathogen or restricting the severity of disease, is an essential virulence mechanism that allows EHEC to persist in the host. Here, we will focus on manipulation of the innate immune response by EHEC that is mediated through the interaction of bacterial effectors with host proteins. The closely related human A/E pathogen, EPEC, and the mouse pathogen, *Citrobacter rodentium*, will also be considered.

The Host Response to EHEC Infection

Upon pathogen infection, the mammalian innate immune system recognizes microbial molecules, including lipopolysaccharide (LPS), peptidoglycan, flagellin and nucleic acid, otherwise known as pathogen-associated molecular patterns (PAMPs) (Akira et al. 2006). The detection of PAMPs via a suite of pattern recognition receptors (PRRs) leads to activation of the innate immune response that provides immediate defense against infection. Based on their localization, PRRs are classified into cell surface receptors, such as Toll-like receptors (TLR) and C-type lectin receptors (CLR), and cytoplasmic receptors, including NOD-like receptors (NLR) and RIG-I-like receptors (RLR) (Takeuchi and Akira 2010). The initiation of innate immune signaling by PRRs leads to the activation of pro-inflammatory gene expression, which results in the production of cytokines, chemokines and antimicrobial peptides that ultimately facilitate elimination of the pathogens either by direct killing or phagocytosis.

Following ingestion, EHEC initiates infection of the surface epithelium of the intestinal mucosa. The intestinal epithelium primarily functions to separate host tissues from the external environment but also plays an essential role in activating the immune response to pathogenic microorganisms (Wong Fok Lung et al. 2014). Infected epithelial cells generally express increased secretion of cytokines, chemokines and antimicrobial peptides, which results from the activation of NF-κB and MAPK signaling pathways. During EHEC and EPEC infection, high levels of Interleukin 8 (IL-8) expression and secretion in epithelial cells accompanied by the infiltration of neutrophils at infected sites have been demonstrated in a number of *in vivo* studies (Tzipori et al. 1985, 1989). This phenotype has been mainly attributed to the recognition of bacterial virulence factors through PRRs. Stx has also been implicated in triggering a marked increase of IL-8 production in polarized T84 human intestinal epithelial cells and Caco2 cells (Thorpe et al. 1999, 2001). However, depletion of H7 flagellin rather than Stx from the EHEC culture supernatant fluid abolished IL-8 induction in epithelial cells, suggesting flagellin is the principal factor that contributes to the EHEC-induced inflammatory response (Zhou et al. 2003; Berin et al. 2002). Consistently, EHEC H7 flagellin significantly enhanced transcription of IL-8 and Chemokine Ligand 20 (CCL20) in colonic epithelial cells and induced neutrophil infiltration in an *in vivo* study using human intestinal xenografts (Miyamoto et al. 2006). In addition to flagellin, EHEC LPS is associated with the stimulation of immune responses. *In vivo* studies using the

mouse A/E pathogen *C. rodentium* show that *Tlr4*$^{-/-}$ mice, which are insensitive to bacterial LPS, produce lower levels of cytokine and have less neutrophil and macrophage infiltration at the infection site (Khan et al. 2006).

Although Stx and LPS are not essential to induce pro-inflammatory cytokine secretion in intestinal epithelial cells, their roles in inducing inflammation systemically are recognized as important. In children with HUS, EHEC LPS binds and activates platelets, resulting in the expression of CD40 Ligand (CD40L) which can in turn increase the amount of Toll-like Receptor (TLR4) on neutrophils and monocytes, and induce the production of inflammatory cytokines in vascular endothelial cells (Stahl et al. 2006). In addition, translocation of Stx across the intestinal epithelium is promoted by neutrophil transmigration, although the mechanism is not fully characterized. Hence Stx can access the host circulatory system and induce pro-inflammatory reactions (Hurley et al. 2001; Walters et al. 1989). Stx also binds to monocytes through Globotriascylcermide 3 (Gb3) receptors in patients with HUS, while *in vitro* studies have revealed that stimulation of monocytes with Stx leads to a significant increase in the production of IL-6, IL-8, TNF and IL-1β (van Setten et al. 1996).

In addition to inflammation, immune detection of bacterial pathogens can trigger apoptotic signaling cascades that are associated with the clearance of infected cells. Stx appears to be the major virulence factor responsible for inducing apoptosis of epithelial cells during EHEC infection *in vitro* (Smith et al. 2003; Schuller et al. 2004; Barnett Foster et al. 2000; Kashiwamura et al. 2009), and evidence from *in vivo* studies in rabbits suggests that loss of microvilli is associated with Stx-induced apoptosis (Smith et al. 2003; Schuller et al. 2004; Kashiwamura et al. 2009). Additionally, inflammatory cytokines, such as Tumor Necrosis Factor (TNF), can activate cell death signaling by binding death receptors and the activity of some T3SS effector proteins; namely EspF, Map and Cif, are associated with apoptosis induction.

Inflammation and Cell Death Signaling

NF-κB signaling is a crucial part of the host immune response that induces pro-inflammatory gene expression upon stimulation (Mukaida et al. 1990). The NF-κB proteins comprise a family of transcription factors that includes p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel and RelB which regulate gene expression by binding to promoters/enhancers of target genes (Ghosh et al. 1998). All NF-κB transcription factors possess an N-terminal Rel homology domain that mediates homo- and hetero-dimerization with other NF-κB proteins, nuclear translocation and DNA-binding. The C-terminal transcription activation domains (TADs) of p65, c-Rel and RelB control the activation of target gene transcription (Vallabhapurapu and Karin 2009), whereas p50 and p52 lack TADs and can either down-regulate gene expression by directly binding with NF-κB-responsive promoters or activate transcription through recruitment of p65, c-Rel, RelB or other TAD-containing proteins (Vallabhapurapu and Karin 2009).

Although NF-κB may be activated by various pathways, most converge at IKK (IκB Kinase) complex activation, which is comprised of two kinase subunits, IKKα (IKK1) and IKKβ (IKK2), and a regulatory subunit NEMO (Liu and Chen 2011). Upon stimulation by pathogen-derived molecules, PRRs recruit various adaptor proteins to form signaling complexes that can activate IKK kinase and direct IKK phosphorylation. Activation of the IKK complex mediates phosphorylation and ubiquitination of IκB proteins that serve as NF-κB inhibitors by retaining NF-κB dimers in the cytoplasm of inactive cells. Ubiquitinated IκB proteins are targeted for degradation by the host 26S proteasome, following which NF-κB subunits are released and translocated into the host cell nucleus where they enhance transcription of inflammatory genes (Hayden and Ghosh 2008).

MAPK signaling functions to coordinate cell responses to numerous physical and chemical stimuli and also contributes to IL-8 production in epithelial cells during EHEC and EPEC infection. MAPK represents a group of conserved proteins that are subdivided into three classes of serine/threonine kinases, ERK, p38 and JNK. The activation of JNK, which involves phosphorylation at threonine residue (Thr_{183}) and serine residue (Ser_{185}), is induced through MAP3Ks in response to TLR, IL-1R (IL-1 receptor) or TNFR (TNF receptor) stimulation (Johnson and Lapadat 2002). Previous studies have shown that A/E pathogen infection triggers JNK activation which in turn activates the transcription factor c-Jun, a member of the activation protein-1 (AP-1) family that has similar functions to NF-κB in regulating gene transcription during inflammation (Mukaida et al. 1994).

Apoptosis in the epithelium has recently emerged as an important host immune defense mechanism that combats intestinal bacterial infection. Killing of infected cells helps to control and eliminate pathogens, clear damaged cells and induce an adaptive immune response. Intrinsic signals such as DNA damage as well as extrinsic signals such as the stimulation of death receptors can trigger caspase-dependent cell death. Cell death receptors, including TNFR1, TRAIL (TNF-associated apoptosis-inducing ligand) receptor and FAS (TNFRSF6), are stimulated upon infection with their respective ligands TNF, TRAIL and FasL. These receptors activate intracellular cell death signaling by recruiting a range of adaptor proteins through their conserved cytoplasmic death domains (DD) (Park et al. 2007). Activation of death receptors can also lead to non-apoptotic signaling, namely activation of inflammatory signaling, indicating that there is substantial crosstalk between the components of these pathways.

Pathogen Response to the Host: Inflammation

Inflammation pathways involving NF-κB and MAPK signaling and inflammatory cytokine production are strongly influenced by EHEC virulence factors in the early stages of infection. The activity of some T3SS effectors, such as EspT and NleF, leads to enhanced NF-κB activation (Raymond et al. 2011; Pallett et al. 2014), whereas other T3SS effectors suppress the inflammatory response through specific targeting of host signaling proteins.

Inhibition of NF-κB Signaling

EHEC and other A/E pathogens employ multiple T3SS effectors to inhibit IκB degradation and nuclear translocation of NF-κB. One of these is NleE, a highly conserved T3SS effector encoded on the virulence-associated O-island (OI) 122 in EHEC O157:H7 EDL933. NleE from EPEC O127:H6 E2348/69 is encoded by integrative element 6 (IE6) (Nadler et al. 2010; Iguchi et al. 2009). NleE is highly conserved, with NleE proteins from EPEC, EHEC and *C. rodentium* displaying more than 85% amino acid identity (Zurawski et al. 2008). NleE has homologues in *Shigella* spp., known as OspZ, and amino acid similarity between NleE and OspZ is also high (ca. 86% similarity). Notably, *Shigella flexneri* serogroup 2a carries a truncated OspZ that lacks 36 amino acids at the C-terminus and is non-functional for NF-κB inhibition (Newton et al. 2010).

Early studies revealed that ectopically-expressed NleE from EHEC, EPEC and *C. rodentium* and full length OspZ from *Shigella* spp. can block IκB degradation and p65 nuclear translocation in response to either TNF (tumor necrosis factor) or IL-1β stimulation, ultimately resulting in a decrease in the expression and production of inflammatory cytokines such as IL-8 (Newton et al. 2010). Consistently, the presence of NleE during EPEC infection significantly reduces the level of pro-inflammatory cytokine production from infected epithelial cells. In addition, the ability of NleE to control NF-κB activation can also be seen in human dendritic cells (DCs), where DCs infected with the *nleE* mutant of EPEC E2348/69 secreted markedly higher levels of IL-8, TNF and IL-6 compared to wild type E2348/69 (Vossenkamper et al. 2010).

Recently, NleE was characterized as a S-adenosyl-L-methionine (SAM) dependent methyltransferase that specifically modifies the zinc coordinating cysteine in the Npl4 Zinc figure (NZF) domain of human TAB2 and TAB3 (TAK1-binding proteins 2 and 3) (Zhang et al. 2012). TAB2 and TAB3 are adaptor proteins with redundant functions that play an essential role in signaling pathways via Toll-like, IL-1 and TNF receptors. Upon activation, the NZF domains of TAB2/3 recognize and bind to K63-linked polyubiquitin chains on upstream receptor-associated proteins TRAF6 and TRAF2 (Takaesu et al. 2000; Sato et al. 2009). Normally, upstream signals, transduced via the interaction of TAB2/3 with ubiquitinated TRAF proteins, activate TAK1 and subsequently induce IKKβ phosphorylation and IκB degradation (Takaesu et al. 2000). Cysteine-methylated TAB2-NZF and TAB3-NZF lose their polyubiquitin chain binding activity, indicating that NZF-domain methylation abolishes signal transduction by interrupting the interaction between TAB2/3 and TRAF2/6 (Zhang et al. 2012). The methyltransferase activity of NleE is dependent on a conserved C-terminal motif $_{209}$IDSYMK$_{214}$, which is explained by the crystal structure of NleE where tyrosine (Tyr$_{212}$), together with arginine (Arg$_{107}$) and glutamine (Glu$_{191}$), form the binding pocket for SAM (Yao et al. 2014). Despite the potent *in vitro* activity of NleE, *in vivo* studies with *C. rodentium* revealed no significant differences in bacterial load between wild type *C. rodentium* and an *nleE* null mutant strain, probably due to redundant functions with other T3SS effectors that target NF-κB activation (Kelly et al. 2006).

The non-LEE encoded effectors NleH1 and NleH2 have also been associated with the inhibition of NF-κB activation. NleH1 and NleH2 share 84% amino acid sequence identity and both are present in EHEC and EPEC. This is in contrast to *C. rodentium,* which carries only one copy of *nleH* (Gao et al. 2009; Feuerbacher and Hardwidge 2014). Although NleH1 and NleH2 are closely related effectors that both contain a C-terminal kinase domain, they interfere with NF-κB activation through different mechanisms. Recent studies revealed that the host target of NleH1 is ribosomal protein 3 (RPS3), a known co-activator of NF-κB that can be phosphorylated by IKKβ on serine (Ser_{209}). Upon phosphorylation, RPS3 translocates to the nucleus and activates gene transcription (Gao et al. 2009). Upon binding to NleH1, not NleH2, phosphorylation of RPS3 by IKKβ is inhibited, and, even though the N-terminal region of NleH1 is sufficient for the binding, the C-terminal kinase activity of NleH1 appears to be important for this inhibition (Pham et al. 2012). NleH1 and NleH2 share 30% and 27% amino acid identity, respectively, with the *Shigella* effector OspG (Kim et al. 2005). OspG is a Ser/Thr protein kinase that subverts NF-κB by preventing ubiquitin-conjugating enzyme-mediated ubiquitination and degradation of phospho-IκBα (Kim et al. 2005). Similar to NleH1, the kinase activity of OspG is critical for modulating NF-κB activation (Gao et al. 2009; Kim et al. 2005). NleH2 suppresses NF-κB activation in cultured epithelial cells when IKKβ is overexpressed, and appears to not to have any influence on RPS3 nuclear translocation (Wan et al. 2011). While NleH1/NleH2-induced NF-κB inhibition has been attributed to the subversion of IκBα phosphorylation/degradation, another study revealed that neither NleH1 nor NleH2 was able to modulate the phosphorylation and degradation of IκBα individually (Pham et al. 2012). This study further demonstrated that NleH1 and NleH2 bound to each other, and that this interaction regulated their ability to inhibit NF-κB signaling through an unknown mechanism (Pham et al. 2012).

In vivo studies using gnotobiotic piglets have revealed that deletion of *nleH1* results in reduced bacterial load, more severe inflammatory responses and higher levels of phosphorylated RPS3 in the colon of infected piglets than those infected with wild type EHEC O157:H7. Infection with the *nleH2* mutant strain, however, only showed diminished colonization (Wan et al. 2011). Similarly, *C. rodentium* infection of mice has revealed that *nleH* contributes to full intestinal colonization, and that this phenotype can also be mediated by EHEC *nleH1* but not *nleH2* (Feuerbacher and Hardwidge 2014). Another study revealed that a *C. rodentium nleH* mutant was attenuated for colonization in the early stages of infection and induced less activation of NF-κB activation than wild type *C. rodentium* (Hemrajani et al. 2008).

NF-κB signaling is further influenced by the zinc metalloprotease T3SS effector NleC that directly degrades NF-κB subunits, p65 and p50 (Baruch et al. 2011; Muhlen et al. 2011; Pearson et al. 2011; Sham et al. 2011). NleC is encoded by OI-36 in EHEC EDL933 and PP4 in EPEC E2348/69, and is highly conserved among all A/E pathogens. The amino acid sequences of NleC from EHEC and EPEC are 100% identical and share 95% similarity with NleC from *C. rodentium* (Iguchi et al. 2009; Perna et al. 2001). The conserved zinc metalloprotease motif $_{183}$HEIIH$_{187}$ is essential for the proteolytic activity of NleC, as NleC, which carries a mutated metalloprotease motif, loses its ability to cleave the NF-κB subunits p65 and p50 (Marches et al. 2005).

NleC-mediated cleavage of p65 can be observed in cultured epithelial cells by ectopic expression or during EPEC infection. Although different cleavage sites of p65 were originally proposed, namely between proline (Pro_{10}) and alanine (Ala_{11}) or between cysteine (Cys_{38}) and glutamic (Glu_{39}), both sites are located within the N-terminal Rel homology domain that mediates the binding of p65 to promoters or enhancers of target genes (Pearson et al. 2011; Baruch et al. 2011; Yen et al. 2010). Subsequent studies confirmed the cleavage site as Cys_{38}/Glu_{39} and a recent molecular analysis of NleC revealed that two motifs in the Rel homology domain of p65, $_{22}EIIE_{25}$ and $_{177}PVLS_{180}$, were critical as the substrate recognition and binding sites for NleC (Giogha et al. 2015). In addition, other NF-κB subunits, such as p50, c-Rel, IκBα, and coactivator of transcription regulators p300, have been reported as substrates of NleC; however, some of these targets remain a topic of debate among certain research groups (Muhlen et al. 2011; Pearson et al. 2011; Shames et al. 2011a).

The role of NleC in pathogenesis has been examined *in vivo* using an EHEC O157:H7 infection model in lambs and calves. However, an EHEC strain lacking NleC did not show any significant differences in colonization when compared to wild type EHEC O157:H7 (Marches et al. 2005). Similarly, C57BL/6 mice infected with either wild type *C. rodentium* or an *nleC* mutant had equivalent numbers of the pathogen in the colon, however, *nleC* deletion resulted in increased tissue damage in mice, suggesting that NleC functions *in vivo* to reduce tissue pathology (Kelly et al. 2006; Sham et al. 2011; Marches et al. 2005).

The LEE-encoded effector, Tir, which is critical for A/E lesion formation, has also been implicated in inhibiting host innate signaling. The C-terminal region of Tir shares sequence similarity with host cellular immunoreceptor tyrosine-based inhibitory motifs (ITIM), which are on the cytoplasmic tail of many immunoreceptors. Phosphorylation of a conserved tyrosine in ITIM-containing proteins allows the receptor to bind to protein tyrosine phosphatases, such as SHP-1 and SHP-2. These phosphatases, in turn, down-regulate host immune responses by decreasing the phosphorylation of a variety of immune signaling-related proteins (Zhang et al. 2000; Neel et al. 2003). The phosphorylation of Tir on amino acid residues Tyr_{483} and Tyr_{511} leads to an interaction between Tir and SHP-1, resulting in suppression of cytokine production including TNF and IL-6 (Yan et al. 2012, 2013). Apart from ITIM similarity, Tir also binds to the NF-κB adaptor protein TRAF2 and consequently inhibits TNF-induced NF-κB signaling (Ruchaud-Sparagano et al. 2011).

Inhibition of MAPK Signaling

In addition to NleC, A/E pathogens encode another conserved zinc metalloprotease, termed NleD. NleD is a 30-kD T3SS effector encoded on OI-36 in EHEC EDL933 and PP4 in EPEC E2348/69, respectively, and the amino acid sequence similarity between them is 99% (Pearson and Hartland 2014). NleD contains a conserved zinc metalloprotease motif, $_{141}HELLH_{145}$, which is crucial for proteolytic activity (Baruch et al. 2011). In contrast to NleC, NleD targets MAPK signaling through direct

cleavage of the conserved activation loop in the MAPKs, JNK and p38 (Baruch et al. 2011). Targeting of MAPK by NleD may be a mechanism that contributes to the profound decrease in IL-8 secretion during EPEC infection, since an *nleBECD* mutant induced higher levels of IL-8 in infected epithelial cells than an *nleBEC* mutant (Baruch et al. 2011). NleD may also contribute to promoting cell survival, as the cleavage of JNK can arrest JNK-induced apoptosis (Shaulian and Karin 2001).

Pathogen Response to the Host: Apoptosis

Effectors that Induce Apoptosis

Programmed cell death pathways can be induced directly upon the secretion of T3SS effectors by EHEC during infection. All of EspF, Map and Cif have been associated with induction of cell death. Due to carriage of a mitochondrial targeting sequence (MTS) located at the N-terminus, EspF localizes to host mitochondria and induces disruption of mitochondrial membrane potential, which in turn initiates apoptosis signaling by stimulating cytochrome *c* release and caspase-9 cleavage (Nougayrede et al. 2007; Dean et al. 2010). EspF also binds Abcf2 in mitochondria which may explain the induction of caspase-9 cleavage since lower Abcf2 levels in mitochondria correlate with caspase-9 cleavage (Nougayrede et al. 2007). The T3SS effector Map also harbors an MTS in the N-terminal region and Map is predicted to activate intrinsic cell death during A/E pathogen infection (Kenny and Jepson 2000). *In vitro* studies have revealed that Map leads to mitochondrial membrane disruption; however, the precise function of Map *in vivo* is still unclear (Ma et al. 2006). Finally, the T3SS effector Cif can arrest cell-cycle progression at G1/S and G2/M in host cells by inhibiting the ubiquitination and degradation of proteins that regulate cell cycle. This induces a delayed form of apoptosis (Marches et al. 2003; Taieb et al. 2006; Samba-Louaka et al. 2008, 2009). Cif inactivates Cullin Ring E3 ligases (CRL) by deamidation of NEDD8 (Crow et al. 2012; Toro et al. 2013; Cui et al. 2010). Neddylation is usually required to activate CRLs by inducing a conformational change that allows the E3 ligase to ubiquitinate target proteins. In addition, Cif prevents perforin-2 ubiquitination and hence its activation and bactericidal activity (McCormack et al. 2015).

Another conserved T3SS effector, NleB1, is encoded directly upstream of *nleE* in OI-122 of EHEC EDL933 or IE6 of EPEC E2348/69 and a close homologue, NleB2, is encoded in OI-36 (EHEC EDL933) or PP4 (EPEC E2348/69). When expressed ectopically in cultured epithelial cells, NleB1 can dampen NF-κB signaling in response to TNF but not IL-1β (Newton et al. 2010). However, during infection, NleB1 has little impact on the inhibition of pro-inflammatory cytokine production (Pearson et al. 2013; Li et al. 2013). Instead, NleB1 has a key role in blocking extrinsic apoptosis signaling stimulated by death receptor activation through FAS or TNFR1. NleB1 possesses *N*-acetylglucosamine (GlcNAc) transferase activity that specifically modifies the death domains (DD) of death receptor adaptor proteins by

transferring GlcNAc to a conserved arginine in FADD (Arg_{117}), TRADD and RIPK1 (Pearson et al. 2013; Li et al. 2013). This activity blocks caspase-8 activation and subsequent cell death by preventing formation of the death-inducing signaling complex (DISC). These findings are consistent with previous discoveries revealing that Arg_{117} of FADD is an essential residue that mediates death domain interactions between FAS-FADD and FADD-TRADD (Imtiyaz et al. 2005; Wang et al. 2010). TRADD is an adaptor protein involved in the NF-κB pathway where it forms a component of the TNFR1 complex I with RIPK1 and TRAF2. Thus the modification of Arg_{235} in TRADD may explain the role of NleB1 in suppressing NF-κB activation under certain experimental conditions (Newton et al. 2010).

The GlcNAc transferase activity of NleB1 depends on a catalytic motif, DxD, that is conserved in NleB1 and NleB2, and also the SseK T3SS effectors from *Salmonella* spp. (Gao et al. 2013). *In vivo* studies using *C. rodentium*, which carries only one copy of *nleB* revealed, that either deletion of *nleB* or mutation of the DxD motif results in attenuation of colonization and increased caspase-8 cleavage in the colon of infected mice (Kelly et al. 2006; Pearson et al. 2013). Currently, the host targets and functions of NleB2 and the SseK effectors are not clear, although some activity against DD containing proteins has been observed (Kelly et al. 2006; Pearson et al. 2013).

NleF is also a conserved T3SS effector that is encoded on OI-71 in EHEC EDL933, and PP6 in EPEC E2348/69. The amino acid sequences of NleF from EPEC and EHEC are 100% identical (Iguchi et al. 2009; Deng et al. 2004). NleF inhibits intrinsic and extrinsic apoptosis *in vitro* by binding caspase-4, caspase-8 and caspase-9 (Blasche et al. 2013). The inhibition appears to occur through the binding of NleF to the active site of these caspases. However, during infection, deletion of *nleF* from EPEC E2348/69 does not lead to a significant increase in caspase-3/7 activity in cultured epithelial cells, although this experiment did not induce apoptosis in infected cells using an exogenous signal (Blasche et al. 2013). NleF also promotes NF-κB nuclear translocation and IL-8 production, although the mechanism by which NleF activates NF-κB is not clear (Pallett et al. 2014).

NleH1 and NleH2 also modulate intrinsic cell death signaling pathways. The function of NleH in inhibiting apoptosis was initially determined *in vitro* where a higher number of cultured epithelial cells died when infected with an *ΔnleH1/nleH2* double mutant compared to cells infected with wild type EPEC E2348/69 (Hemrajani et al. 2010). NleH was then implicated in the inhibition of caspase-3 cleavage which was observed in EPEC-infected epithelial cells and *C. rodentium*-infected mice, which may arise from an interaction between NleH and the apoptosis inhibitor protein, Bax inhibitor-1 (BI-1) (Hemrajani et al. 2010; Robinson et al. 2010).

Additionally, the LEE-encoded effectors, EspZ and EspO, have been implicated in maintaining epithelial integrity during A/E pathogen colonization. EspZ (SepZ) is a conserved T3SS effector carried by all A/E pathogens and the amino acid sequences of EspZ from EPEC and EHEC are 71.7% identical (Kanack et al. 2005). Initial research revealed that deletion of *espZ* from *C. rodentium* significantly reduced the colonization of *C. rodentium in vivo* (Deng et al. 2004) and *in vitro* studies further revealed that EspZ protects epithelial cells from cell death during

EPEC infection. The mechanism of EspZ activity in enhancing cell survival was originally attributed to its ability to bind CD98 and upregulate integrin signaling and focal adhesion kinase (FAK) signaling (Shames et al. 2010). More recently, EspZ has been observed to localize to mitochondria and may reduce cell cytotoxicity through binding the inner mitochondrial membrane 17b (TIM17b), thereby maintaining mitochondrial membrane potential (Shames et al. 2011b). Rabbit enteropathogenic *E. coli* (REPEC) *ΔespZ* mutants also exhibit attenuated virulence during infection and induce markedly higher levels of host cell death (Wilbur et al. 2015). EspZ assists in regulating T3SS effector translocation from a plasma membrane location which may contribute to its virulence function (Berger et al. 2012), but, to date, the biochemical basis of EspZ activity is unknown.

Another LEE-encoded effector, EspO, which is homologous to OspE from *Shigella flexneri*, enhances host cell-cell adherence during infection. Previous studies have revealed that OspE promotes the stability of focal adhesions (FA) by interacting with integrin-linked kinase (ILK), and also by binding to the PDZ-containing protein PDLIM7 to modulate the activity of protein kinase C (PKC), which is associated with cell spreading and adherence (Kim et al. 2009; Yi et al. 2014). EspO carries the Trp68 equivalent that is involved in OspE-ILK interaction and has also been observed to localize with FA during EHEC infection in cultured epithelial cells, indicating that EspO may have a similar role to OspE in bacterial gut infection (Kim et al. 2009; Morita-Ishihara et al. 2013).

Inflammasomes and Immunity to A/E Pathogens

Mice deficient in certain inflammasome components, including Nlrc4, Nlrp3, Asc and Caspase-1 (but not Caspase-11), are more susceptible to *C. rodentium* infection, suggesting that inflammasome sensing plays a role in defense against A/E pathogens (Lupfer et al. 2014; Nordlander et al. 2014). Inflammasomes of the intestinal epithelium play a general protective role during infection by removing damaged enterocytes and producing inflammasome dependent cytokines (Sellin et al. 2015). Findings from a recent study suggest that A/E pathogens counter this using the T3SS effector, NleA/EspI, which binds directly to Nlrp3 and blocks deubiquitination, thereby preventing inflammasome activation (Yen et al. 2015).

Conclusions

The manipulation of host signaling pathways, especially those associated with host immunity, is a key strategy utilized by EHEC and other A/E pathogens for successful infection. A growing number of host proteins have been identified as targets of T3SS effectors that are inactivated through specific post-translational modifications. These discoveries have not only expanded our understanding of pathogen-host interactions, but, in some cases, have also helped to define the function of host proteins

in signaling pathways. Indeed, identification of the host proteins modified by T3SS effectors during infection has greatly informed us about the immune responses important for combating infection, as these are precisely the pathways targeted by the pathogen for inhibition. Overall, there is still much to learn about roles of T3SS effectors during infection; in particular, those where no enzymatic activity has been identified (Table 1). Given this, some reassessment of T3SS biology in EHEC and other A/E pathogens is needed. Future research must also be cautious about inferring the function of homologous bacterial effectors from different pathogens as these may display different activities and target distinct host signaling pathways.

Table 1 EHEC and EPEC T3SS effectors, reported to influence host inflammatory and cell death-signaling pathways

Effector Protein	Host target relevant to inflammation or cell death	Enzymatic activity	Function	References
NleE	TAB2/TAB3 ZRANB3	Cysteine methyltransferase	Potently blocks NF-κB activation	Newton et al. (2010), Zhang et al. (2012), Yao et al. (2014)
NleB1	FADD, TRADD, RIPK1, TNFR1	Arginine glycosyltransferase	Inhibits death receptor signaling and formation of the DISC	Newton et al. (2010), Pearson et al. (2013), Li et al. (2013)
NleH1/NleH2	RPS3, Bax inhibitor 1	Kinase activity	Inhibits NF-κB signaling and apoptosis	Gao et al. (2009), Pham et al. (2012), Hemrajani et al. (2010), Robinson et al. (2010)
NleC	p65, p50	Zinc metalloprotease	Inhibits NF-κB signaling	Pearson et al. (2011), Giogha et al. (2015)
NleD	JNK, p38	Zinc metalloprotease	Inhibits MAPK signaling	Baruch et al. (2011)
NleF	Caspase-4, −8, −9	None identified	Inhibits apoptosis by blocking caspase activation, increased NF-κB activation	Pallett et al. (2014), Blasche et al. (2013)
Tir	SHP-1, SHP-2, TRAF2	None identified	Essential for A/E lesion formation, inhibits NF-κB signaling	Yan et al. (2012, 2013), Ruchaud-Sparagano et al. (2011)

(continued)

Table 1 (continued)

Effector Protein	Host target relevant to inflammation or cell death	Enzymatic activity	Function	References
EspF	Abcf2	None identified	Induces apoptosis by activating caspase-9	Nougayrede et al. (2007), Dean et al. (2010)
Map	Unknown	WxxxE family guanine nucleotide exchange factor for Cdc42	Induces apoptosis by disruption of the mitochondrial membrane	Kenny and Jepson (2000), Ma et al. (2006), Orchard et al. (2012)
Cif	Cullin Ring E3 Ligases (CRLs)	NEDD8 glutamine deamidase	Arrest of cell-cycle progression	Marches et al. (2003), Taieb et al. (2006), Samba-Louaka et al. (2008, 2009), Cui et al. (2010)
EspZ	CD98, TIM17b	None identified	Inhibits host cell apoptosis	Shames et al. (2010, 2011b)
EspO	PDLIM7, ILK	None identified	Promotes host cell survival and cell-cell interactions	Kim et al. (2009), Yi et al. (2014)
NleA/EspI	NLRP3	None identified	Inhibition of NLRP3 activation	Yen et al. (2015)

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In Defense of the European 100 CFU of *Listeria monocytogenes*/g in Ready-to-Eat Foods

Phil Voysey and Roy Betts

Abstract *Listeria monocytogenes* is a pathogenic bacterium which may be present in foods derived from the natural environment, as well as foods that have been processed. Vulnerable foods are those that are not processed further, immediately prior to being consumed (i.e., ready-to-eat (RTE) foods). The frequency of contamination of RTE foods with *L. monocytogenes* is estimated to be 0–10%, with high levels of contamination (>1000 CFU/g) reported to be associated with foods that have been linked to foodborne illness episodes. A number of risk assessments suggest that low levels of contamination (<100 CFU/g), may be an acceptable risk. With food safety systems such as Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) programs in place and operating, high levels of contamination likely would rarely be associated with foods entering the market. So, given that RTE foods will occasionally be contaminated with *L. monocytogenes,* it can be argued that low level contamination is an acceptable risk to the consumer. A system such as the 'European 100 CFU of *L. monocytogenes*/g' system described here protects the health of consumers while ensuring fair practices in food trade.

Keywords *Listeria monocytogenes* • Criteria • Ready-to-eat foods • Specifications • European

In order to understand the need for appropriate controls to be placed on the presence of the pathogen *Listeria monocytogenes* in foods, it is necessary to understand the organism and its behavior with respect to foodstuffs. A brief outline of these aspects are given here.

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Listeriosis

'Listeriosis' is a foodborne illness caused by the bacterium *L. monocytogenes.* It is an infrequent and severe infection predominantly affecting the elderly (over 60 years of age), people with weakened immunity, and pregnant women and their unborn or new-born babies. In England and Wales, and in Europe, although it causes a small number of illnesses annually, compared to other pathogens; however it is one of the major causes of death due to foodborne illness.

Between the years 2005 and 2014, an average of 180 cases of listeriosis was reported annually in England and Wales. This is a 23% increase from the previous decade (1995–2004), during which an average of 137 cases was reported. It is suggested (Public Health England 2015) that this increase may be attributed to more cases from ethnic minority groups and cases of bacteremia in the elderly. In 2014, the number of reported cases increased by 5.3% from 2013, with 169 cases reported.

In the United States of America (USA) according to the CDC (2014), 582 listeriosis cases were reported in 2012. Of these, 13% was pregnancy-associated and 87% not associated with pregnancy. Of the total number of listeriosis cases, 63% were associated with people 60 years old or more. This reflects a general increasing trend of cases in the elderly population. For example in 2007, 311 cases were reported, with 21% being pregnancy-associated and 78% non-pregnancy associated. Fifty eight percent of the total number of cases of listeriosis were associated with people 60 years old or more.

Food microbiologists should be well aware of the characteristics of *L. monocytogenes* and the available measures for its control in foods. It is generally believed that most cases are foodborne and may be preventable.

Taxonomy

The genus *Listeria* is currently comprised of 17 species, with *L. floridensis, L. aquatica, L. cornellensis, L. riparia* and *L. grandensis* reported in 2014 (den Bakker et al. 2014), and *L. booriae* and *L. newyorkensis* (Weller et al. 2015) being added in 2015 to *L. fleischmannii, L. grayi, L. innocua, L. ivanovii, L. marthii, L. monocytogenes, L. rocourtiae, L. seeligeri, L. weihenstephanensis* and *L. welshimeri.*

Cases of human listeriosis are almost exclusively caused by the species *L. monocytogenes.* Although some rare cases of hospitalized patients with underlying diseases have been associated with *L. ivanovii* and *L. seeligeri,* these bacteria are not considered to be a foodborne hazard.

Listeria monocytogenes can be divided into 13 different serotypes all of which are able to cause listeriosis. However, most cases are caused by serotypes 1/2a, 1/2b and 4b.

Listeria monocytogenes Foodborne Illness

Manifestations of listeriosis include gastroenteritis, meningitis, septicemia and meningoencephalitis. The incubation period can vary from 3–70 days, although for gastroenteritis, symptoms usually occur after 18–27 h (Montville and Matthews 2008).

For pregnant women, the onset of listeriosis is often a flu-like illness, which leads to infection of the fetus, and in turn can result in miscarriage, stillbirth or birth of a very ill infant. When listeriosis was first observed in large numbers in the 1980s, typically 25% of cases were pregnancy-related. More recently, with controls put in place that warn pregnant women not to eat particularly risky foods, the balance of those ill tend to be 'the elderly' (60 years old and over) (Gillespie et al. 2010).

One of the reasons that listeriosis is considered a serious illness, is that the mortality rate is at 30%, which is considered very high. In highly susceptible individuals, however, the rate can be as high as 40% (Rocourt 1996).

There is considerable uncertainty regarding the infective dose for listeriosis, which to some extent is because of the potentially long incubation period for the disease. Foods recovered from households of patients who developed listeriosis were often heavily contaminated (>1000 CFU of *L. monocytogenes*/g), suggesting that a typical infective dose is likely to be high (McLauchlin 1996). Investigation of an outbreak of listeriosis associated with butter in the UK in 2003, revealed a small number of samples with a count of ca. 180 CFU of *L. monocytogenes*/g, whereas most of the samples had a count of <100 CFU of *L. moncytogenes*/g (ACMSF 2003). However, frankfurters implicated in a listeriosis outbreak in the USA in 1998 were reported to contain <0.3 CFU of *L. monocytogenes*/g, although it has been suggested that this may be indicative of a strain with enhanced virulence (CDC 1999). The infective dose of *L. monocytogenes* resulting in gastroenteritis is reported to be much higher, at 1.9×10^5 to 1×10^9 CFU/g (Montville and Matthews 2008).

Foods Implicated

Many cases of listeriosis have not been linked to a specific food source. However, the first significant food-associated outbreak occurred in 1981 in Canada and implicated coleslaw salad. There were at least 41 cases and 7 deaths in this outbreak, which resulted from using sheep manure-contaminated cabbage in the coleslaw (Schlech et al. 1983).

Over the years, a wide variety of foodstuffs have been associated with outbreaks of listeriosis, with examples listed in Table 1.

Meat, dairy products and fresh produce are all well represented as being foodstuffs associated with listeriosis.

Foods can become contaminated with *Listeria* at any stage in the food chain, from processing on the farm (e.g. cantaloupes associated with listeriosis in the USA in 2011 (CDC 2011)), through distribution, to the consumers kitchen, especially in moist/wet environments.

Table 1 Some examples of listeriosis outbreaks linked to contaminated foodstuffs

Year	Location	Cases	Deaths	Food(s)
1947–57	Halle, Germany	?	?	Unpasteurized milk
1979–81	Maritime Provinces, Canada	66	18	Coleslaw
1983–8	Switzerland	122	>30	Vacherin soft cheese
1985	California, USA	142	48	Mexican-style cheese
1987–9	UK	>350	>90	Belgian paté
1992	France	>279	85	Jellied pork tongues
1993	France	33	8	Pork rillettes
1994	USA	45	0	Chocolate milk
1994–5	Sweden	9	2	Vacuum-packed gravid and cold-smoked rainbow trout
1995	France	20	4	Raw milk soft cheese
1997	Italy	748	0	Corn
1998–9	USA	100	>10	Hot dogs/Deli meats
1998–9	Finland	25	4	Butter
2000	USA	29	4	RTE Turkey
2003	UK	17	0	Butter
2008	Canada	57	22	Cured meats
2010	Austria and Germany	14	7	Prolactal – acid-curd cheese
2011	USA	123	25	Cantaloupes
2014	Denmark	24	13	Sausage
2014	USA	8	1	Cheese
2014	USA	35	7	Caramel apples
2015	USA	10	3	Ice cream

Numerous studies have revealed that *L. monocytogenes* can be found in a wide variety of foods, including milk, soft cheese, raw and pre-cooked poultry, meats, paté, fermented sausage, fruits and vegetables, smoked and lightly processed fish products (e.g. Ryser and Marth 2007), and seafoods (Jay 1996).

In the UK, *L. monocytogenes* has been found in many foods, usually in very low numbers, but occasionally in numbers exceeding 10,000 CFU/g. Highly contaminated foods include paté and soft cheese (Roberts 1994). In recent years, surveys of foodstuffs for the UK Food Standards Agency and others, have determined the prevalence of *Listeria* in such foods, and these findings are summarized in Table 2.

Recent work in the UK has identified sandwiches served as meals in hospitals as being potential sources of listeriosis. Many of these cases were among the sick and elderly (Little et al. 2012).

Although *Listeria* species other than *L. monocytogenes* are not pathogenic, their presence in foods, may be indicative of poor hygienic practices. Their presence is also considered by many as indicative of the potential presence of *L. monocytogenes,* since the different species generally share similar requirements for growth.

Table 2 Summary of survey findings for presence of *Listeria* in a number of foodstuffs

Foodstuff	Year	Number of samples tested	Percentage samples positive for *Listeria* spp.	Percentage samples positive for *L. monocytogenes*	Reference
Pre-packed mixed vegetable salads	2005	2686	10.8	4.8	Little et al. (2007)
Sandwiches	2007	3249	8	3	Little et al. (2008)
Cooked meats	2007	1127	–	7	FSA (2007)
Retail smoked fish	2008	3227	12	9	FSA (2008)

Hence, the presence of any *Listeria* species should be treated as important, and should trigger cleaning and other precautionary measures in food producers' hygiene systems.

Control of *L. monocytogenes* in Foods

It is generally accepted that "*the total elimination of the organism [L. monocytogenes] from all food is impractical and may be impossible*" (WHO 1988). The relatively common occurrence of *L. monocytogenes* in the environment and in some foods would suggest that exposure to this bacterium does not usually lead to infection. In fact, the prevalence of listeriosis, in comparison with other foodborne illness such as salmonellosis or campylobacteriosis, is infrequent. However, with a mortality rate as high as 30%, it is a requirement to reduce the prevalence of *L. monocytogenes* in foods to an absolute minimum. The presence of *Listeria* spp. in a food – especially a cooked food – may be an indication of poor hygiene during manufacture, distribution or retailing. The United States Department of Agriculture (USDA) and the US Food and Drug Administration (FDA) have adopted a 'zero tolerance policy for *L. monocytogenes* in RTE (ready-to-eat) foods, but in Europe counts of up to 100 CFU/g at end of shelf life are generally considered acceptable (EC 2005).

The HACCP (Hazard Analysis and Critical Control Points) approach to food safety assurance should be applied to all levels of the food industry. With respect to controlling *Listeria*, particular attention should be paid to temperature control, practices to prevent cross-contamination between raw and processed foodstuffs, and from wet processing/manufacturing or packaging environments. In addition, storage times for sensitive foods should be carefully controlled.

The UK Department of Health advises vulnerable individuals (e.g., pregnant women) to take appropriate precautions against *Listeria* infection, including avoiding some delicatessen foodstuffs, and to re-heat cook-chill foods adequately (Roberts 1994). In the US, the FDA has added refrigerated ready-to-eat meats, hot dogs, and smoked seafood, (unless 'properly reheated'), to the list of foods that 'at risk' consumers should avoid (FDA 2016).

History of Criteria Relating to *Listeria monocytogenes* in Foods

Given that *L. monocytogenes* is a common contaminant of the environment and foodstuffs derived from the environment, setting a meaningful microbiological criterion for this pathogen in these foods has proved difficult. Many of these RTE foods will not receive a listericidal treatment immediately before being consumed. Items such as salads may be washed, but this process will not eliminate all *Listeria* spp. or other pathogens that might be present. For example, Fishburn et al. (2012) suggested that 1–3 log reductions in pathogen counts were typical for vegetable washing processes. The ability for pathogens, including *L. monotocygenes* to become internalized in plant tissues (e.g., Chitarra et al. 2014), protects them from the action of the sanitizing chemicals that are used in produce wash waters. Hence, pathogens can survive the wash treatments that are used.

In 1996, the UK Public Health Laboratory Service (PHLS) revised its document entitled '*Provisional microbiological guidelines for some ready to eat foods sampled at point of sale*'. The revised document included the following criteria for *L. monocytogenes*: '**satisfactory**' = Not Detected in 25 g; '**fairly satisfactory**' = 10–200 CFU/g; '**unsatisfactory**' = 100–1000 CFU/g; '**unacceptable**' = greater than 1000 CFU/g. These guidelines suggested that low levels of *Listeria monocytogenes* are 'fairly satisfactory' in some ready-to-eat foods that naturally could contain *L. monocytogenes*. The overlap in 'fairly satisfactory' and 'unsatisfactory' counts underlines the difficulty in determining what is acceptable and what is not (in terms of counts) for different scenarios and foodstuffs.

The document was updated in 2000 and in this version (Gilbert et al. 2000) the following guideline criteria were suggested: '**satisfactory**' = Not Detected in 25 g or <20 CFU/g; '**acceptable**' = 20–99 CFU/g; '**unacceptable**' = ≥100 CFU/g. These documents suggest that a 'zero tolerance' criterion for these types of products is not appropriate and/or not achievable for some ready-to-eat products.

The next 'step' in developing useable microbiological criteria for *L. monocytogenes* in foods followed the route of developing decision trees, e.g., Codex (2001). In this document, it was suggested that the number of samples taken and the frequency of testing was dependent on the ability of *L. monocytogenes* to: be present; to grow; and to cross-contaminate different foodstuffs. An example of the type of decision tree derived from this is presented in Fig. 1.

European Commission Regulation 2073/2005 on Microbiological Criteria for Foodstuffs

In order to evaluate the actual risk posed by *L. monocytogenes* from RTE foods, a microbiological risk assessment study was carried out by the European Union Scientific Committee for Veterinary Measures. Its findings (EC 1999) concluded that:

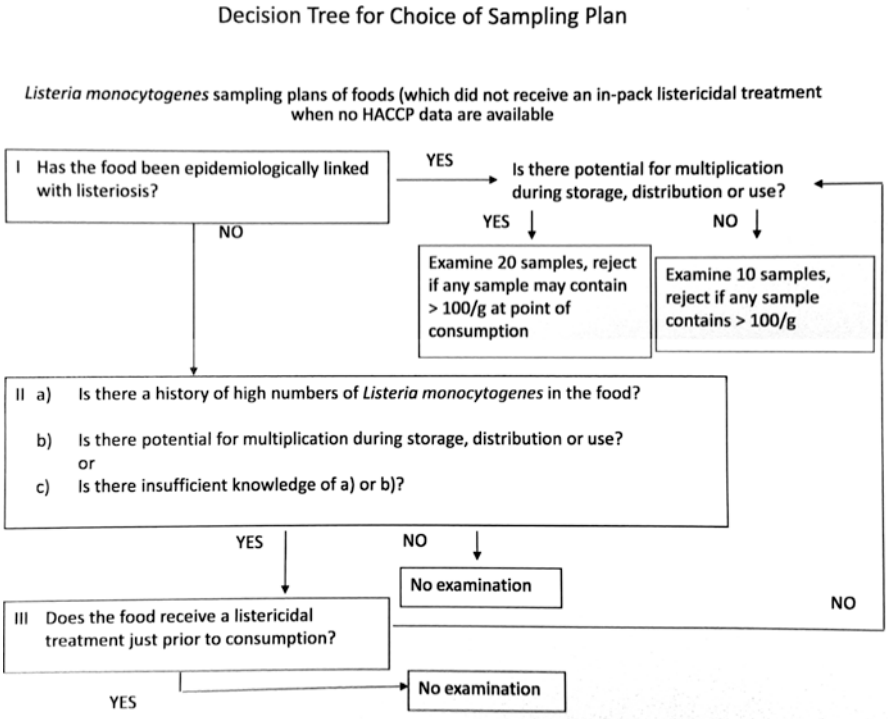

Fig. 1 Decision tree for *Listeria monocytogenes* testing of foodstuffs

less than 100 colony-forming units (cfu) of* L. monocytogenes *per gram of food at point of consumption should be considered low risk to consumers.

With this conclusion in mind, and other relevant information and data, the *Listeria monocytogenes* section of + EC Regulation 2073/2005 (EC 2005) was written. EC Regulation 2073/2005 came into effect in January 2006 as a legal requirement in all European member states of the European Union. This stipulated that it was acceptable to have low levels (<100 CFU/g) of *L. monocytogenes* present in certain RTE foods, providing the Food Business Operator (FBO) could demonstrate that the level would not increase above 100 CFU/g at the point of consumption. In other words, providing it could be demonstrated to the Competent Authority (CA) that *L. monocytogenes* could not grow in the foodstuff, or that if *Listeria* were present, its growth would not result in a level greater than 100 CFU/g by the end of its shelf life. If this could not be demonstrated, the product had to be withdrawn from the market if *L. monocytogenes* were present.

Microbiological testing of food products is carried out by FBOs as one way of determining that the food is acceptable. Regulation 2073/2005 (with the exception of minced meat, meat preparations and mechanically separated meat) does not specify the sampling or microbiological testing frequency. That is decided by the FBO. The principles of Good Manufacturing Practices (GMP) and Hazard Analysis & Critical Control Point (HACCP) must be applied when manufacturing all products.

Regulation 2073/2005 legally sets Food Safety Criteria and Process Hygiene Criteria. These criteria are in the form of a series of microbiological testing requirements and allowable levels of various microorganisms within different food types. Food Safety Criteria are established for a number of pathogenic bacteria, including *Listeria monocytogenes.* The criteria for *L. monocytogenes* cover all ready-to-eat (RTE) foods.

It is suggested in Regulation 2073/2005 that a decision tree summarizing which *Listeria monocytogenes* criteria should be used for specific RTE foods. An example of this is provided in Table 3.

There are three categories of foodstuffs covered in Regulation 2073/2005 in which *L. monocytogenes* criteria are defined. These are shown in the Decision Tree (Table 3) and are based on the risk posed by this pathogen.

(i) **RTE foods intended for infants or special medical purposes**

(Table below is taken from EC Regulation 2073/2005)

Food category and criterion number	Microorganisms/their toxins, metabolites	Sampling plan		Limits		Analytical reference method	Stage where the criterion applies
		n	c	m	M		
1.1 Ready-to-eat foods intended for infants and ready-to eat foods for special medical purposes	*Listeria monocytogenes*	10	0	Absence in 25 g		EN/ISO 11290-1	Products placed on the market during their shelf-life

In the table:
Limit *m*: This level is the target normally achieved using HACCP, Good Manufacturing and Good Hygienic Practices
Limit *M*: This is the maximum acceptable level
Sampling Plan *n*: The number of samples from the batch which are tested
Sampling Plan *c*: The number of samples that are allowed to have results between *m* and *M*

Regulation 2073/2005 states that regular testing against the criterion is not required for the following RTE foods:

- those that have been processed (included heat treatment) effective to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g. products that are packed and then fully heat-treated).
- fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds
- bread, biscuits and similar products
- bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products
- sugar, honey and confectionery, including cocoa and chocolate products
- live bivalve molluscs.

Table 3 Decision tree: which *L. monocytogenes* criteria to use for RTE foods[a]? (FSA 2006)

Category 1.1	Is food intended for humans or for special medical purposes?	Yes →	*L. monocytogenes* absent in 25 g during its shelf life (1.1)
↓ No			
Is shelf life 5 days or less?		Yes →	*L. monocytogenes* should not exceed 100 CFU/g during its shelf life (1.3)
↓ No			
Is it impossible for *L. monocytogenes* to grow within the product: i.e. is the foodstuff unable to support the growth of *L. monocytogenes*?		Yes →	*L. monocytogenes* should not exceed 100 CFU/g during its shelf life (1.3)[b]
↓ No			
Category 1.2	Has growth of *L. monocytogenes* been taken into account when setting shelf life?	Yes →	*L. monocytogenes* should be absent at the point of manufacture and at a level that should not exceed 100 CFU/g at any point during its shelf life (1.2)[c]
↓ No			
Category 1.3	*L. monocytogenes* should be absent in 25 g of the product, both at the point of manufacture and throughout the shelf life of the product		

Notes:
[a]Regular testing is not useful for the following RTE foods:
Foods processed sufficiently to eliminate *L. monocytogenes* when recontamination not possible (e.g. foods heat-treated in final packaging)
Fresh uncut and unprocessed fruit and vegetables (excluding sprouted seeds)
Bread, biscuits, breakfast cereals and similar products
Bottled or packaged waters, soft drinks, beer, cider, wine, spirits and similar products
Sugar, honey and confectionery, including cocoa and chocolate products
Live bivalve molluscs
[b]Includes products with:
pH less than or equal to 4.4
water activity (a_w) less than or equal to 0.92
pH less than or equal to 5.0 and a_w less than or equal to 0.94
[c]If shelf-life studies reveal 100 CFU/g is likely to be exceeded before the end of the shelf life, options include: reviewing the shelf life; reviewing food safety management procedures; or ensuring absence in 25 g before the food leaves the immediate control of the food business operator.

Results indicating the presence of *L. monocytogenes* in 25 g of these products are considered to be an unacceptable food safety risk at the point of production or at any time within the shelf life of the product. So, products with *L. monocytogenes* detected within 25 g must not be placed on the market or must be removed from the market and the CA notified.

The sampling plan given above (i.e., ten samples taken per batch (*n*)) may be revised if the FBO can demonstrate by shelf-life assessment, challenge test and historical data and documentation that effective HACCP-based procedures are in place. A preventative approach such as environmental monitoring for *L. monocytogenes* should be demonstrated.

(ii) **RTE foods able to support the growth of *L. monocytogenes***

(Table below is from Regulation 2073/2005)

Food category and criterion number	Microorganisms/their toxins, metabolites	Sampling plan		Limits		Analytical reference method	Stage where the criterion applies
		n	c	m	M		
1.2 Ready-to-eat foods able to support the growth of *L. monocytogenes,* other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2	Products placed on the market during their shelf-life
		5	0	Absence in 25 g		EN/ISO 11290-1	Before the food has left the immediate control of the food business operator, who has produced it

Criterion 1.2 should be applied if either:

- It is known that *L. monocytogenes* can grow within the product, or
- The manufacturer has no knowledge as to whether it could grow or not

The criterion of 'absence in 25 g' applies to products before they have left the immediate control of the FBO. If *L. monocytogenes* is detected within 25 g of the product, it must not be placed on the market.

The criterion of less than 100 CFU/g applies when the product has left the control of the FBO and is on the market and within its shelf life. If *L.monocytogenes* is found at levels greater than 100 CFU/g in products that are on the market, then they must be withdrawn and the Competent Authority informed.

If *L. monocytogenes* is detected in 25 g or at levels of no more than 100 CFU/g in product when already on the market, the producer must have evidence to validate that the level will not exceed 100 CFU/g within the shelf life under expected storage conditions. Failure to do this should result in a serious consideration of withdrawal from the market and informing the Competent Authority.

The Regulation states that *L. monocytogenes* **should not be considered able to** grow in the following product types:

- those that have been processed (including heat treatment) to eliminate *L. monocytogenes*, when recontamination is not possible after this treatment (e.g., in-pack treatment).
- fresh, uncut and unprocessed vegetables and fruits, excluding sprouted seeds
- bread, biscuits and similar products
- bottled or packed waters, soft drinks, beer, cider, wine, spirits and similar products
- sugar, honey and confectionery, including cocoa and chocolate products
- live bivalve molluscs
- products with a pH of less than or equal to (≤)4.4, or

- products with an a_w of ≤0.92, or
- products with a chilled shelf life of less than 5 days, or
- products with a pH of ≤5.0 and an a_w of ≤0.94.
- frozen foods (<−1.0 °C)

Products that do not comply with any of the above should be considered able to support the growth of *L. monocytogenes* unless it can be proven scientifically that they cannot.

If it is believed that a product supports the growth of *L. monocytogenes*, it is suggested that:

- studies be carried out to confirm if *L. monocytogenes* growth is possible, or not. This will usually require expert scientific input but may consider pH, a_w, salt content, concentration of preservatives and the type of packing system, taking into account the storage and processing conditions, the possibilities for contamination and the foreseen shelf life; or
- mathematical modelling, existing scientific literature and research data regarding the growth and survival of *L. monocytogenes* within the product be used to formulate and support an opinion.

If it is still considered that *L. monocytogenes* could grow within the product, then it important to understand how quickly listeriae are able to grow. An understanding of this is required in order to correctly establish a safe shelf life with respect to *L. monocytogenes.* If there is no knowledge of growth rate of *L. monocytogenes*, then the detection of any level of *L. monocytogenes* in product that is on the market and within its shelf life should result in consideration of notification of the Competent Authority.

In order to understand how *L. monocytogenes* behaves in a product, the Regulation gives a number of options, of which all may require the input of expert microbiologists. These options include:

- Understanding the physico-chemical characteristics of the product (pH, water activity, salt content, preservatives, packaging type, processing conditions, possibilities for contamination and shelf life) and how these relate to survival and growth of *L. monocytogenes.*
- Consultation with available scientific literature/data regarding survival and growth characteristics of *L. monocytogenes.*
- Predictive mathematical modelling established for the food in question, using critical survival or growth factors for the microorganisms of concern in the product;
- Use of tests to determine the ability of the appropriately inoculated *L.monocytogenes* to survive or grow in the product after different reasonably foreseeable storage conditions (challenge testing);
- studies to determine the survival or growth of *L. monocytogenes* that may be present in the product during the shelf life under reasonably foreseeable conditions of distribution, storage and use (shelf-life or durability testing).

- Data obtained from previous testing of normally produced product may provide an indication of the ability and rate of *L. monocytogenes* growth within a product. This is known as the use of Historic Data and can be used to show ability of the organism to grow, or not, within the product and to define a safe shelf life (i.e., levels of less than 100 CFU/g and the end of life.

Historical Data

Historical data relate to results that have been obtained for a given microorganism in a given product over time. Historical data are only useful if they apply to the product at the end of its shelf life.

If *L. monocytogenes* has been found in a product of interest at levels of <100 CFU/g but never at levels of greater than or equal to (≥) 100 CFU/g, then that suggests that *L. monocytogenes* will not grow to levels of ≥100 CFU/g, but it is not evidence that it won't. There is no way of knowing for sure what would happen to the level of *L. monocytogenes* found in a sample at the beginning of shelf life, unless the same sample was again tested at the end of shelf life. (This is what is done in Predictive Mathematical Modelling and Challenge testing.)

If *L. monocytogenes* has never been detected in the product under question, then historical data are not useful to give an indication of the fate of *L. monocytogenes* if it was present on one occasion.

So, historical data can give some indication of whether *L. monocytogenes* has ever been found at unacceptable levels in a given product, but it cannot be used to determine if the pathogen can grow in the food to unacceptable levels.

Outbreak and Scientific Data

There is considerable information in the scientific literature relating to growth of *L. monocytogenes* in different foodstuffs. The difficulty, is finding information and data relating to foods which exactly match the foodstuff being made by a FBO. If there is not a precise match, it is not possible to definitively say that information in the literature relates directly to the FBOs product.

There may be information that a particular foodstuff has never been linked to a *L. monocytogenes* outbreak. This is useful information, but cannot replace a HACCP scheme, an effective *Listeria* management/monitoring plan, and the establishment of a safe shelf life through testing.

Predictive Mathematical Modelling

Predictive models allow key physico-chemical properties (e.g., pH, a_w, salt content, storage temperature) of the foodstuff to be entered into a computer program. A prediction is then obtained for relevant microorganisms (pathogens and spoilage

microbes), in terms of their anticipated behavior in that foodstuff. The behavior addressed includes both growth and death rate.

Predictive models cannot take into account all the stimulatory or inhibitory factors a microorganism might encounter in a given foodstuff. Hence, any prediction should always be supported by experimental evidence. Models of this type can be "fail safe" (e.g. conservative) if they predict a shorter shelf life than is achievable in reality.

Predictive models are inexpensive and quick to use; however, they need some interpretation in their use. A number of models are available such as ComBase (see: http://modelling.combase.cc/membership/ComBaseLogin.aspx?ReturnUrl=%2f).

Challenge Testing

Challenge testing is the process of inoculating the microorganism of interest into the product in question and observing its growth over time, by monitoring at selected intervals.

This work must be carried out in an appropriate laboratory. Conducting a challenge test requires expert knowledge to ensure that the strain(s) selected are relevant, the inocula are correctly applied with minimal interference to the products characteristics, and ensuring that the inoculum is viable and fully recoverable.

Shelf-Life Testing (Durability Studies)

Shelf-life testing is the process of holding a food under conditions that it would be exposed to in reality and measuring the level of targeted microorganisms present over time. The time needed for the level of microorganisms or a specific microorganism to reach an unacceptable level can be interpreted as its shelf life. So for example, if the shelf life of a ready-to-eat food with respect to *Listeria monocytogenes* is being determined, because a count of greater than 100 cfu/g is deemed unacceptable, the workers would look for the time for an increase of two logs on the inoculum level.

In order to do a durability study, the microorganism used should be those likely to be found associated with the foodstuff under test.

To establish a shelf life with respect to *L. monocytogenes* is difficult, because usually *L. monocytogenes* will not be present in the food at the beginning of shelf life. Hence, challenge testing approaches have to be used to determine the shelf life. There are a number of useful references to help with this, including:

- *Shelf life of ready to eat food in relation to L. monocytogenes – Guidance for food business operators (CFA/FSA/BRC)* and
- SANCO/1628/2008. EC Guidance Document on *Listeria monocytogenes* shelf life studies for ready-to-eat foods, under Regulation (EC) No 2073/2005 of 15

November 2005 on microbiological criteria for foodstuffs. See: http://ec.europa.eu/food/food/biosafety/salmonella/docs/guidoc_listeria_monocytogenes_en.pdf

(iii) **RTE foods unable to support the growth of L. monocytogenes**

(Table below is taken from Regulation 2073/2005)

Food category and criterion number	Microorganisms/their toxins, metabolites	Sampling plan		Limits		Analytical reference method	Stage where the criterion applies
		n	c	m	M		
1.3 Ready-to-eat foods unable to support the growth of *L. monocytogenes,* other than those intended for infants and for special medical purposes	*Listeria monocytogenes*	5	0	100 cfu/g		EN/ISO 11290-2	Products placed on the market during their shelf-life

In order for a food to belong to this category (i.e., 1.3), it is necessary for the manufacturer to establish that *L. monocytogenes* will not be able to grow in the product.

If the product being considered is neither:

- A RTE food intended for infants or RTE food for special medical purposes (category 1.1); nor
- A RTE food able to support the growth of *L. monocytogenes*, other than those intended for infants and for special medical purposes (category 1.2)

then the food manufacturer must have other scientific evidence that *L. monocytogenes* cannot grow in its product in order to apply to this category (i.e. category 1.3).

Levels of *L. monocytogenes* of more than 100 CFU/g in a product are considered to be a food safety risk. Such products must be withdrawn from the market and the CA notified.

International Work Complimentary to the European 100 CFU/g *L. monocytogenes* in Ready-to-Eat Foods

In 2004, the FAO/WHO stated that the risk of acquiring foodborne listeriosis increases depending on factors such as host susceptibility, the amount and frequency of consumption of a food contaminated with *L. monocytogenes*, the frequency, distribution and level of *L. monocytogenes* in the food, the potential for growth of *L.monocytogenes* in the food during refrigerated storage, the refrigerated storage temperature, and/or the duration of refrigerated storage before consumption (FAO/WHO 2004a).

Consumers of RTE foods are likely to eat foods that are contaminated with *L. monocytogenes* on a regular basis, however, the incidence of listeriosis is low.

Various surveys have been carried out (e.g., Ryser and Marth 2007), which reveal that the prevalence of *L. monocytogenes* in RTE foods ranges from 0% to 10%. A large US study revealed that the prevalence of *L. monocytogenes* in RTE products such as smoked seafood, luncheon meats, salads, and cheeses ranged from 0.17% to 4.7% (Gombas et al. 2003). In the UK, Little et al. (2009) determined that the prevalence of *L. monocytogenes* in RTE foods ranged from 0% to 7%.

Based on case data, the likelihood of any one food contaminated with low numbers of *L. monocytogenes* resulting in illness is considered to be remote (FAO/WHO 2004b). Foods containing low levels of *L. monocytogenes* (e.g. <100 CFU/g) pose very little risk (Chen et al. 2003; FAO/WHO 2004b). The testing of foods associated with outbreaks has often revealed the presence of relatively high levels of *L. monocytogenes* (i.e. >1000 CFU/g). Thus, these outbreaks were due to noncompliant samples. The consequence of this is that lower priority should be placed on products in which *L. monocytogenes* cannot grow to levels above 100 CFU/g throughout their stated shelf life.

Following the microbiological risk assessment carried out by the joint FAO/WHO Expert Meetings on Microbiological Risk Assessment (JEMRA) on *L. monocytogenes* (JEMRA 2004), guidelines were published by the Codex Committee on Food Hygiene. These defined the microbiological criteria in RTE foods depending on the growth potential of *L. monocytogenes* (Codex 2007).

Following the publication of these Codex guidelines (Codex 2007), Codex member states have revised their regulations accordingly (e.g. FSANZ 2011; Health Canada 2011). However, in the USA, the USDA-FDA published an alternative approach of 'zero-tolerance' of *L. monocytogenes* in RTE foods (USDA-FDA 2008), although the classification versus growth potential was considered in a prior risk assessment (USDA-FDA 2003).

Presently, the risk posed by *L. monocytogenes* in RTE foodstuffs is regarded far greater in the USA (with the "zero-tolerance policy) compared to Europe (with the <100 CFU/g policy) and specific other parts of the world. When looked at logically, it should be possible to isolate *L. monocytogenes* from most samples of environmentally-derived foods such as bagged salads. It doesn't make scientific sense to accept samples of a commodity such as this, in which *L. monocytogenes* have not been detected, as a microbiologist knows that the organism is likely to be present in other portions of the lot which were not tested.

Conclusions

Listeria monocytogenes is a pathogenic bacterium that is widely occurring in the natural environment. Hence, it is anticipated that this foodborne pathogen would occasionally be associated with foods derived from the environment such as fresh produce. Unless stringent cleaning regimes are in place, which are operated correctly each time, *L. monocytogenes* can populate food factory environments. Its ability to produce biofilms helps *Listeria* spp. to achieve this, by making it more resistant to cleaning techniques. The consequence of this is that *L. monocytogenes*

may be present in foods derived from the natural environment, as well as foods which have been processed. Especially vulnerable are foods that are not processed further prior to being consumed i.e., RTE foods.

Although relatively few cases of listeriosis are diagnosed each year, the organism can cause death. It is estimated that the mortality rate associated with listeriosis is approximately 30% (2004b). Hence, *L. monocytogenes* and listeriosis must be taken seriously.

The frequency of *L. monocytogenes* contamination of RTE foods has been estimated to be 0–10%, with high levels of contamination (>1000 CFU/g) being associated with foods which have been linked to food poisoning episodes. The conclusions of a number of risk assessments (EC 1999; JEMRA 2004) are that low levels of *L. monocytogenes* contamination (<100 CFU/g), is an acceptable risk. With food safety systems such as Good Manufacturing Practices (GMP) and Hazard Analysis and Critical Control Point (HACCP) in place and operating, high levels of contamination should be rarely associated with foods entering the market. Hence, given that RTE foods will occasionally be contaminated with *L. monocytogenes,* it can be argued that low level contamination is an acceptable risk to the consumer. A tolerance such as the 'European 100 CFU/g' tolerance described here protects the health of consumers while ensuring fair practices in food trade.

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Science to Support the Prevention of *Listeria monocytogenes* in Ready-to-Eat Foods

Janell R. Kause, Daniel L. Gallagher, and Daniel L. Engeljohn

Abstract *Listeria monocytogenes* (*Lm*) is a foodborne pathogen of international public health concern. It is a leading cause of food-related hospitalization, fetal loss, and death in the United States (U.S.). Most exposures result from foods that do not undergo further consumer cooking and are considered ready-to-eat (RTE). In most healthy individuals, exposure to *Lm* results in flu-like symptoms; however, among growing susceptible populations (e.g., older adults, pregnant women, and the immunocompromised), exposure to *Lm* can result in spontaneous abortion (in pregnant women), septicemia, meningitis, and death. Control of *Lm* can be difficult, since it is widely distributed and persists along the entire food supply chain, can re-contaminate RTE foods, and can grow to high levels at typical refrigeration temperatures. Despite these challenges, the U.S. has successfully reduced the incidence of listeriosis by over 42% in the past decade – primarily as a result of a regulatory risk management strategy that motivated industry adoption of more effective *Lm* processing controls and implementation of enhanced sanitation programs to prevent *Lm* contamination of meat and poultry products. This chapter explores the systematic conduct and use of microbiological risk assessments to effectively guide federal policies and programs, creation of a regulatory environment that supported industry efforts to prevent *Lm* contamination of RTE meat and poultry products, and the resulting shared public-private success in reducing the risk of listeriosis in the U.S. The chapter ends with the consideration of several recent outbreaks of invasive listeriosis and recognition of continued gaps in knowledge to fully assess the food safety risk that supports maintenance of a "zero tolerance" for *Lm*.

Keywords *Listeria monocytogenes* • Risk assessment • Risk management strategy • Process controls • Enhanced sanitation programs • Regulatory environment • Prevention

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Public Health Regulatory Context

Listeria monocytogenes (*Lm*) is a foodborne pathogen of public health concern in the United States (U.S.). It is responsible for an estimated 1,600 cases of severe illness (invasive listeriosis), resulting in almost 1,500 hospitalizations, and 260 deaths, annually (Scallan et al. 2011). The high hospitalization (>90%) and mortality (over 20% compared to 0.5% for either *Salmonella* or *E. coli* O157:H7) associated with this foodborne pathogen results in an economic burden of $2.8 billion per year – the third highest of all foodborne pathogens in the U.S. (Scallan et al. 2011; Hoffmann et al. 2015). In most healthy individuals, exposures to *Lm* in foods results in flu-like symptoms; however, among growing susceptible populations (older adults (>65 years), pregnant women, and the immunocompromised), exposure to *Lm* can result in spontaneous abortion (in pregnant women), septicemia, meningitis, and death (Mook et al. 2011; Goulet et al. 2012; Silk et al. 2012). Both the proportion of older adults and immunocompromised (e.g., diabetics) are expected to dramatically increase over the next few decades, increasing the vulnerability of the U.S. population to invasive listeriosis (Colby and Ortman 2015; Ortman et al. 2014). These factors make prevention of *Lm* a continued food safety priority for industry and a driver for regulatory action in the U.S.

Prevention and control of *Lm* can be a challenge. It is widely distributed and persists along the entire food supply chain, including processing establishments and retail delicatessens, and readily grows in many foods under refrigerated temperatures (Lin et al. 2006; Ryser and Marth 2007; Saunders et al. 2012; Ferreira et al. 2014). Foods that do not undergo consumer cooking to achieve lethality, i.e., those considered "ready-to-eat" (RTE), present the greatest challenge. Cooking and other lethality treatments (e.g., fermentation, drying, salt curing) generally eliminate *Lm* during processing; however, foods may be re-contaminated through cross-contamination during further processing, at retail, and at consumers' homes (Tompkin 2002; Kennedy et al. 2005; Lianou and Sofos 2007; Carpentier and Cerf 2011; Pouillot et al. 2015b, Gallagher et al. 2016).

Despite these challenges, the U.S. has successfully reduced the incidence of listeriosis by over 42% in the past decade – primarily as a result of a regulatory risk management strategy that supported and helped motivate industry adoption of more effective *Lm* processing controls and implementation of enhanced environmental surveillance and sanitation programs to prevent *Lm* contamination of RTE meat and poultry products (Cartwright et al. 2013; DHHS-CDC 2014). This chapter explores the systematic conduct and use of microbiological risk assessments (MRAs) to effectively guide federal policies and programs, the creation of a regulatory environment that supported industry efforts to look for and to prevent *Lm* in RTE meat and poultry products and the processing environment, and the resulting shared success of public and private sectors in reducing the risk of listeriosis in the U.S. The chapter ends with the consideration of several recent outbreaks of severe listeriosis and knowledge gaps in risk assessments that support maintenance of a "zero tolerance" for *Lm*.

Risk-Informed Policies and Programs to Prevent *Lm* Contamination of Foods

For the past two decades, the U.S. has relied on the use of MRAs to guide food safety decisions related to *Lm* (DHHS/FDA-USDA/FSIS 2003; USDA-FSIS 2003a, 2007; Endrikat et al. 2010; Pouillot et al. 2015b; Gallagher et al. 2016) and other foodborne pathogens. MRA is a systematic and scientifically-based approach for assessing the likelihood of exposure and subsequent impact of a pathogen on human health. The reliance on MRA in food safety has been recognized both nationally and internationally as a powerful and practical public health tool for improving food safety policies and programs because it allows risk managers to identify the most effective strategies to control hazards to prevent foodborne illness (Hathaway and Cook 1997; IOM/NRC 1998; CAST 2006; FAO/WHO 2006). While widely used, MRA is still a relatively new scientific discipline that presents many challenges. These challenges are due, in part, to the dynamic nature (e.g., growth, decline, antimicrobial resistance) of microbiological pathogens as they move through the food supply chain from the farm to the table, lack of regular testing for the presence and, perhaps more importantly, the levels of pathogens across a wide array of foods, and limited information on host susceptibility and virulence of *Lm* strains in consumer exposures (DHHS-FDA/USDA-FSIS 2003; Hoelzer et al. 2013). As more data become available, along with a better understanding of host susceptibility and the conditions that support growth of pathogens in foods, and as scientific techniques evolve (e.g., whole genome sequencing), more robust scientific information will be available to further the application of MRA to inform decision-making (Brul et al. 2012; Hoelzer et al. 2013).

Currently, the lack of robust information on host susceptibility and strain variability results in a highly uncertain *Lm* dose-response relationship (i.e., Pouillot et al. 2015a) and limits the ability to accurately estimate, with any reasonable certainty, the level of *Lm* that results in illness (i.e., absolute risk estimate) or establish an appropriate level of protection (Gallagher et al. 2013). It is widely believed that most invasive listeriosis cases result from consumer exposure to high levels of *Lm* in RTE foods (McLauchlin et al. 2004; Pouillot et al. 2015a), although there is some past evidence of illness from exposure to low *Lm* doses (Lyytikäinen et al. 2000; Mead et al. 2006). A recent outbreak of invasive listeriosis involved low levels of *Lm* in ice cream served to more susceptible hospitalized patients (DHHS-CDC 2015b). It is also widely believed that invasive listeriosis occurs only among those with weaker immune systems (e.g., the very young, the very old, or those with comorbidities (e.g., diabetes, AIDS)). Yet, the epidemiological evidence suggests otherwise – that healthy individuals are also at risk. Another recent *Listeria* outbreak included healthy children developing listerial meningitis (DHHS-CDC 2015a), presumably from the growth of *Lm* to high levels in foods previously thought not to readily support the growth of this pathogen and therefore a food considered as posing a low to moderate risk (DHHS/FDA-USDA-FSIS 2003; DHHS-CDC 2015a; Glass et al. 2015).

In one dose-response model developed by FAO/WHO (2004), the estimated ID_{50}s (those doses with a corresponding 50% probability of illness) were 2.9×10^{13} and 6.5×10^{11} cells for healthy and susceptible subpopulations, respectively. Recently, Pouillot et al. (2015a) incorporated strain variability and population subgroups. This updated dose-response model suggests that *Lm* poses a 300-fold greater risk than previously estimated by FAO/WHO, with an ID_{50}s 8.8×10^{10} for healthy adults less than 65 years old, 4.6×10^{9} for elderly, and 7.2×10^{7} for hemolytic cancer patients – the most susceptible subgroup studied. The authors also note that doses above 6.3×10^{7} cells were not seen in the exposure data. Thus most cases of invasive illness occur from low probability events. The authors conclude that, if highly virulent strains are involved, even relatively low doses of *Lm* can lead to illness among those already at risk of developing listeriosis.

Despite these limitations of food safety MRAs for making predictions of "absolute risk," they are often anchored to robust epidemiologic foodborne illness surveillance data and can be useful for comparing the "relative risk" of foods (DHHS-FDA/USDA-FSIS 2003) or evaluating the predicted relative reduction in illnesses when various intervention strategies are implemented (USDA-FSIS 2003a). In these decision-making contexts, food safety MRAs are considered "fit for purpose," that is, they are appropriately used (Dearfield et al. 2014). The information on the relative risk of foods and ability to evaluate and compare the public health impact of control strategies has made MRA a valuable tool for systematically prioritizing and targeting national and international resources and informing policies to improve food safety. Over the past 15 years, the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA), often in partnership with the U.S. Food and Drug Administration (FDA), have conducted several quantitative MRAs for *Lm* that have been successfully used to develop effective food safety policies and target inspection resources to reduce the risk of listeriosis in the U.S.

Emergence of Lm in the U.S. and Establishment of a "Zero Tolerance"

In the 1980s, *Lm* emerged in the U.S. as a recognized foodborne pathogen with outbreaks associated with post-lethality exposed pasteurized milk, soft cheese, *pâté*, seafood, deli meats, and turkey hot dogs (Schlech et al. 1983; DHHS-CDC 1985; Fleming et al. 1985; Linnan et al. 1988). An outbreak of listeriosis associated with soft cheese (i.e., Queso blanco) resulted in 52 deaths, including 19 stillbirths and 10 infant deaths – the largest number of *Lm*-related foodborne deaths in U.S. history since the Centers for Disease Control and Prevention (CDC) began tracking in 1970 (DHHS-CDC 1985). Since 1987, USDA-FSIS has randomly sampled and tested for *Lm* in RTE meat and poultry products produced in federally inspected establishments (USDA-FSIS 1987, 2016; Ryser and Marth 2007). In 1989, the CDC directly linked consumption of contaminated turkey hot dogs to a case of listerial meningitis), the first direct association between listeriosis and a RTE meat or poultry product in

the U.S. (DHHS-CDC 1989). This resulted in a nationwide recall, enhanced routine monitoring and verification sampling of RTE products produced in the U.S., and the adoption of a "zero tolerance" (i.e., no detectable levels of a viable pathogen in, at that time, a 25-gram portion) for *Lm* in RTE products (Crawford 1989; USDA-FSIS 1989). RTE meat and poultry products testing positive for *Lm* are considered "adulterated" under the Federal Meat Inspection Act and Poultry Products Inspection Act (21 U.S. Code 601(m) and 453(g), respectively). These initial efforts led to a decline in invasive listeriosis cases from 0.7 per 100,000 cases in the 1980s to 0.4 per 100,000 cases by the mid-1990s (Tappero et al. 1995). While establishment of a "zero tolerance" with corresponding enhanced federal oversight reduced the incidence of listeriosis in the U.S., large outbreaks of invasive listeriosis, primarily associated with dairy products and RTE meat and poultry products, continued throughout the 1990s (Ryser and Marth 2007). There was a need to better understand where to focus efforts along the supply chain and which interventions would most effectively prevent the risk of invasive listeriosis.

Science-Based Prevention: PR/HACCP and Microbiological Risk Assessments (MRAs)

In 1996, USDA-FSIS implemented the Pathogen Reduction and Hazard Analysis and Critical Control Point (PR/HACCP) regulatory program to support efforts to prevent foodborne illness in the U.S. (USDA-FSIS 1996). Under this program, industry analyzes its processes to determine at what points foodborne biological, chemical, or physical hazards might exist that could affect the safety of its products, establishes science-based mitigation measures, and monitors and documents the effectiveness of its food safety controls and sanitation programs. PR/HAACP encouraged the use of a systems approach to prevent, eliminate, or reduce microbiological hazards to an acceptable level and ensure safe food by controlling production processes from the beginning to the end, rather than detect problems at the end of the line. As a complement to PR/HACCP, USDA-FSIS began conducting food safety MRAs to guide the establishment of risk-based policies, including industry pathogen reduction performance standards, to achieve national food safety goals (e.g., Healthy People goals) (Buchanan and Whiting 1998; Hulebak and Schlosser 2002). These initial steps to modernize food safety focused on utilizing scientific risk-based approaches to prevent foodborne illness.

The motivation to strengthen efforts to prevent *Lm* in RTE meat and poultry products resulted from a nationwide outbreak in 1998 of invasive listeriosis primarily associated with the consumption of hot dogs (Mead et al. 2006). This outbreak resulted in 108 illnesses and 21 deaths and was attributed to insanitary conditions with products being contaminated with *Lm* from the processing environment. In February 2001, USDA-FSIS issued a federal notice advising manufacturers of RTE meat and poultry products of the need to reassess their PR/HACCP plans to ensure they were adequately addressing *Lm,* taking into consideration that this pathogen

was reasonably likely to occur in the production of most RTE meat and poultry products (USDA-FSIS 2001). In an effort to better target federal resources, USDA-FSIS also partnered with FDA, in consultation with CDC, to conduct a large-scale quantitative MRA (i.e., the "FDA-FSIS MRA") (DHHS-FDA/USDA-FSIS 2001). This MRA was undertaken to identify which RTE foods posed the greatest public health risk of invasive listeriosis (DHHS-FDA/USDA-FSIS 2003).

The FDA-FSIS MRA was designed to evaluate the relative risk of invasive listeriosis and deaths from 23 categories of RTE foods among three age groups (i.e., older adults (>60 years), fetuses and newborns up to 1 month, and an intermediate age group (between 1 month and 60 years old). This MRA modeled the growth and decline of *Lm* from retail, through consumer refrigeration (Yang et al. 2006), to consumption, and included an exposure assessment pathway for each category of RTE food. This quantitative risk ranking enabled comparison of the relative exposure to *Lm* among various types of RTE foods. These comparisons were dependent on available data for the presence and level of *Lm* in various foods and the quality of the predictive microbiological models to accurately predict the growth of *Lm* in these foods. For some categories of RTE foods, such as deli meats, there were robust data on the presence and levels of *Lm* in foods from routine USDA-FSIS verification testing programs (DHHS-FDA/USDA-FSIS 2003; USDA-FSIS 2016), resulting in greater certainty on the estimated consumer exposure to *Lm* from these products. For other categories of RTE foods, such as fruits and vegetables, there were significant data gaps on the presence and levels of *Lm* in these foods as a result of limited testing data. This resulted in substantive uncertainty in estimated consumer exposure to *Lm* from these categories of RTE foods. Moreover, predictive microbiological data were more robust for some categories of RTE foods than others in this MRA, resulting in significant variability in the certainty of exposure estimates. Consumption data from dietary surveys (i.e., Continuing Survey of Food Intakes by Individuals and the Third National Health and Nutrition Examination Survey) provided nationally representative data on the amount of food consumed by an individual, the number of servings per year, and demographic characteristics of the consumer. Estimated levels of contamination along with information on serving size and number of servings for each category of RTE food were used to derive estimates of *Lm* exposure among the various age-based sub-groups in this MRA.

To predict the risk of invasive listeriosis in the FDA-FSIS MRA, exposure estimates for each of the 23 categories of RTE foods were anchored to the epidemiologic surveillance data, with the total number of listeriosis cases apportioned among the categories based on MRA estimates of *Lm* exposure (DHHS-FDA/USDA-FSIS 2003). The animal dose-response model was shifted to fit the number of listeriosis fatalities observed in national epidemiologic data to derive a dose-response relationship for each age group considered. It is important to note that the relative risk rankings in this MRA do not represent an absolute risk of illness from each category of RTE food, but provide a comparison of the risk of invasive listeriosis or death relative to each other. The per-serving risk of a RTE food category is heavily dependent on the available data for the presence, levels,

and predicted growth of *Lm* in the particular food. The per-annum risk is heavily dependent on the frequency with which foods are consumed over the year (e.g., hot dogs versus pâté).

Despite the inability to accurately predict the absolute risk of invasive listeriosis for each category of RTE food, this MRA provided: (1) useful estimates of the potential level of exposure of three age-based population groups to *Lm* in various categories of RTE foods; and (2) related the level of *Lm* in these foods to the likelihood of listeriosis among these populations. This MRA, completed in 2003, provided the first systematic evaluation of the available scientific data and information to estimate the relative risk of invasive listeriosis and death that may be associated with the consumption of different types of RTE foods contaminated with *Lm*. This information was valuable given that Batz et al. (2011) estimated that there are 44 sporadic cases of listeriosis for every outbreak case with the majority of sporadic cases not linked to a specific food. As a result, listeriosis outbreaks may not be representative of the relative risk posed by RTE foods overall.

Prior to the preliminary results of the FDA-FSIS MRA, first made public as a 2001 draft, deli meats were not the primary vehicle of concern – hot dogs had been the primary vehicle implicated by a case-control study related to the 1998–1999 nationwide listeriosis outbreak and in prior outbreaks of invasive listeriosis in the U.S. (DHHS-CDC 1989, 1999; Mead et al. 2006). In 2001, the preliminary findings of the MRA relative risk ranking pointed to deli meats as posing the greatest risk of invasive listeriosis per serving and per annum. A year later, in October 2002, a second large outbreak of listeriosis (resulting in 46 culture-confirmed cases, 7 deaths, and 3 stillbirths) in Northeastern U.S. was linked to the consumption of turkey deli meat (DHHS-CDC 2002; Olsen et al. 2005). The 2001 draft FDA-FSIS MRA had correctly predicted that deli meat posed a significant risk a year in advance of the Northeastern outbreak, highlighting the value of these types of MRAs (i.e., quantitative risk-rankings) to predict potential problems (e.g., an outbreak) before they occur. These types of MRAs also serve as a useful microbiologically-based exposure-based approach for attributing foodborne illness. This approach to attribution, often referred to as a "bottom-up" approach (compared to a "top-down" approach based on foodborne outbreak data) is most useful when cases of foodborne illness are sporadic (i.e., not linked in an outbreak to a foodborne vehicle) (Batz et al. 2005; EFSA 2015; IFSAC 2015).

In 2003, USDA-FSIS and FDA completed the FDA-FSIS MRA quantifying the relative risk of RTE foods based on public comment and additional industry consumer behavior data (DHHS-FDA/USDA-FSIS 2003). The results are summarized in Fig. 1. Overall, deli meats were estimated to account for 67% of all listeriosis cases per year in the U.S. On a per serving basis, this MRA identified several foods as posing a high risk, including deli meats, soft cheeses, pâté, and smoked seafood. Fruits and vegetables were identified as posing a low to moderate risk per serving risk, whereas ice cream and other frozen dairy products were estimated to pose a very low relative risk. In general, the categories of RTE foods that did not support or supported only limited growth of *Lm* were those that posed a low to moderate relative risk. Recent outbreaks of invasive listeriosis associated with vegetables

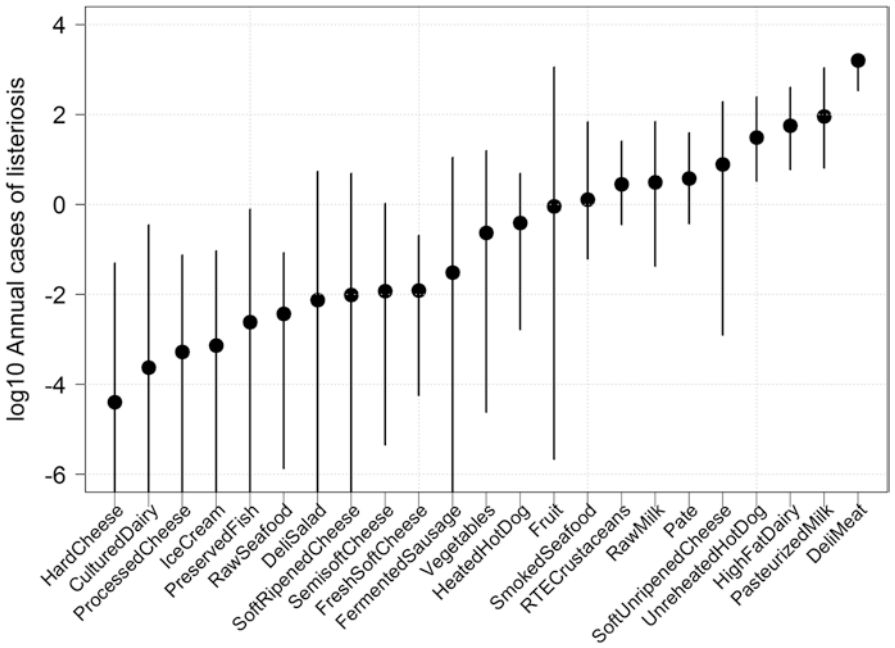

Fig. 1 Estimated annual cases of listeriosis for the 23 food categories of the 2003 FDA-FSIS risk ranking. Median and 95% confidence intervals shown

(e.g., prepackaged salads (DHHS-CDC 2016a) and frozen vegetables (DHHS-CDC 2016b) and fruits (e.g., cantaloupe and caramel apples; DHHS-CDC 2011, 2015a), however, have led to more recent reconsideration of the general "rule of thumb" for the conditions under which *Lm* grows in various RTE foods (Glass et al. 2015; Bronstein et al. 2015). The recent outbreak of invasive listeriosis associated with the consumption of caramel apples highlights how specific details of a particular process may contribute to the contamination and growth of *Lm* in foods generally considered to not support the growth of *Lm* and, as a result, be a "low risk" (Glass et al. 2015). These outbreaks have also pointed to the need for risk managers to more fully consider the characterized uncertainty provided with MRA estimates to better anticipate potential food safety risks and guide risk management decisions.

In response to findings of insanitary conditions in RTE meat and poultry processing establishments producing deli meat products associated with the 2002 Northeastern listeriosis outbreak (DHHS-CDC 2002; Gottlieb et al. 2006) and preliminary results of the FDA-FSIS MRA, USDA-FSIS conducted a second quantitative MRA, deemed the "2003 USDA-FSIS deli meat MRA," to evaluate which *Lm* controls and sanitary measures would be most effective in preventing contamination of deli meats during processing (USDA-FSIS 2003a). A year prior, USDA-FSIS had published a proposed rule to establish food safety controls for various RTE foods (USDA-FSIS 2001). One of the food safety controls was to address *Lm* cross-contamination concerns for post-lethality exposed RTE meat and poultry

products during processing. In the proposal, USDA-FSIS required all establishments producing post-lethality exposed RTE meat and poultry products, including those that already addressed *Lm* as part of their PR/HACCP plan, to conduct environmental testing of both food contact surfaces and nonfood contact surfaces for *Listeria* species after the lethality treatment of certain products and before final packaging. *Listeria* species rather than *Lm* were identified as the target class of indicator microorganisms for the possible insanitary condition because its presence does not directly implicate end product safety. *Listeria* species as a class of microorganisms is not deemed pathogenic.

In the USDA-FSIS proposal, establishments were given the option to forego testing if they established controls to help eliminate possible *Lm* contamination following lethality treatments in their food safety system. For establishments that did not incorporate a control step to eliminate possible *Lm* contamination, but incorporated an agent or process to inhibit growth; or simply control cross-contamination through enhanced sanitary hygiene measures, verification testing for *Listeria* species is required. The establishment and USDA-FSIS would use testing results to verify the efficacy of the establishment's "Sanitation Standard Operating Procedures" (Sanitation SOPs) in preventing *Lm* contamination in post-lethality-exposed RTE product. USDA-FSIS also suggested an increased frequency of *Listeria* species testing of food contact surfaces for larger establishments, as well as those that produced a larger volume of product, particularly for those establishments that relied more on Sanitation SOPs than an agent or process to limit growth of *Lm.* The public health benefits for increased product and food contact surface testing were initially unknown. The 2003 USDA-FSIS deli meat MRA would provide this information (i.e., the public health benefits of increased product and food contact testing) in addition to quantifying the number of annual listeriosis cases prevented by the adoption of various *Lm* processing controls (i.e., product formulation with growth inhibitors and post-lethality interventions) (USDA-FSIS 2003a).

In 2003, USDA-FSIS completed the deli meat MRA (USDA-FSIS 2003a), which provided a strong public health basis for its 2003 Interim Final Rule for *Listeria* (USDA-FSIS 2003b). This second MRA included a novel dynamic in-plant processing module that linked to the previously developed retail-to-table exposure pathway and *Lm* dose-response relationship used in the risk-ranking model (DHHS-FDA/USDA-FSIS 2003). The 2003 USDA-FSIS deli meat MRA was developed to evaluate the effectiveness of product testing and lot removal, food contact surface testing and sanitation, product formulation with growth inhibitors, and post-lethality processing controls in mitigating the risk of invasive listeriosis from deli meats. Results are illustrated in Fig. 2, which depicts the estimated number of lives saved among the elderly, the largest at-risk group, for different sampling and intervention strategies. Prior to conducting the 2003 USDA-FSIS deli meat MRA, both federal risk managers and stakeholders were focused on product and food contact surface testing as primary measures to mitigate the risk of listeriosis from RTE meat and poultry products (USDA-FSIS 2003a). This MRA revealed that formulating deli meats with growth inhibitors (i.e., to limit the growth of *Lm* to 1-log or less over the shelf life of this product) combined with the use of a post-lethality intervention

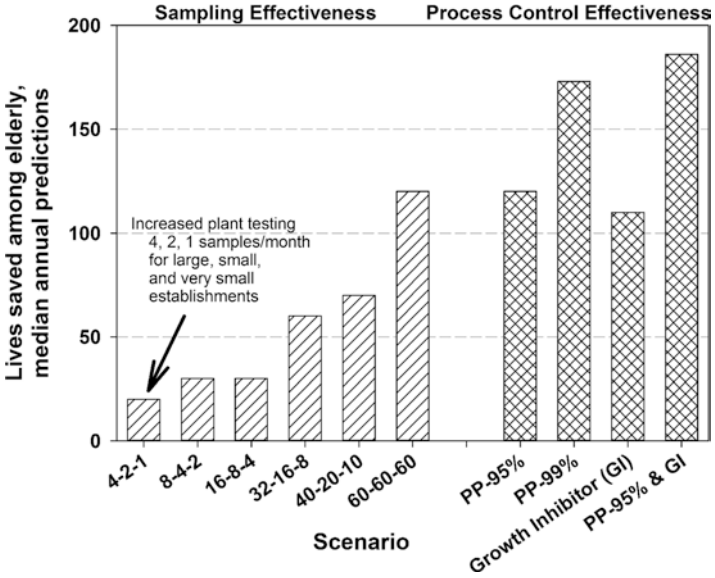

Fig. 2 Estimated annual lives saved among the elderly for different levels of lot testing and process controls. The "4-2-1" scenario represents USDA-FSIS baseline verification sampling frequency per month for large, small, and very small establishments. The *PP* indicates a post-processing lethality step with the lethality given as a percentage. *GI* stands for "growth inhibitor"

(e.g., high pressure pasteurization) to eliminate *Lm* (Alternative 1) would prevent 186 listeriosis deaths annually (see Fig. 2). Use of either of these *Lm* interventions alone was the next most effective processing control [Alternative 2a (post-lethality intervention) and Alternative 2b (formulation with growth inhibitors)]. Product testing and lot removal and food contact surface testing and sanitation were much less effective in plant interventions (Alternative 3). The findings of the 2003 USDA-FSIS deli meat MRA directly formed the public health basis for USDA-FSIS's Interim Final Rule for *Lm*, which encourages industry adoption of more effective food safety interventions (i.e., adoption of Alternative 1 > Alternative 2 > Alternative 3) during the production of RTE meat and poultry products (USDA-FSIS 2003a).

Successful Industry Reduction of Lm *Incentivized by U.S. Regulatory Policies*

USDA-FSIS's *Listeria* rule encouraged processing establishments producing post-lethality-exposed RTE meat and poultry products (i.e., those under 9 CFR 430) to adopt more effective *Lm* controls and to enhance environmental surveillance and monitoring to prevent *Lm* contamination of RTE meat and poultry products (USDA-FSIS 2003a, 2004). In the *Listeria* rule, USDA-FSIS announced that it would

inspect establishments producing post-lethality-exposed RTE meat or poultry product based on the effectiveness of *Lm* controls they had in place and the risk presented by the products they produced. Those establishments producing a higher risk RTE meat and poultry products (i.e., deli meats or hot dogs) with less effective *Lm* controls (e.g., those that relied solely on environmental testing and sanitation rather than a lethality treatment or an agent or process to inhibit growth) would have an increased frequency of USDA-FSIS verification inspection activity, including product testing, compared to those that had more effective *Lm* controls in place. [Note: Throughout, USDA-FSIS continued to encourage the prevention of *Lm* contamination coupled with a non-detectable *Lm* standard (i.e., below 0.04 colony-forming units (CFU) per gram in a 25-gram test portion) for all RTE meat and poultry products because of the need to minimize the presence of this controllable food safety hazard in the processing environment, the high case-fatality rate associated with *Lm* (>20%), and continued concerns regarding susceptibility of consumers to invasive listeriosis. USDA-FSIS has since increased the sample test portions to 125 grams (USDA-FSIS 2006).]

To aid in the implementation of its 2003 Interim Final Rule (USDA-FSIS 2003b), USDA-FSIS developed compliance guidelines and conducted outreach to support industry efforts to adopt more effective *Lm* processing controls and enhance sanitary SOPs (USDA-FSIS 2006, 2014 (updated)). USDA-FSIS also developed a statistical risk-ranking algorithm to guide the allocation of USDA-FSIS's substantive inspection resources among establishments producing post-lethality exposed RTE meat and poultry products based on public health risk (USDA-FSIS 2007). This statistical algorithm provided a monthly ranking of establishments producing RTE meat and poultry products that could be exposed to *Lm* after the lethality step during processing. The risk-based statistical algorithm was driven by the following factors in order of weight: effectiveness of the type of *Lm* control an establishment had in place (as determined by the quantitative 2003 USDA-FSIS deli meat MRA), the type of product it produced (as determined by the 2003 FDA-FSIS MRA risk-ranking), USDA- FSIS *Lm* product test results, and the volume of post-lethality exposed RTE product produced by the establishment. Information on the type of intervention, product produced, and volume were initially provided at regular intervals by the industry in response to an Office of Management and Budget-approved survey. USDA-FSIS's Interim Final Rule (USDA-FSIS 2003b) enhanced its oversight of RTE meat and poultry establishments, while encouraging industry to strengthen its *Lm* processing controls (USDA-FSIS 2003b, 2015a). USDA-FSIS no longer requires the industry to submit production volume information because USDA-FSIS inspectors are now able to capture this information as part of an establishment's profile in an integrated information technology system – the Public Health Information System (PHIS) developed in the late 2000s (USDA-FSIS 2014).

In January 2005, USDA-FSIS launched its risk-based sampling verification program. Approximately 10,000 post-lethality exposed RTE meat and poultry product samples were tested for *Lm* annually. Establishments held tested product lots until laboratory results were confirmed as testing negative for *Lm*. Often, negative results took up to 3 days to receive, with positive results taking longer. More frequent

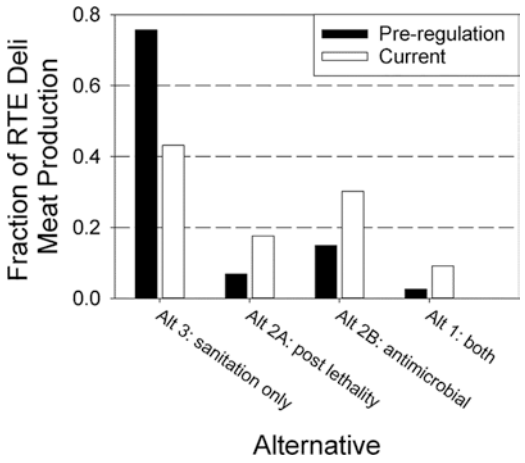

Fig. 3 Increased adoption of more stringent food safety practices before and after the Interim Final Rule (Adapted from Endrikat et al. (2010))

testing was correlated with a delay in releasing product into commerce. Product lots testing positive for *Lm* would not be allowed into commerce without reprocessing (e.g., re-cooking) or would be recalled if already released. Moreover, USDA-FSIS *Lm*-positive sample results for an establishment would be weighed into a 6-month rolling average with *Lm* findings factored into the risk-based statistical ranking of the establishment, increasing the frequency with which the establishment's product would be federally tested. USDA-FSIS's risk-based federal inspection program created an incentive for industry to adopt more stringent controls to prevent *Lm* on products. The concurrent development and availability of validated growth inhibitors enabled industry to adopt additional *Lm* controls to reduce risk (see Fig. 3). USDA-FSIS-regulated deli meat processing establishments voluntarily moved from the riskier sanitation and sampling only category (Alternative 3) to the more public health protective categories (Alternatives 1, 2A and 2B shown in Fig. 3). Before the Interim Final Rule, approximately 75% of establishments relied on sampling and sanitation only. After the Rule's promulgation, this dropped to just over 40%.

In April 2006, USDA-FSIS expanded its risk-based verification sampling program from solely product testing to also include environmental testing. As part of this expanded risk-based *Lm* inspection program, USDA-FSIS considered RTE meat and poultry products adulterated if they tested positive or if they came into contact with food contact surfaces that tested positive for *Lm*. To further support industry food safety efforts to prevent *Lm* contamination of RTE meat and poultry products, USDA-FSIS encouraged environmental testing for the indicator microorganism, *Listeria* species (USDA-FSIS 2013, 2014). Food contact surfaces found positive were an indication of suboptimal sanitation and the industry was afforded the opportunity to "find and fix" the problem before USDA-FSIS conducted follow-up testing (USDA-FSIS 2013). USDA-FSIS's expanded verification testing program was instrumental in incentivizing industry to take steps to know where *Lm* may be present in processing operations and to take corrective action (Butts 2012; Malley et al. 2015). The RTE meat and poultry industry implemented enhanced

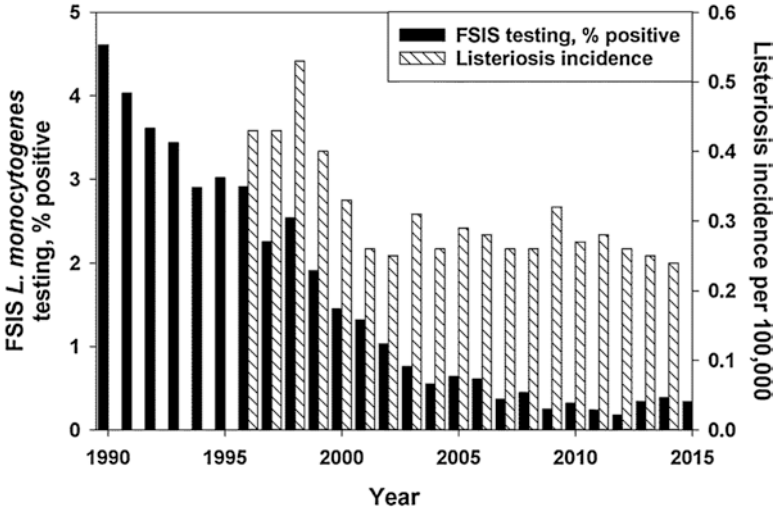

Fig. 4 USDA-FSIS regulatory testing results in RTE products and incidence of foodborne listeriosis (http://www.fsis.usda.gov/wps/wcm/connect/d2aedcb2-4890-42fc-9960-57a6a5bf3ecd/Figure-1-RTE-Testing.pdf?MOD=AJPERES and http://www.cdc.gov/foodnet/trends/2014/number-of-infections-by-year-1996-2014.html#table2b)

surveillance programs (e.g., zone testing), including "seek and destroy" programs designed to identify the root cause of potential problems before they occur – often seeking and eliminating the sources of *Lm* in processing establishments (Butts 2012; Malley et al. 2015). The industry also increased the sharing of best *Lm* control and prevention practices, having declared the control of *Lm* a "non-competitive" issue since 2001, and invested in long-term enhancements, including advancements in sanitary facility design and new equipment to prevent harborage of *Lm* in RTE meat and poultry processing establishments.

U.S. risk-based policies and programs to address *Lm* in RTE meat and poultry products have been extremely successful. USDA-FSIS has observed a steady decline in the percentage of RTE meat and poultry products testing positive through its routine sampling verification program – from 0.72% in 2005 to 0.34% in 2015 (USDA-FSIS 2015c, 2016) as depicted in Fig. 4. This dramatic decline of *Lm* in RTE meat and poultry products has been recognized by CDC as contributing to the reduction in the incidence of invasive listeriosis in the U.S. (0.26 per 100,000 cases) (Cartwright et al. 2013; DHHS-CDC 2014). Correspondingly, federally-inspected RTE meat and poultry products (i.e., foods identified as being a "high risk" based on the FDA-FSIS risk ranking (DHHS-FDA/USDA-FSIS 2003) and implicated in listeriosis outbreaks from the late 1980s through the early 2000s) have not been associated with an outbreak in more than a decade (Cartwright et al. 2013). Today, both the meat and poultry processing industries and the federal government claim shared success for reducing the risk of invasive listeriosis from RTE meat and poultry products in the U.S. Safety of RTE meat and poultry products produced in

the U.S. is a direct result of the effectiveness of risk-informed food safety policies and inspection programs combined with creation of a regulatory environment that motivates and supports industry efforts to prevent *Lm* contamination.

Efforts to Further Prevent Listeriosis in the U.S.: Mitigating Risk at Retail

With the success of risk-based policies and programs in preventing *Lm* contamination of RTE meat and poultry products during processing, the U.S. expanded this approach to address *Lm* at retail. Despite the dramatic decline in the percentage of *Lm*-positive RTE meat and poultry products in the 2000s, which were the primary foodborne vehicles for *Lm* exposure based on the risk ranking attributed to the 2003 FDA-FSIS MRA (DHHS-FDA/USDA-FSIS 2003), there was not a concurrent decline in listeriosis cases in the U.S. (Silk et al. 2012; DHHS-CDC 2013) (See Fig. 4). Surveys conducted by industry and academia revealed that RTE foods prepared in retail delicatessens were more frequently and heavily contaminated (Gombas et al. 2003; Draughon 2006; Ahmed et al. 2015). For example, deli meats sliced and sold at retail had a seven-fold higher prevalence and level of *Lm* compared to prepackaged product. This difference in *Lm* contamination was further evaluated in a comparative MRA, the "2010 USDA-FSIS MRA." This MRA estimated that approximately 83% of listeriosis cases attributed to deli meat were associated with product sliced and sold in retail delicatessens (Endrikat et al. 2010). An independent MRA conducted by academia had similar findings, i.e., that the majority of deli meat-related listeriosis cases were associated with retail prepared product (Pradhan et al. 2010). While it was recognized that *Lm* was present and persisted in the retail environment (Simmons et al. 2014; Wang et al. 2015) and that retail-prepared RTE foods could be contaminated (i.e., from the retail environment, from other RTE foods contaminated with *Lm*, or both (Pradhan et al. 2011), the extent to which retail preparation of RTE food contributed to the risk of invasive listeriosis was greater than commonly thought. Moreover, the extent to which retail practices and food safety interventions influenced this risk was not well understood.

Based on the results of the 2010 USDA-FSIS MRA, USDA-FSIS partnered with FDA to develop the most recent *Lm* MRA, deemed the "2013 Interagency Retail *Lm* MRA." This MRA was designed to help guide retail food safety efforts to further minimize the public health burden of invasive listeriosis in the U.S. (Pouillot et al. 2015b). The 2013 Interagency Retail *Lm* MRA, developed with extensive input from industry and consumer advocacy groups, is a discrete-event model that mimics an operating retail deli department and tracks *Lm* that may potentially be present in various environmental niches (e.g., slicer or utensils) and in select food products (i.e., deli meat, deli cheese, or deli salad). This MRA model simulates worker behavior, the contamination and cross-contamination of RTE foods, and the growth or decline of *Lm* in deli-prepared RTE foods, and predicts

the subsequent risk of invasive listeriosis among consumers. Data for the 2013 Interagency Retail *Lm* MRA was based on several targeted studies, conducted in collaboration with academia. These studies examined survey observations of deli employees' work routines (Lubran et al. 2010), presence and persistence of *Lm* strains in the retail deli environment (Simmons et al. 2014), the ability of *Lm* to spread in retail delis (Hoelzer et al. 2011), such as from a slicer to food, and transmission of *Lm* in a mock deli (Maitland et al. 2013). This 2013 Interagency Retail *Lm* MRA quantified the public health impact of how changes in current retail practices affect the risk of invasive listeriosis from retail-prepared RTE foods (Gallagher et al. 2016). Overall, the 2013 Interagency Retail *Lm* MRA reinforced the importance of FDA's Food Code recommendations and demonstrated the public health benefit of preventing *Lm* from entering the retail environment, particularly on incoming RTE foods, and mitigating the spread and growth of *Lm* (e.g., through regular sanitation and maintenance of proper storage temperature for RTE foods at retail) in retail delicatessens.

A key finding of the 2013 Interagency Retail *Lm* MRA was the benefit of maintaining current efforts to prevent *Lm* contamination on RTE foods products, including those that do not support growth. In this MRA, it was found that even preventing low levels of *Lm* on incoming RTE foods (i.e., by reducing the mean incoming *Lm* concentration on all RTE products by a factor of 2, from the observed mean of − 9.2 log CFU per gram to a mean of −9.5 log CFU per gram) was predicted to reduce the risk of invasive listeriosis from retail prepared RTE foods by 10–24%. Incoming RTE foods to be prepared at retail represent a primary vehicle for "seeding" the retail environment, serving as a major source for contamination of retail food contact surfaces and subsequent cross-contamination of other RTE foods (including those that do support growth and may result in illness) (Gallagher et al. 2016). Even in retail deli settings in which *Lm* is already present in niches, a reduction in the levels of *Lm* on incoming RTE products was predicted to have a substantive impact in reducing the risk from deli-prepared RTE foods.

The 2013 Interagency Retail *Lm* Risk Assessment also explored the public health impact of allowing 100 CFU *Lm* per gram for products that support the growth of *Lm* and those that do not (results are shown in Fig. 5). For RTE foods that support the growth of *Lm,* allowing 100 CFU per gram increases the average predicted risk of invasive listeriosis from RTE food prepared at retail from 1.4×10^{-7} to 300×10^{-7} per serving. The MRA revealed that this substantial increase in risk is primarily a result of the higher levels of *Lm* on incoming RTE foods that support *Lm* growth because the model predictions remained unchanged whether or not cross-contamination was occurring at retail. In contrast, when the incoming RTE products that do not support *Lm* growth were contaminated at 100 CFU per gram, there was almost no added risk of invasive listeriosis (viz., increasing only slightly from 1.1×10^{-7} to 1.2×10^{-7} per serving) under the hypothetical condition of no retail cross-contamination. Under typical conditions in which retail cross-contamination does occur, an increase to 100 CFU per gram of *Lm* on in-coming RTE products that do not support growth, leads to a substantial increase in the risk of listeriosis, from 1.1×10^{-7} to 66×10^{-7} per serving. Overall, these findings reveal that preventing

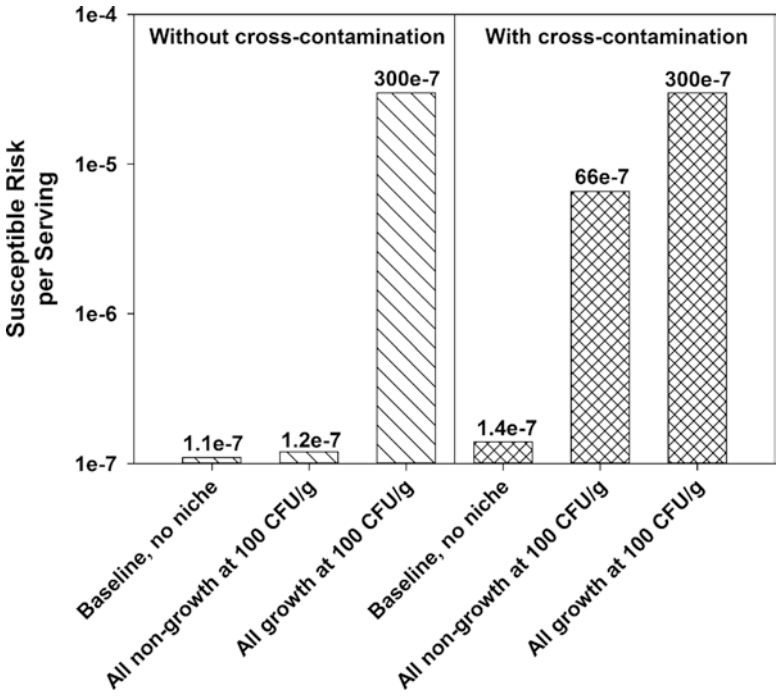

Fig. 5 Simulation results showing the impact of allowing *Lm* at 100 CFU/gram in retail deli products that do not support *Lm* growth. The panel to the left shows the results when cross-contamination does not occur. The panel to the right includes cross-contamination (Adapted from Gallagher et al. (2016))

even low levels of *Lm* on RTE foods to be prepared at retail, including those that do not support the growth of *Lm*, can substantially reduce the risk of invasive listeriosis.

There has been broad acceptance and use of the 2013 Interagency Retail *Lm* MRA findings. The Food Marketing Institute used the assessment to update its guidance to retailers (FMI 2012, 2016). Also, USDA-FSIS recently developed additional guidance to support industry efforts to control *Lm* in retail delicatessens (USDA-FSIS 2015b, c). In addition, there has been increased private sector investment in research to further prevent and control *Lm* at retail, and cross-sector collaboration to leverage the lessons learned (e.g., enhanced environmental *Lm* monitoring).

The successes in RTE meat and poultry safety and the corresponding reduction of listeriosis in the U.S. demonstrate the value of risk-based policies and inspection programs that motivate and support industry-wide efforts to prevent *Lm* contamination of RTE foods.

Recent Listeriosis Outbreaks Highlight Knowledge Gaps

Over the past 15 years, the food vehicles implicated in outbreaks of invasive listeriosis have shifted from being associated with RTE foods previously identified as "high risk," which correlated to those that supported *Lm* growth, to those previously considered to pose a "low risk" (those RTE food generally thought not to readily support *Lm* growth) (DHHS-FDA/USDA-FSIS 2003; Cartwright et al. 2013). RTE meat and poultry products were commonly implicated in listeriosis outbreaks until the mid-2000s. For the past decade, however, there have been no outbreaks associated with federally-inspected RTE meat and poultry products (Cartwright et al. 2013) – evidence that the USDA-FSIS risk-based policies and industry efforts to prevent *Lm* contamination of these products have made them safer. In contrast, RTE foods previously identified as being "low risk" are increasingly being implicated in outbreaks of invasive listeriosis (Fig. 6). In 2011, the U.S. experienced one of the largest and deadliest outbreaks of listeriosis, with 147 illnesses and 33 deaths linked to the consumption of contaminated cantaloupes (DHHS-CDC 2011; McCollum et al. 2013). In 2014, an outbreak traced to caramel apples caused 35 cases of invasive listeriosis,

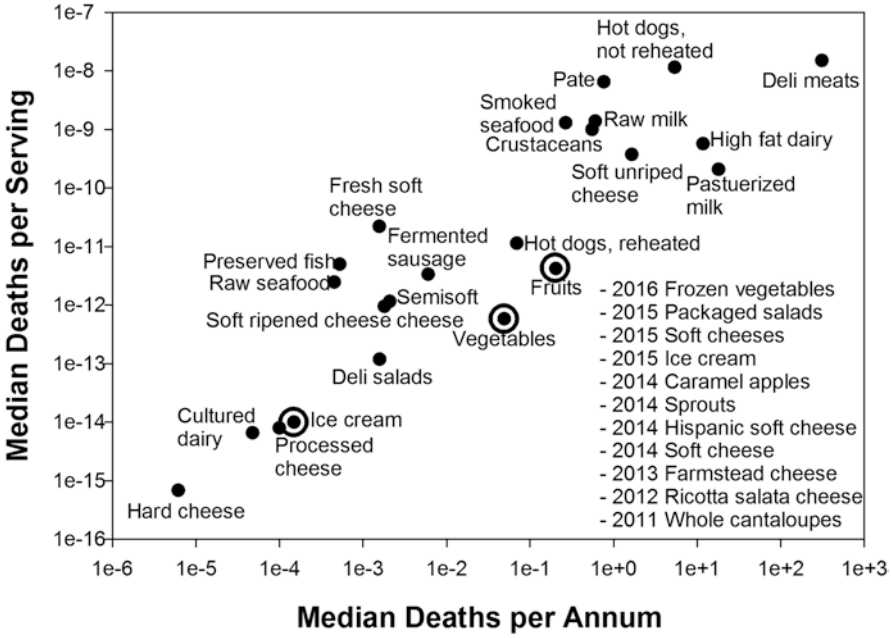

Fig. 6 Estimated median deaths per serving versus deaths per annum from the 2003 FDA-FSIS MRA risk ranking. Recent outbreaks of invasive listeriosis are listed in the side table. Examples involving illnesses among healthy children or at low *Lm* doses are highlighted. The recent shift in *Lm* outbreaks from deli meats in the late 1990s to the fruit, vegetable, and cheese categories since 2010 indicates successful policies for RTE meat and poultry products

including meningitis among otherwise healthy children – those not considered to be susceptible (DHHS-CDC 2015a). In 2015, low levels of *Lm* in ice cream were associated with deaths among hospitalized patients (DHHS-CDC 2015b). More recently, another outbreak of invasive listeriosis, this one associated with prepackaged salad, led to another unexpected case of listerial meningitis in a healthy child (DHHS-CDC 2016a). An outbreak of listeriosis that led to one death was associated with frozen vegetables (DHHS-CDC 2016b). All of these recent outbreaks involved RTE foods that were previously thought not to generally support *Lm* growth, based on generalizations from prior MRAs (DHHS-FDA/USDA-FSIS 2003). Recent studies suggest, however, that post-harvest processing of produce may result in specific changes to the produce that support *Lm* growth in these foods, which are not always predictable (Hoelzer et al. 2012; Danyluk et al. 2014; Glass et al. 2015).

These outbreaks underscore the continued knowledge gaps and the complexity of factors (e.g., *Lm* virulence, conditions for pathogen growth, food matrix, and host susceptibility) that contribute to the risk of listeriosis. The result is a highly uncertain dose-response relationship, which makes setting an appropriate level of protection at some fixed level of *Lm* at processing facilities or retail environments infeasible (Gallagher et al. 2013). These outbreaks also highlight the lack of evidence of a safe level of *Lm* on any food, including those that do not support *Lm* growth. Therefore, the U.S. maintains a "zero tolerance" for the presence of *Lm* in RTE foods (i.e., below 0.04 CFU per gram) (Williams 2008), and continues to implement and maintain federal policies and programs focused on preventing *Lm* contamination of RTE foods.

A timeline summary of the major outbreaks, MRAs, and regulatory actions for *Lm* is presented in Fig. 7. The 1980s were characterized by initial recognition of the pathogen, the beginning of surveillance, and adoption of the "zero tolerance" standard. The 1990s were characterized by the introduction of PR/HACCP and the beginning of MRA development. The 2000s marked the first of several successful MRAs including: (1) the 2003 FDA-FSIS MRA risk ranking, which identified deli meats as the food category of highest concern; (2) the 2003 USDA-FSIS deli meat MRA, which helped facilitate the voluntary industry shift toward adoption of better processing controls under the Interim Final Rule; and (3) the 2005 risk-based verification sampling approach, which allocated more sampling resources to establishments producing higher risk RTE meat and poultry products. In addition to the success in using MRAs to effectively guide policies and verification inspection programs, USDA-FSIS set up a regulatory environment that motivated, without penalizing, industry to enhance environmental testing to find *Listeria* species in the processing environment and implement controls to prevent the contamination of RTE meat and poultry products. Together, these actions led to a 75% decline in FSIS RTE foods testing positive for *Lm* and a 42% reduction in the incidence of listeriosis. In the 2010s, outbreaks from "low" or "moderate" risk RTE foods further highlighted the uncertainties in the *Lm* dose-response relationship. During this same period, MRAs pointed to the need to prevent RTE products from becoming contaminated at retail (Endrikat et al. 2010; Pradhan et al. 2010). Further analyses revealed that even small increases of *Lm* on in-coming RTE foods prepared at retail, including those that do not support growth of *Lm*, substantially increased the risk of

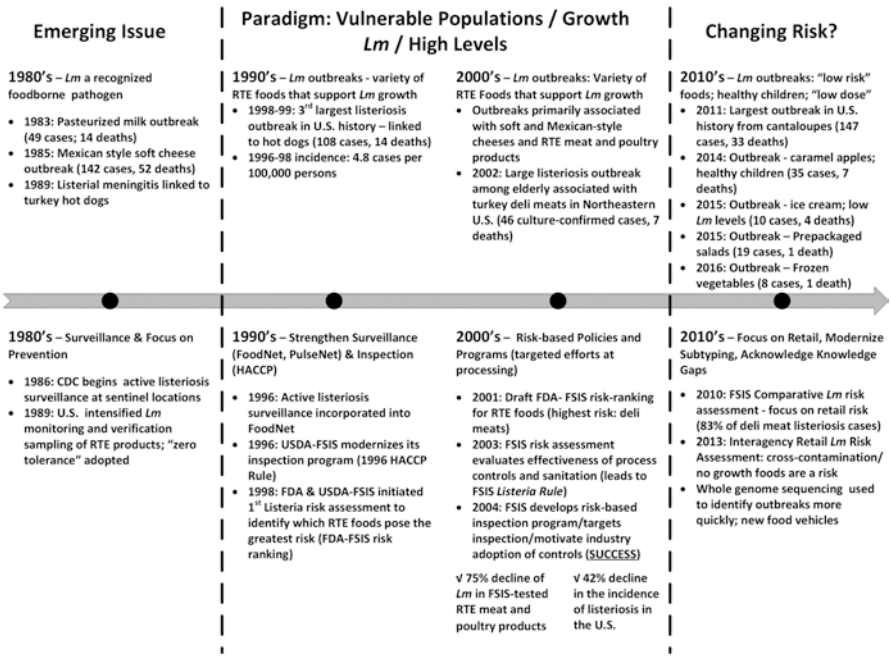

Fig. 7 Timeline of key events related to *Lm*: risk-based prevention

invasive listeriosis (Gallagher et al. 2016). The primary driver of the increase in the risk was through cross-contamination of retail-prepared RTE products that support the growth of *Lm*, either directly from other foods or indirectly from contaminated food contact surfaces. The scientific findings of these MRAs, recent outbreaks of listeriosis, and the success and broad recognition (USDA-FSIS 2015c) of USDA-FSIS's regulatory strategy and industry prevention efforts support continued maintenance of a "zero tolerance" policy for *Lm.*

Summary

- *Lm* continues to be a foodborne pathogen of public health concern in the U.S. and internationally.
- The U.S. has conducted and utilized MRAs to guide policies and programs that have been recognized as contributing to the reduction of listeriosis to 0.26 cases per 100,000.
- The U.S. has implemented a risk management strategy focused on prevention and created a regulatory environment that motivates and supports the food industry to adopt more effective processing controls and implement enhanced surveillance and sanitation programs to prevent *Lm* contamination of RTE foods.

- Both the U.S. Federal policies and programs and industry practices have resulted in a dramatic decline in the presence of *Lm* in RTE meat and poultry products, with no outbreaks associated with USDA-FSIS-inspected products in over a decade.
- Recent outbreaks underscore the current knowledge gaps highlighted in prior risk assessment dose-response relationships, make it unfeasible to set an appropriate level of protection at some level of *Lm* in RTE foods (both that support *Lm* growth and those that do not) (Gallagher et al. 2013).
- Given the success in reducing the risk of listeriosis from RTE meat and poultry products, the U.S. remains committed to a risk management strategy focused on prevention to protect public health.

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Variability in Human Host Susceptibility to *Listeria monocytogenes* Infections

Rahat Wadhwa Desai and Mary Alice Smith

Abstract The risk for listeriosis among humans is increased in certain physiological states, such as during pregnancy and advanced age (≥65 years), as well as in immunocompromised-states, resulting from immune-suppressing conditions such as those with Human Immunodeficiency Virus (HIV) infection, diabetics, transplant patients, those on immunosuppressive therapy, or medications such as corticosteroids. Higher listeriosis-related mortality is correlated to lower gestational age (<28 weeks of gestation) during pregnancy; increasing age (>70 years); use of immune-suppressive medications; and, the severity of any underlying condition(s). However, in cases where multiple risk factors are present, the amount of risk that each factor contributes to the total individual risk for listeriosis remains unknown. Physiological changes that occur in advanced age and pregnancy, as well as immune-suppression lead to lowering of critical aspects of cell-mediated immunity (or T cell immunity), thus increasing the risk for listeriosis. Co-morbidities seem to increase the risk for listeriosis as well as related mortality, especially in people who are otherwise considered low risk, such as those under 65 years of age. Much less is known about the role of other factors such as genetic makeup, nutritional status, frequent use of nonsteroidal anti-inflammatory drugs (NSAIDs), and gut microbiome composition in increasing the risk for contracting listeriosis. Carefully designed studies in human cell lines, appropriate animal models, and, when possible, more complete epidemiologic studies in outbreaks and individual cases of human listeriosis are needed to understand and predict the risk for listeriosis.

Keywords Human susceptibility • Listeriosis • Pregnancy • Aging • Neonates • T cell Immunity • Microbiome

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Abbreviations

AA	Antacids
AIDS	Acquired Immune Deficiency Syndrome
CFUs	Colony Forming Units
CI	Confidence Interval
GI	Gastrointestinal
NSAID(s)	Nonsteroidal Anti-inflammatory Drug(s)
OR	Odds Ratio
PPIS	Proton Pump Inhibitors
RR	Relative Risk
Th1	T-helper type 1
Th2	T-helper type 2
TNFα	Tumor necrosis factor α

Introduction–Susceptibility to Listeriosis

Human listeriosis presents primarily in two forms: non-invasive or invasive listeriosis (Franciosa et al. 2001). Non-invasive listeriosis commonly presents as gastrointestinal (GI) illness and is usually self-limiting (Franciosa et al. 2001). According to the Centers of Disease Control and Prevention (CDC), invasive listeriosis is defined as isolation of *Listeria monocytogenes* (*L. monocytogenes*) from a site such as blood, cerebrospinal fluid or placental and/or fetal tissue that is normally considered sterile (Silk et al. 2012). While the first appearance of non-invasive listeriosis-related GI symptoms begin at about 20 h after exposure, the incubation time for onset of invasive listeriosis can vary between 2 and 67 days (Dalton et al. 1997; Goulet et al. 2013). Listeriosis-associated meningitis in the invasive form is defined as isolation of *L. monocytogenes* from cerebrospinal fluid, whereas in pregnancy-related cases, *L. monocytogenes* has been isolated from the mother, fetus, or neonate (newborn <31 days old) (Silk et al. 2012).

The estimated average human exposure to *Listeria monocytogenes* is 2.4 times per year for concentrations of 10^3–10^6 colony forming units (CFU), once in 2 years for 10^6–10^9 CFU, and once in 3 years at levels higher than 10^9 CFU (US Food and Drug Adminstration [FDA] 2003). Even under such estimated widespread exposure, invasive listeriosis primarily occurs in specific subpopulations, including the elderly, immunocompromised individuals, and fetuses or neonates in pregnancy-related cases (Silk et al. 2012; Pouillot et al. 2012; Centers for Disease Control and Prevention [CDC] 2013; Goulet et al. 2012; Painter and Slutsker 2007). In this chapter we will describe the current scientific evidence about the differences in susceptibilities to listeriosis observed in humans. The listeriosis cases reported from 2004 to 2011 in the US are summarized in Table 1.

Listeriosis outbreaks vary with respect to the number of cases occurring in each high risk group. Typically, about 14–17% of the cases in a listeriosis outbreak are

Table 1 Reported cases of invasive listeriosis from 2004 to 2011 in the US

Year of data (Source)		Overall	Pregnancy-related[a]	Elderly ≥65 years	Others (under 65 years)
2009–2011 (Centers for Disease Control and Prevention [CDC] 2013)	Incidence[b]	0.29	3	1.3	NR
	Number of Cases (% of total cases)[c]	1651	227 (14%)	950 (58%)	434 (29%)
	Case fatality rate	292 (21%)	46 (21%)	193 (24%)	53 (14%)
2004–2009 (Silk et al. 2012)	Incidence[b]	0.27	3.42	1.21	0.10
	Number of Cases (% of total cases)[c]	760[d]	126 (17%)	400 (53%)	234 (31%)
	Case fatality rate	140 (18%)	30 (29%)	79 (20%)	31 (13%)

[a]Fetal loss or neonatal deaths in pregnancy-related cases based on available data
[b]Incidence per 100,000 individuals
[c]Percentages have been rounded and may therefore not add exactly to 100%
[d]Two cases were excluded from the overall cases because the pregnancy status was unknown in women of reproductive age
NR–Data not reported

related to pregnancy; however, the recent largest listeriosis outbreak in the US that occurred in 2011 was associated with 147 cases and 33 deaths, but only seven pregnancy-related cases (Silk et al. 2012; Centers for Disease Control and Prevention [CDC] 2011, 2013). Most of the 2011 outbreak cases occurred in people aged >60 years, with a median of 78 years, whereas only 4.7% (7 out of 147) of cases, including one miscarriage, were pregnancy-related (CDC 2011).

Figure 1 depicts the potential factors that may affect the host's susceptibility for listeriosis. In addition to the well-established risk factors such as pregnancy, advanced age and immune-compromised state, less understood factors such as genetic makeup, nutritional status, nonsteroidal anti-inflammatory drug (NSAID) use and gut microbiome composition (and placental microbiome during pregnancy) may also affect the risk for listeriosis (Fig. 1). For example, when compared to the general population, higher overall relative risk (RR) has been observed in adults ≥65 years (4.4 RR) and pregnant women [10.1 RR] (Centers for Disease Control and Prevention [CDC] 2013). Additional factors such as ethnicity and disease status also increase the risk for listeriosis. In the US, a higher incidence (7 per 100,000) of pregnancy-related cases have been reported to occur among Hispanic women than the pregnancy-related cases in non-Hispanic ethnicities (3 per 100,000) (Silk et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). The higher rate in pregnant Hispanic women is thought to be related to cultural food preferences such as consumption of unpasteurized as well as pasteurized soft cheese (ILSI Research Foundation/Risk Science Institute – Expert Panel on Listeria monocytogenes in Foods 2005). Several chronic diseases related to liver and kidney, alcoholism, achlorhydria and diabetes mellitus have also been linked to increased risk for the invasive form of listeriosis (Mook et al. 2011).

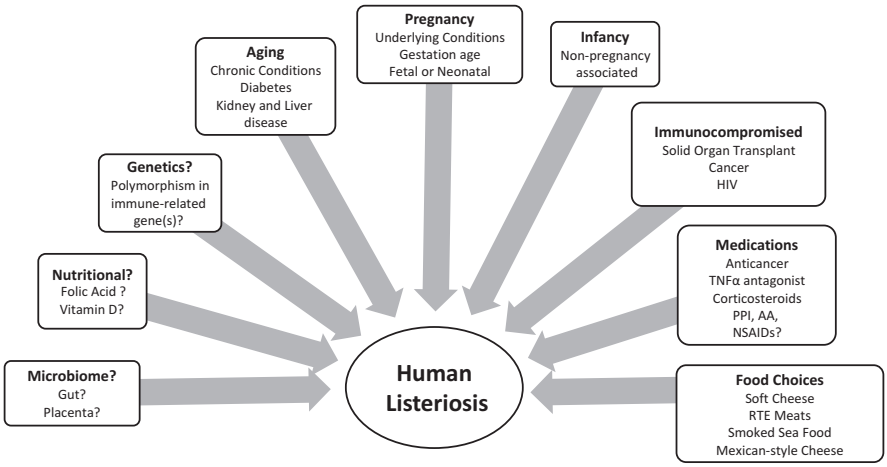

Fig. 1 An overview of susceptibility factors in human listeriosis. Factors affecting susceptibility to listeriosis are depicted here. ? = Represents factors in which clear evidence has not been established. Abbreviations: *TNFα* tumor necrosis factor α, *PPI* proton pump inhibitors, *AA* antacids, *NSAIDs* nonsteroidal anti-inflammatory drugs, *RTE* ready to eat

Susceptible Populations

Epidemiologic data reveals that elderly, pregnant women, neonates, immune-compromised individuals such as HIV-positive patients, and people consuming immune-suppressive medication(s) are at increased risk for listeriosis (Silk et al. 2012; Pouillot et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). The sections (below) will address the scientific evidence related to physiological changes and the underlying mechanisms that lead to increased susceptibility to listeriosis among older adults, during pregnancy, and the neonatal period. Also addressed are factors known or suspected to increase the susceptibility to listeriosis, knowledge gaps in current scientific evidence and new research that is needed to fill those crucial gaps. Unless otherwise stated, all results and data refer to the invasive form of listeriosis, including listeriosis-related meningitis and pregnancy-related cases.

Physiological Conditions

The human body undergoes physiological changes to meet the changing demands during life's stages or in advanced age (≥65 years). While these physiological changes are sometimes necessary for optimum functioning (e.g. in pregnancy), they can lead to reduction in immunity, thus altering a human's risk for infections including listeriosis (Vazquez-Boland et al. 2001).

Aging and Listeriosis

Most listeriosis cases in non-pregnant adults occur among people over ≥65 years old (Centers for Disease Control and Prevention [CDC] 2013). As evidenced by the high number of people ≥65 years that were affected in the 2011 listeriosis outbreak as well as the rise in sporadic cases of listeriosis in people aged ≥65 years, listeriosis poses a risk to the aging population, even in those without any underlying conditions (Centers for Disease Control and Prevention [CDC] 2013, 2015; Muñoz et al. 2012). In comparison to the relative risk of listeriosis in people aged 14–44 years (RR = 1), the relative risk for listeriosis among people aged 45–49 years increases to 4.7, and to 53.8 in people over 85 years (Pouillot et al. 2012).

Immunity, as well as general responsiveness to vaccination, are known to decrease with advancing age in humans (Smithey et al. 2011). With increasing age, there is an increase in disease burden which confounds our understanding of the contribution of aging alone towards the increase in risk for listeriosis (Pouillot et al. 2012). Underlying diseases are thought to be a major contributor to the increase in susceptibility to listeriosis (Adams et al. 2009). The reduction of innate and adaptive arms of immunity due to advanced age is called immunosenescence (Kumar and Burns 2008). Thus, immunosenescence combined with an increase in disease burden, likely contributes to the overall susceptibility observed in the aging population (Adams et al. 2009).

Recently, there have been reports of increases in the number of cases of listeriosis in several European countries (Allerberger and Wagner 2010). This increase in incidence may be due to an increase in the number of people >60 years of age (Goulet et al. 2008; Shetty et al. 2009). From 2001 to 2008, only 35% of 1959 cases of listeriosis in France were reported to occur without an underlying condition (Goulet et al. 2012). In comparison to adults under 65 years of age, the incidence of listeriosis among people aged 65–74 years and people >74 years was 8 times and 20 times higher, respectively (Goulet et al. 2012).

In the US, between 2009 and 2011, among the 1424 non-pregnancy related listeriosis cases, 58% were associated with a median age of 72 years [range 61–81] (Table 1) (Centers for Disease Control and Prevention [CDC] 2013). The increase in chronic diseases such as cancers, kidney disease, diabetes and HIV/AIDS among the elderly is thought to contribute to an increase in risk for listeriosis (Silk et al. 2012; Mook et al. 2011). An important point to consider is that the age (≥45 years) at which vulnerability for listeriosis starts to increase, the number of people living with one chronic condition also increases from 19.4% in the 19–44 year age group to 30.6% in people aged ≥45 years (Pouillot et al. 2012; Ward et al. 2014). Altogether, these statistics reveal an increase in the number of adults aged ≥45 years who are at a higher risk for listeriosis. According to the National Health Interview estimates from 2012, among the age group (viz., ≥65 years) most vulnerable to listeriosis, approximately 25% have at least one chronic condition(s); an additional 27.6% live with 2 or more conditions; whereas another 33.2% have been diagnosed with three or more chronic conditions (Centers for Disease Control and Prevention [CDC] 2013; Ward et al. 2014). Therefore, it is possible that a portion of

the increase in listeriosis among elderly is driven by an increase in chronic diseases (Pouillot et al. 2012; Ward et al. 2014). For example, according to the US Renal System Data Report, the rate of chronic kidney disease increases rapidly at ≥65 years, and the incidence has doubled from 2000 to 2008 (Saran et al. 2015). Nonetheless, a review of 299 cases from 1994 to 2003 in Denmark revealed that while chronic diseases may increase susceptibility to listeriosis in people >70 years old, the presence of a chronic disease did not significantly increase listeriosis-related mortality within the same individuals (Gerner-Smidt et al. 2005). Conversely, in patients less than 70 years old, the presence of underlying condition(s) increased the listeriosis-related mortality significantly. In comparison to the patients without predisposing illness, blood stream infections or bacteremia was significantly more common in patients with predisposing condition(s). The limitations of the Denmark study included possible underdiagnoses of underlying diseases in the sample elderly population and thus their underreporting in the data, and potential misclassification of deaths as listeriosis-related (Gerner-Smidt et al. 2005).

Epidemiologic data from the US suggests that in the presence of underlying conditions, fatal complications of listeriosis in the elderly (≥65 years) such as bacteremia and meningitis may lead to higher listeriosis-related mortality (Silk et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). Among non-pregnancy related cases, meningitis was more common in patients under 65 years of age (25%) of age than patients aged ≥65 years (10%), whereas, bacteremia was more common in patients aged ≥65 year (87%) than patients aged less than 65 years [70%] (Silk et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). However, significantly higher mortality from complications like meningitis have been observed in patients aged ≥65 years in the presence of an underlying condition (Silk et al. 2012). Therefore, while the patients aged ≥65 years may be less susceptible to listeriosis-related meningitis, in the presence of an underlying disease, these patients may have a greater risk for mortality from listeriosis-related meningitis than patients less than 65 years of age. In summary, the results reveal that the presence of underlying diseases could lead to a significant increase in listeriosis-related mortality in people aged less than 70 years as well as ≥65 years old when listeriosis-related meningitis occurs (Silk et al. 2012; Gerner-Smidt et al. 2005).

Differences in study design make it difficult to draw general conclusions from these data, as the Denmark study compares mortality between people with preexisting conditions aged >70 years and less than 70 years, whereas, the US data compares mortality and presentation of listeriosis (either meningitis or bacteremia) between people aged ≥65 years and less than 65 years of age with preexisting conditions (Silk et al. 2012; Goulet et al. 2012; Gerner-Smidt et al. 2005). The incidence of listeriosis is known to increase as age advances and the use of different age groups, >70 vs. ≥65 years old, makes it difficult to draw definitive conclusions from the abovementioned data (Pouillot et al. 2012).

Based on the US FoodNet data from 2004 to 2009, the relative risk for listeriosis increased significantly and steadily as age advanced, with the highest rate for people aged 85 years or older (Pouillot et al. 2012). The continuous increase in listeriosis in the non-pregnant population aged 45 to 84 years reveals that the use of the

commonly employed threshold age (60 or 65 years) for assessing listeriosis risk offers little accuracy in depicting this risk (Pouillot et al. 2012). An international group of scientists from academia, government and industry have also suggested that instead of an age-related threshold, the use of 5-year intervals is more suitable for categorizing the age-related increase in risk for listeriosis (Luber et al. 2011). Categorizing age in this manner could help identify specific risk factors related to aging, and standardize data to allow for comparisons.

Pregnancy and Listeriosis Risk

The CDC has defined pregnancy-related listeriosis as the isolation of *L. monocytogenes* from a pregnant woman, fetus or infant aged ≤31 days with the mother-infant pair counted as a single case (Centers for Disease Control and Prevention [CDC] 2013). Currently, the most common diagnostic test for pregnancy–related listeriosis is culture of blood, placenta or other products of conception (Centers for Disease Control and Prevention [CDC] 2013; Mylonakis et al. 2002). In a case review of 222 cases of pregnancy-related cases of listeriosis in four Boston hospitals, the most common symptoms were fever, followed by flu-like symptoms (Mylonakis et al. 2002).

Data from the US, England and Wales and Western France reveal that approximately 16% of listeriosis cases occur during pregnancy (Centers for Disease Control and Prevention [CDC] 2013; Girard et al. 2014; Awofisayo et al. 2015). During 2004–2009, in comparison to other non-pregnant women of reproductive age, the RR for listeriosis during pregnancy in the US was 114.6 [95% CI, 68.9–205.1] (Pouillot et al. 2012). The incidence of listeriosis has also increased in the UK since the 1960s, likely due to an increase in the availability and consumption of high-risk ready-to-eat foods, among other factors (Lamont et al. 2011). In comparison to the general population, upon consumption of contaminated food, the RR for listeriosis in pregnant women ranges from 10 to 18 (Centers for Disease Control and Prevention [CDC] 2013; Lamont et al. 2011). The maternal-fetal susceptibility to listeriosis is thought to occur because of immune-modulation during pregnancy to protect the fetus from allogenic rejection (Jamieson et al. 2006; Mor and Cardenas 2010). Co-morbidities such as diabetes as well as other diseases such as AIDS that affect cell-mediated immunity are also likely to increase the risk of listeriosis during pregnancy, although few such cases have been reported (Silk et al. 2012; Girard et al. 2014).

Epidemiologic data indicates that fetal outcomes are related to the gestational age at which the mother is diagnosed with listeriosis (Girard et al. 2014; Awofisayo et al. 2015). Fewer fetal deaths have been reported after 28 weeks of gestation (Girard et al. 2014; Awofisayo et al. 2015). In addition, pregnant women that exhibit listeriosis-related symptoms reported fewer live births than women that did not report such symptoms (Awofisayo et al. 2015). These symptoms may be reflective of the severity of listeriosis in pregnant women.

Neonatal listeriosis is defined as *L. monocytogenes* infection in newborns aged less than 31 days or 28 days, depending on the study (Centers for Disease Control

and Prevention [CDC] 2013; Girard et al. 2014; Awofisayo et al. 2015). Neonatal listeriosis can result in meningitis, granulomatosis infantiseptica or death (Silk et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). Neonatal survival also appears to improve with (increasing) gestation age at birth (Awofisayo et al. 2015). Meningitis is common in neonatal cases of listeriosis (Silk et al. 2012). Evidence suggests that meningitis is less fatal in neonates older than 15 days as compared to neonates that are less than 15 days of age (Silk et al. 2012). For further details on fetal and neonatal susceptibilities, please refer to our review on pregnancy-related listeriosis (Wadhwa Desai and Smith 2017).

The challenges in interpreting the pregnancy-related listeriosis data include inconsistencies in data collection and assessment among different studies, long incubation time (>20 days) in pregnancy-related cases, and determination of gestational age at the time of the infection in women who have experienced mild or no symptoms (Goulet et al. 2013; Kaur et al. 2007). Elucidating underlying mechanisms associated with differences in maternal and fetal (or neonatal) immunity could help understand abovementioned susceptibilities, and design suitable prevention and treatment strategies.

Underlying Condition(s), Immune Status and Related Medication Use

Presence of underlying (infectious and chronic) disease(s) increases the risk for listeriosis (Goulet et al. 2012). Studies have revealed that the presence and severity of chronic conditions are also important risk factors in determining the outcome for listeriosis (Pouillot et al. 2012; Gerner-Smidt et al. 2005; Barber et al. 2005). This section addresses the risk for listeriosis posed by the presence of underlying disease in people aged less than 65 years as well as older than 65 years; the impact of these conditions on the immune status; and, the medication(s) used to treat these disease(s) as it affects susceptibility towards listeriosis. The risk among patients aged ≥65 years with underlying conditions has been discussed in the aging section. Most (74%) of the cases of listeriosis in people aged less than 65 years are in individuals with underlying conditions (Centers for Disease Control and Prevention [CDC] 2013).

Of the 96 invasive listeriosis cases in the US between 2009 and 2011 among non-pregnant adults less than 65 years of age, 32 were associated with immune-suppressive therapy administered to manage an underlying condition (i.e., steroids, radiation or chemotherapy), followed by 24 malignancy-associated cases, 11 diabetes-related cases, 7 cirrhosis/liver disease-associated cases, 7 renal failure-associated cases, 6 alcoholism-associated cases, and 6 HIV-related cases (Centers for Disease Control and Prevention [CDC] 2013). Of the 1612 cases of listeriosis in France from 2001 to 2008, 65% had underlying disease, and 41% (~661 cases) had undergone immunosuppressive therapy (Goulet et al. 2012). In this French cohort, chronic lymphocytic leukemia increased the risk for listeriosis by 1000 fold, and in patients with other cancers such as multiple myeloma, acute leukemia, pancreatic,

esophageal, stomach, liver, lung, and brain cancer, as well as conditions such as dialysis, cirrhosis, and organ transplants, the increased risk was by 100–1000 fold (Goulet et al. 2012). In patients with underlying conditions, the listeriosis-associated case fatality ranged from 20% to 40% (Goulet et al. 2012).

Interference with cell-mediated immunity and tumor necrosis factor α (TNFα) production by immunosuppressive medication increases the risk for a severe form of listeriosis (Vazquez-Boland et al. 2001; Slifman et al. 2003; Schuchat et al. 1991). TNFα is produced by monocytes and macrophages, and serves to activate both T and B cells, among other crucial functions (Bazzoni and Beutler 1996; O'Dell 1999; Choy and Panayi 2001). It plays an important role in host defense, specifically against intracellular pathogens, and mediates local inflammation to control the infection (Bazzoni and Beutler 1996). Several cases of listeriosis in dermatological, rheumatic, and inflammatory bowel disease patients receiving TNF-antagonists have been reported (Slifman et al. 2003). Due to the role of TNFα in inflammation, the drug infliximab (Remicade), a human-murine chimeric monoclonal antibody displaying a high affinity for the human TNFα, was approved by the US Food and Drug Administration (FDA) for treatment of Crohn's disease, and later for rheumatoid arthritis in conjunction with methotrexate (Slifman et al. 2003). Methotrexate is primarily an anti-neoplastic drug (Cutolo et al. 2001). It is also thought to indirectly inhibit TNFα in human monocytic cell lines (Seitz et al. 1998; Sajjadi et al. 1996; Merrill et al. 1997). Due it's anti-inflammatory effects, methotrexate is used in combination with standard anti-TNFα drugs like etanercept, which is thought to be more effective than monotherapy with anti-TNFα drugs alone (Cutolo et al. 2001; van der Heijde et al. 2006). Etanercept (Enbrel) also binds to TNFα, acting as a false receptor thereby preventing the binding of TNF to the cellular TNF receptors (Slifman et al. 2003). From 1998 to 2001, 14 patients on infliximab and one patient on etanercept were reported to have listeriosis (Slifman et al. 2003). The median age of the patients was 65.9 years (range: 17–80 years). All patients with available information in the study were on concurrent immunosuppressive drugs, among which, 10 patients received corticosteroids (current or previous use), seven patients received methotrexate, three patients received a cyclooxygenase-2 inhibitor, and one patient received methotrexate and cyclosporine. All patients presented with sepsis and/or meningitis. From January 2002 to September 2002, 11 additional cases of listeriosis were reported in patients taking anti-TNF drugs, infliximab and etanercept. Of these, 10 cases were associated with infliximab, and one was associated with etanercept (Slifman et al. 2003). These reports reveal that immunosuppressive drugs may increase susceptibility of these patients to listeriosis, and future investigations could help identify specific mechanisms resulting in susceptibility to listeriosis.

Immunosuppressive therapy can cause cellular immune deficiencies which can lead to a higher risk of listeriosis in solid-organ transplant recipients (Fishman 2007; Skogberg et al. 1992). To formally assess the listeriosis risk factors for solid-organ transplant recipients, a 1:2 matched case-controlled study was conducted in 15 Spanish tertiary care hospitals (Fernàndez-Sabé et al. 2009). In 25,997 patients identified from 1995 to 2007, 30 (0.12%) cases of listeriosis were confirmed. Based on univariate analysis, significant risk factors for listeriosis included diabetes

mellitus, cytomegalovirus infection, high prednisone dose, and allograft rejection all within the preceding 6 months. Conditional logistic regression revealed an odds ratio (OR) of 5.6 (95% CI = 1.6–19.6; p = 0.007) for diabetes mellitus; 35.9 OR (95% CI = 2.1–620; p = 0.014) for CMV infection; and, 6.2 OR (95% CI = 1.8–21.1; p = 0.003) for high-dose prednisone use (Fernàndez-Sabé et al. 2009). All of these were independent risk factors for listeriosis in solid-organ transplant recipients (Fernàndez-Sabé et al. 2009).

Since 2000, the World Health Organization has established the use of trimethoprim-sulfamethoxazole as the standard of care for preventing bacterial infections in HIV patients (Gilks and Vitoria 2006). Trimethoprim-sulfamethoxazole is a broad-spectrum antibiotic that is routinely given to organ-transplant and HIV patients to prevent bacterial infections, including listeriosis (Gilks and Vitoria 2006; Horwedel et al. 2014). A trimethoprim-sulfamethoxazole-based prophylactic antibiotic treatment at the time of the transplant was found to be protective against listeriosis [OR = 0.07; 95% CI = 0.006−0.76; p = 0.029] (Fernàndez-Sabé et al. 2009). In the study, eight of the 30 patients diagnosed with listeriosis died within 30 days of their hospital stay (Fernàndez-Sabé et al. 2009). Four patients died due to sepsis, two died due to multiple organ failure, one due to respiratory failure, and one from neurological complications (Fernàndez-Sabé et al. 2009). As compared to patients presenting with other symptoms, meningoencephalitis was linked to a significantly higher (OR = 13.5; 95% CI = 1.99–93.25; p = 0.007) mortality rate in the 30-day hospitalization period (Fernàndez-Sabé et al. 2009).

Despite higher risk, listeriosis is infrequent in patients with cancer, possibly due to prophylactic broad spectrum antibiotics and improved food safety (Safdar and Armstrong 2001; Lorber 1997). In a retrospective review of patients from 1955 to 1997 from Memorial Sloan–Kettering Cancer Center, 94 cases of listeriosis were reported (Safdar and Armstrong 2001). Out of the 94, 97% had an underlying malignancy, 78% received chemotherapy, 68% were on corticosteroids, and 36% had pre-existing liver disease. Listeriosis was most often associated with anti-neoplastic therapy for advanced or refractive malignancy which results in a significant immune dysfunction. Thirty-nine percent (n = 37) of total cases died of listeriosis-related causes in this group (Safdar and Armstrong 2001).

HIV also increases the risk for listeriosis, with a 29% mortality rate (Jurado et al. 1993; Lorber 2000). In an Atlanta metropolitan cohort, the listeriosis incidence was 52 cases per 100,000 in HIV infected and 115 cases per 100,000 in AIDS patients, which is about 65–115 times higher than that of the general population (Jurado et al. 1993).

Disease status has also been linked to atypical listeriosis presentations, including localized infections such as endocarditis, osteoarticular and cutaneous infections brought on by subclinical systemic spread of the bacteria (Allerberger and Wagner 2010; Charlier et al. 2012, 2014; Godshall et al. 2013). Sixteen out of 20 (of the total 3231) cases with listeriosis-related biliary tract infections and cholecystitis in France from 1996 to 2013, had 1–4 associated comorbidities, including four cases each of hypertension, cirrhosis, and rheumatoid arthritis; three cases of diabetes; two cases of aortic patch tube; and, several other single cases of various diseases

(Charlier et al. 2014; Medoff et al. 1971; Gordon and Singer 1986; Allerberger et al. 1989; Gluck et al. 2002; Wagner and Allerberger 2003; Descy et al. 2012; Bruminhent et al. 2013). Another four patients were prescribed corticosteroids, and three patients were receiving anti-TNF therapies. Diagnosis was confirmed in each case by testing a bile or gallbladder swab for *L. monocytogenes* wherein it was the only pathogen cultured. Only three of the 5 patients in whom blood culture was performed, tested positive for *L. monocytogenes* illustrating that such focal atypical listeriosis infections may occur in humans without the infection being detected in the blood. Among 15 patients that were followed, five cases resulted in an adverse outcome with three related deaths, one recurrence, and one cerebellous stroke that was unrelated to *L. monocytogenes*. Death from listeriosis-related complications was significantly ($p = 0.03$) associated with inadequate or altogether lack of treatment (Charlier et al. 2014).

The presence of underlying diseases is especially important relative to occurrences of listeriosis in patients less than 65 years of age (Silk et al. 2012; Pouillot et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). It remains to be investigated whether a patient with underlying disease and concurrent immune-suppressive medication use presents a higher risk compared to the risk posed by these factors individually. One of the challenges in the US is that FoodNet does not collect information on underlying conditions, and their reporting is only voluntary (Centers for Disease Control and Prevention [CDC] 2013). Aforementioned cases suggest that TNF antagonists with concurrent use of corticosteroids could further compromise the host immune response, thereby potentially leaving them at an additional risk for listeriosis. This concept of additional risk posed by medication requires further study to more accurately assess the risk for compromised patients so that proper education and safety measures can be put in place.

Gender-Based Differences

There have been very few studies directly examining gender differences in response to *L. monocytogenes* exposures in humans. Studies in mice have revealed a difference in male and female susceptibility to listeriosis, with females being more susceptible (Charlier et al. 2014, 2012). However, because mice are not susceptible to listeriosis by oral exposure, the relevance of this finding in humans is unknown. A shift in the cytokine profile from T-helper type 1 (Th1) to T-helper type 2 (Th2) is thought to increase the risk for listeriosis in humans (Szekeres-Bartho 2002; Grutz 2005). Studies on gender-based differences in the Th1 and Th2 profile between 20 women and 15 men revealed a higher IFN_{γ} to IL-4 ratio in men than women (Giron-Gonzalez et al. 2000). This ratio is reflective of the Th1 cytokine profile, which is thought to be protective against listeriosis (Giron-Gonzalez et al. 2000; Corr et al. 2007a; Swain et al. 1991). To confirm these findings, this study needs to be repeated in a larger sample size.

Although the previously described studies are suggestive of gender-based differences, epidemiologic data do not support a gender-based difference in susceptibility to listeriosis (Silk et al. 2012; Pouillot et al. 2012; Centers for Disease Control and Prevention [CDC] 2013). Furthermore, it is still unclear whether any gender-based differences affect recovery and/or mortality related to listeriosis. It is also not known whether differences in food-related behavior among women and men such as a higher frequency of eating out or choosing more high-risk foods (e.g. ready-to-eat meats and cheese) play a role in their susceptibility to listeriosis (Driskell et al. 2006). For example, higher immunity in men than women may be mitigated by the greater consumption of high-risk food by men vs. women, thereby resulting in equitable number of annual cases.

Immunity Against *L. monocytogenes*

Data on immune response to *L. monocytogenes* infection in humans are lacking, and most human immune data are confined to results from *in vitro* experiments (Sherrid and Kollmann 2013). Other immune data on listeriosis are primarily from studies using a mouse model, which is different from humans both in exposure and internalization mechanisms. However, what we do know based on mouse studies is that upon intravenous injection (~10^3–10^4 CFU), *L. monocytogenes* is readily internalized and killed by local macrophages in the mouse liver and spleen (Sherrid and Kollmann 2013; Conlan 1996). These macrophages secrete TNFα and IL-12 which together stimulate natural killer (NK) cells to produce IFN_{Υ}, which in turn further activate macrophages, thereby increasing their bactericidal activity (Havell 1987; Tripp et al. 1993; Hsieh et al. 1993). The innate response is followed by a T cell response which is maintained through a memory T cell population that protects the host from subsequent exposures (Jiang et al. 2003). An effective T cell response requires the production of adequate levels of pro-inflammatory cytokines such as TNFα, IFN_{Υ} and IL-12, and proper antigen presentation by dendritic cells, which is needed to stimulate naive $CD8^+$ T cells into primed functional $CD8^+$ T cells (Byun et al. 2007; Rocha et al. 1995). The memory T cell response is controlled via the secretion of regulatory immunosuppressive cytokines TGF_{β} and IL-10 by $CD4^+CD25^+$ T cells (T-helper type 2 response) which downregulate the expansion and response of memory T cells (Kursar et al. 2002).

While many immune mechanisms elucidated in the murine model have homologous functions in humans, key differences between human and mouse immune responses such as defensin secretion by neutrophils, IFN induction of Th1 response, and IL-10 secretion by both Th1 and Th2 cells have been found (Del Prete et al. 1993; Risso 2000; Waterston et al. 2002). Mouse immune data also present other challenges in extrapolating the data to humans because there is a single amino acid difference at position 16 in mouse and human E-cadherin, whereby the mouse is generally not susceptible to the oral route of *L. monocytogenes* infection, whereas most human cases of listeriosis are thought to be foodborne (Lecuit et al. 1999; Ryser and Marth 2007).

Changes in Human Immune Response with Aging

Aging is associated with a reduction in the function of epithelial barriers such as in the GI tract, which is the first line of defense against *L. monocytogenes* (Gomez et al. 2005). As age advances, this functional reduction in the GI tract places an ever increasing demand on the immune system (Ponnappan and Ponnappan 2011). The reduction in human neutrophil response function, namely chemotaxis, phagocytosis and superoxide production in response to pathogen-related stimulatory molecules, have also been associated with aging (Schroder and Rink 2003).

Increases in the pro-inflammatory cytokines IL-6, TNFα and TGFβ, and decline in the expression of macrophage surface receptors such as the major histocompatibility class (MHC)-II occur with advancing age in humans (Ershler and Keller 2000; Bruunsgaard et al. 2003; Zhou et al. 1993; Herrero et al. 2001; Plowden et al. 2004). As a person ages, a reduction in the respiratory burst within the macrophages leads to a reduction in their pathogen-killing capacity (Piazzolla et al. 1998). In response to infections, dendritic cells (DCs) produce less IFNα and IL-12 cytokines, while increasing the production of IL-6 and TNFα cytokines (Shodell and Siegal 2002; Della Bella et al. 2007). Other crucial functions such as phagocytosis, migration and micropinocytosis could also be defective in those over 65 years (Ponnappan and Ponnappan 2011). With aging, there is an increase in memory and effector T cells, thus reducing the overall responsiveness of the immune system to new antigens which is shown by reduced responsiveness to vaccines (Ponnappan and Ponnappan 2011). Data from both human and mice studies reveal that, with increases in IL-4 and IL-10, the cytokine profile in the older population is shifted to Th2 type, and increases in TGF_{β} and IFN_{Υ} are also associated with aging (Carlin et al. 2005; Rink et al. 1998; Weyand et al. 1998).

Taking the evidence in mice and humans together, a shift towards a Th2 cytokine profile, reduction in GI epithelial defenses, along with functional deficits in several crucial immune cells, may be responsible for the reduction in immunity towards listeriosis in aging humans. Despite all we have learned about the effects of aging on the immune system, the specific role of aging and underlying mechanisms by which advancing age leads to alterations in the immune system thus compromising the body's defenses against listeriosis, are not currently understood. Moreover, how the presence of multiple risk factors affects the risk for listeriosis among the aged needs to be elucidated, especially as this population increases globally.

Role of Immune Changes During Pregnancy and Susceptibility to Listeriosis

The changes that a woman's immune system undergoes to accommodate a fetus, where one-half of its genetic material is foreign to hers, increases the susceptibility of the fetus to listeriosis (Jamieson et al. 2006; Mor and Cardenas 2010). This immune adaption in the mother to simultaneously allow the fetal antigen tolerance

and yet be able to mount a needed immune self-defense, is not completely understood (Jamieson et al. 2006). There is evidence that this is achieved by lowering cell-mediated immunity in the mother while maintaining the humoral immune response (Jamieson et al. 2006). However, it is challenging to study pregnancy-related cases of listeriosis, especially due to the lack of an appropriate control group (Jamieson et al. 2006).

Evidence suggests that a lowering of the critical cell-mediated immune response is responsible for the increase in listeriosis risk during pregnancy. The cell-mediated Th1 response is essential to control intracellular infections such as listeriosis, as the intracellular location protects the pathogens from the humoral/antibody response. Cell-mediated immunity is augmented by Th1 type T lymphocytes and their pro-inflammatory cytokines such as IL8 (Corr et al. 2007a). Their key function is to recognize the body's own cells that engulf the pathogen and express pathogen-related antigen on their surface (Jamieson et al. 2006). On the other hand, Th2 T cells and related cytokines in the maternal decidua suppress cytotoxic T lymphocytes or the Th1 T cells response locally, thereby reducing the cell-mediated immune response at the placenta (Szekeres-Bartho 2002). During pregnancy, the Th2-stimulating cytokines dominate, thereby ensuring an adequate humoral immune response while leaving cell-mediated immunity compromised (Thaxton and Sharma 2010). For additional details on immune changes associated with listeriosis in pregnancy, please refer to our review on pregnancy-related listeriosis (Wadhwa Desai and Smith 2017). Altogether, these immune changes lead to increased susceptibility to listeriosis in pregnant women, wherein the woman remains protected while leaving the fetus vulnerable to *L. monocytogenes* infection. The mechanisms behind these immune changes in pregnancy need to be understood both with respect to pregnant women as well as fetuses and neonates. Understanding the differences in vulnerabilities between pregnant women and fetuses/neonates could aid in diagnosis as well as designing new therapies in pregnancy-related *L. monocytogenes* infections.

Neonatal Immune Factors

In human newborns, the highest risk period for a serious *L. monocytogenes* infection is between birth and 6 weeks (Sherrid and Kollmann 2013). Immunodeficiency in human neonates has been extrapolated from study of the ontogeny of the human immune system (Kollmann et al. 2009; Corbett et al. 2010). The neonatal immune neutrophils and monocytes are markedly deficient in adhesion and chemotaxis when compared to adult cells (Klein et al. 1977; Koenig and Yoder 2004). Cytokine response in neonates is also significantly different from adults. Because these factors boost immunity against listeriosis, it is likely that their low levels in infants may predispose them to listeriosis.

Neonatal listeriosis has been studied primarily in the mouse model. A much lower dose of *L. monocytogenes* is required to cause infection in neonatal mice than adult mice, and resistance for infections reaches adult-levels within the first 2 weeks

of life (Franceschi 2007; Tennant et al. 2008). Experiments in mice have revealed a link between age-dependent differences in monocyte function and risk of listeriosis [at birth and 30-day-old mice] (von Wirsing Konig et al. 1978, 1988). Nevertheless, to truly extrapolate these studies, experiments must be conducted in human cell lines and appropriate animal models (Sherrid and Kollmann 2013).

In summary, gestational age may affect the number of live births as well as the timeline (0–6 days vs. 7–28 days of age) of the development of listeriosis in infants. Evidence suggests that fetal (≥22 weeks) susceptibility to listeriosis may be more strongly associated with *in utero* environment and stage of fetal development, whereas susceptibility in infants may be affected by additional parameters such as immune factors and gut microbial composition. Therefore, in order to assess the risk for listeriosis in infants and neonates accurately, records need to clearly distinguish between neonatal (≤31 days of age) and infant case (>31 days of age) data.

Microbiome and Listeriosis

Among conditions that may affect the survival and colonization of *L. monocytogenes* is the gut microbiome and its interactions with pathogens such as *L. monocytogenes*. Composed of over 1000 bacterial species, the gut microbiome is known to provide benefits such as protection against pathogens, immune modulation, and metabolism of toxicants before their systemic absorption (Qin et al. 2010; O'Hara and Shanahan 2006; Sekirov et al. 2010). Over time, this relatively less understood microbial community is altered by antibiotic use, diet, age and other environmental factors which could either have a beneficial or harmful impact on the host (Ley et al. 2008; Guinane and Cotter 2013). The role of gut microbiome in modulating the risk for listeriosis will be discussed with respect to each susceptible population.

Aging and Gut Microbiome

Among the elderly, a reduction in gut microbiome-associated protection against pathogens, and increase in gut microbiome-related chronic inflammatory diseases such as inflammatory bowel disease, Crohn's disease and encompassing ulcerative colitis, have all been reported to occur (Guinane and Cotter 2013; Gahan and Hill 2014; Frank et al. 2007; Grimble 2003). During aging, physiological changes in the GI tract of older people include chronic low-grade inflammation which could affect the gut microbial balance and ultimately their susceptibility to listeriosis (Franceschi 2007; Guigoz et al. 2008). High throughput sequencing analysis has revealed distinctions between the gut microbiome of people aged more and less than 65 years (Claesson et al. 2011). Within the older population, several factors including living in long-term care facilities, diet and health-status affect the gut microbiome (Wang et al. 2011; Claesson et al. 2012). Moreover, the increase in inflammatory markers

(C-reactive protein, serum TNFα, IL-6 and IL-8) among the older individuals living in long-term care facilities were associated with the loss of community-related microbiota (Claesson et al. 2011, 2012). It is important to note that some gut microbes produce bacteriocins, ribosomally synthesized small antimicrobial peptides that have a broad or narrow spectrum of activity against other bacteria including *L. monocytogenes* (Cotter et al. 2005; Corr et al. 2007b).

Two of the most studied intestinal microbiota, *Lactobacillus* and *Bifidobacterium*, have been incorporated into different foods and dairy products (Guerin-Danan et al. 1998). Researchers investigated the ability of facultative anaerobic *Lactobacillus* and obligate anaerobe *Bifidobacterium* strains to prevent *L. monocytogenes* strain EGDe infection in C2Bbe1 cells, which are clonal derivatives of Caco2 human adenocarcinoma cells (Corr et al. 2007a; Peterson and Mooseker 1992). Incubating C2Bbe1 cells with these probiotic bacteria resulted in a 60–90% reduction in invasion by *L. monocytogenes*, and in alterations in pro-inflammatory IL-8 (reduction) and anti-inflammatory IL-10 (increase) related immune response (Corr et al. 2007a). When the probiotic bacteria were separated from the C2Bbe1 cells by an impermeable chamber, probiotic bacteria reduced *L. monocytogenes* invasion to the same extent. Thus, it can be inferred that probiotic bacteria reduced the invasion of *L. monocytogenes* not only through their direct interactions with the C2Bbe1 cells but also via a soluble compound secreted by the probiotic bacteria (Corr et al. 2007b). However, it remains to be determined whether these results can be replicated in *in vivo* models, where host GI defenses may prevent the probiotic bacteria from reaching desired levels (Corr et al. 2007a; Sanders 2011). Nonetheless, this suggests that the presence of probiotics in the gut microbiome may reduce or prevent *L. monocytogenes* infections.

Placental Microbiome

Currently, the interactions between *L. monocytogenes* and the placental microbiome during pregnancy have not been studied. The temporary alterations or fluctuations of physiology during pregnancy also accompany remodeling in the placental microbiome (Cao et al. 2014). Evidence suggests that nearly half of all placentas may harbor intracellular bacteria before 28 weeks of gestations (Stout et al. 2013). Differences in the microbiome of preterm and term placentas have also been reported (Aagaard et al. 2014). Preterm placentas have alterations of *Burkholderia* species of bacteria, whereas the term placenta have fewer *Paenibacillus* bacteria (Aagaard et al. 2014). Therefore, an alteration in the bacterial species of the placental microbiome may make the fetus less or more susceptible to the adverse effects from a *L. monocytogenes* infection. However, these results are limited by the inability to examine the microbiome changes in earlier stages of a term pregnancy (Aagaard et al. 2014). Thus, it is still unknown whether these changes in the placental microbiome are associated with gestational age or particular stages of placental and

fetal development (Cao et al. 2014). Taking into consideration that the presence of certain bacterial species has been associated with preterm placentas, investigations aimed at examining the interactions between *L. monocytogenes* and the placental microbiome are needed. Studies on the gut microbiome and placental microbiome reveal that similar interactions between pathogenic bacteria and commensal bacteria may be possible in materno-fetal infections.

Infant Microbiome

This section describes the role of the microbiome in infants (preterm and term), unless specific age-related vulnerabilities are mentioned. A direct link between the infant gut microbiome and increase in risk for listeriosis has not been established. Abnormal changes such as increase in GI inflammation and abnormal bacteria along with a lower number of commensal bacteria have been reported in the gut of premature infants (Westerbeek et al. 2006). The infant gut microbial composition varies depending on the mode of delivery (vaginal or cesarean section), type of feeding, and antibiotic or prebiotic use (Fouhy et al. 2012). Nevertheless, an infant's microbiome remains far less complex than that of an adult (Guinane and Cotter 2013). By two years of age, an infant's microbiome appears to resemble to that of an adult (Palmer et al. 2007).

Differences have been noted in the gut microbiome of full-term infants compared to low-birth weight infants weighing <1200 gm, which may be associated with the length of gestation (Stout et al. 2013; Aagaard et al. 2014; Sood et al. 2006). Premature infants have highly immune-reactive intestinal epithelium and submucosa in their GI tract (Cao et al. 2014). Considering the protective effect of the gut microbiome in adults and that of full-term infants with normal flora, it may play a crucial role towards protecting the neonates from GI infections. To design therapies, carefully designed studies are needed to investigate the role of the neonatal microbiome in their susceptibility for listeriosis.

Frequently Used Medication(s) and Listeriosis Risk Among Elderly

The use of medications such as antacids and nonsteroidal anti-inflammatory drugs NSAIDs can have a profound effect on the GI tract thereby affecting the host's ability to protect itself from foodborne infections (Bavishi and DuPont 2011; Yoshida et al. 2000; Newton 2006). Consumption of antacids has been associated with increased susceptibility to invasive listeriosis (Silk et al. 2012; Ho et al. 1986). There is no current evidence to suggest that NSAIDs increase the host's risk specifically for listeriosis; however, NSAIDs can erode and ulcerate the GI epithelium

suggesting that they may affect the host's susceptibility for listeriosis (Cole et al. 1999; Hawkey et al. 1991; Stack et al. 2002). The high consumption of these medications in the older population creates a potential for these medications to affect the listeriosis risk for a large portion of this vulnerable population (Bavishi and DuPont 2011; Soni 2007).

Proton pump inhibitors (PPI) or antacids are medications (e.g. Prilosec, Nexium, and Prevacid), that reduce gastric acid and may increase the susceptibility to listeriosis (Bavishi and DuPont 2011). PPIs are the third most prescribed drugs in the United States (Bavishi and DuPont 2011). The gastric acid at pH <4 has potent bactericidal activity, and antacid induced hypochlorhydria increases the pH (>4) thereby increasing the likelihood of microbial survival in the GI tract (Tennant et al. 2008; Giannella et al. 1972). PPIs also have an anti-inflammatory and immunomodulatory effect in *in vitro* tests (Bavishi and DuPont 2011; Yoshida et al. 2000). For example, PPI therapy affects chemotaxis, phagocytosis of microbes and endothelium-related adhesion molecule expression in neutrophils (Suzuki et al. 1996; Zedtwitz-Liebenstein et al. 2002; Wandall 1992). Neutrophils are not only the first line of immune protection against *Listeria,* but they also release chemokines that attract macrophages to the foci of infection (Czuprynski et al. 1994; Rogers and Unanue 1993; Segal 2005; Brinkmann et al. 2004; Guleria and Pollard 2001; Mandel and Cheers 1980).

Animal studies have revealed a link between hypochlorhydria and increased ability of the microbes to cross the GI barrier (Basaran et al. 1998; Dinsmore et al. 1997). A 1979 listeriosis outbreak involving 20 patients in eight Boston hospitals was linked to the consumption of gastric acid-inhibiting H_2-receptor antagonist, Cimetidine (Ho et al. 1986; Ito et al. 2012). The mortality rate was 25% (5 patients) in the Boston outbreak, of which, 10% (2 patients) died due to underlying diseases. The reduction in gastric acid-related local gut defenses could result in a pathogenic bacterium, such as *L. monocytogenes* crossing the GI epithelium with relative ease (Basaran et al. 1998; Dinsmore et al. 1997; Hopkins et al. 2002).

Among older people, another very frequently prescribed medication is aspirin (Soni 2007), an NSAID that is prescribed for arthritic inflammation, and cardiovascular or cerebrovascular disease (Collaboration AT 2002; Hayden et al. 2002). Gastric atrophy (Feldman et al. 1996; James 2000) and deficiencies in gastric mucosa repair capacity may also increase with aging and additional vulnerability brought about by aspirin use may further compromise this protective barrier (Guslandi et al. 1999). Aspirin use-related adverse effects vary from endoscopically visible lesions in the GI tract within 1 h of use to GI erosion and ulcers, and in severe cases, GI bleeding and perforation (Newton 2006; Cole et al. 1999; Hawkey et al. 1991). While these GI effects are dose-dependent, even low doses of NSAIDs like aspirin can cause GI bleeding (Stack et al. 2002).

Even though the role of NSAIDs in increasing the risk for listeriosis among the elderly is not known, their widespread use along with their ability to cause gastric erosion could potentially alter the risk for listeriosis among elderly. Nonetheless, it is difficult to investigate whether old age alone makes the GI tract more susceptible

to aspirin-induced damage thus increasing the risk for listeriosis or whether underlying disease(s) in conjunction with the use of certain high-risk medications leave the aged more susceptible to listeriosis (Solomon and Gurwitz 1997). NSAID and PPIs may be concurrently used by older individuals. Thus, the impact of concurrent use of these medications in increasing risk for listeriosis specifically in the elderly also warrants investigation.

Genetic Susceptibly Factors

Alterations in the human genetic makeup can also affect the susceptibility to infections (Azim et al. 2007). In view of the number of immune factors and their optimum expression that are required for protection against listeriosis, it is possible that genetic polymorphisms involving these crucial genes may lead to alterations in susceptibility towards listeriosis (Yeretssian et al. 2009). Polymorphism in the IL-10 gene promoter and resulting alterations in IL-10 levels enhance the risk for systemic lupus erythematosus (Gibson et al. 2001). As IL-10 levels tilt the Th1 to Th2 balance similar to the events in pregnancy, alterations in IL-10 levels may lead to changes in immunity against listeriosis (Szekeres-Bartho 2002; Kursar et al. 2002; Imrie et al. 1996; Moore et al. 1993). Similarly, genetic polymorphisms in TLR-4 receptor, TNFα, IL1 and other crucial chemokines could also alter susceptibility to listeriosis in humans (Genc and Schantz-Dunn 2007; Jones et al. 2010; Kalish et al. 2004; Murtha et al. 2006).

Nutritional Factors

Adequate levels of nutrients can provide protection against infection. Evidence suggests that riboflavin (vitamin B_2) and vitamin D deficiencies could modulate the risk for listeriosis (Rada and Leto 2008; Schramm et al. 2014; El-Benna et al. 2008; Bounous and Kongshavn 1978). Approximately, 41.6% of the adults in US are deficient in vitamin D, and similar estimates exist worldwide (Forrest and Stuhldreher 2011; Holick and Chen 2008). According to some estimates, about 27% of the older population suffer from riboflavin deficiency (Fanelli and Woteki 1989). Acute deficiency of riboflavin in riboflavin-kinase knockout mice has been shown to result in reduced innate immunity against *L. monocytogenes* infections (Trepka et al. 2007). While vitamin D plays an important role in immune regulation, chemokine production and immune modulation, the direct impact of vitamin D levels on risk towards listeriosis is yet to be determined (Fukuoka et al. 1998; Kamen and Tangpricha 2010). Considering the role of vitamins B_2 and D in human immunity as well as the prevalence of their deficiencies, the impacts of the levels of these vitamins on the human risk for acquiring listeriosis merit investigations.

Social and Behavioral Factors

Identifying and implementing adequate food safety measures is of utmost importance in preventing listeriosis, especially in susceptible populations (Evans and Redmond 2014). The areas of France where the greatest number of listeriosis cases have been reported have also reported significantly higher ($p < 0.05$) consumption of high-risk foods than other areas of the country (Girard et al. 2014). Similarly, studies have revealed that pregnant women, a highly susceptible group, lack sufficient knowledge concerning high-risk foods as well as safe cooking and handling of food, which could result in higher risk of exposure to foods contaminated with *L. monocytogenes* (Athearn et al. 2004; Cates et al. 2004; Ogunmodede et al. 2005; Trepka et al. 2007). While there is great need for similar food safety data on the aged population, especially to develop appropriate food safety initiatives, food-related behavior data for this vulnerable population are also lacking (Evans and Redmond 2014). Preferential consumption of high-risk foods such as unpasteurized Mexican-style soft cheese by people of Hispanic ethnicity is also thought to increase their risk for listeriosis (MacDonald et al. 2005).

Conclusions

Based on epidemiologic studies, it has been known for many years that certain subpopulations have a higher risk for listeriosis. Aging, pregnancy, premature birth and immune suppression are the conditions that result in a higher risk, but other less well-studied factors such as genetics and nutritional status could also affect risk for listeriosis in humans. Relative to other drugs, cytotoxic (or antineoplastic drugs), corticosteroids and drugs affecting the immune system are more likely to increase the risk for listeriosis but more information is needed to directly determine the contribution of each type of drug towards an individual's risk for listeriosis (Mook et al. 2013). Studies aimed at investigating the impact of frequently prescribed drugs such as NSAIDs and PPIs are also needed to understand their possible contribution towards the increase in risk for listeriosis, particularly in older populations. To better predict the risk, the immune response to both low and high doses of *L. monocytogenes* needs to be investigated with respect to each of these risk factors in appropriate laboratory models.

In addition to the susceptibility of these at-risk populations, a better understanding of the factors that increase the severity and mortality associated with listeriosis is also needed. It is important to note that the severity of underlying condition (especially in people aged less than 65 years old), patient age (neonates and ≥65 years aged adults) and gestational stage (<28 weeks) can be important in determining final outcomes in the respective high-risk groups (Silk et al. 2012; Pouillot et al. 2012; Centers for Disease Control and Prevention [CDC] 2013; Goulet et al. 2012; Girard et al. 2014; Lamont et al. 2011; Draper et al. 1999). There is also evidence

that in people aged less than 70 years the presence of underlying conditions lead to a significant increase in listeriosis-related mortality (Gerner-Smidt et al. 2005). More studies are warranted to evaluate the risk factors for listeriosis-related mortality in the elderly.

One of the main challenges in accurately assessing the data for pregnancy-related cases is the inconsistent methods by which the epidemiologic data are collected in different studies (Silk et al. 2012; Centers for Disease Control and Prevention [CDC] 2013; Awofisayo et al. 2015). To better understand the risk of listeriosis during the perinatal period and in infancy, data need to be collected using standardized criteria and assessed separately for each category along with the respective causes (maternal-fetal origin, infant cases etc.), and age (including gestational age).

In conclusion, we need to better define the population at risk of contracting invasive listeriosis as well as the population that is more susceptible to serious listeriosis-related complications such as meningitis. Studies aimed at investigating the impact of frequently prescribed drugs in elderly are needed to understand the contribution of prescription medication towards the increase in risk for listeriosis in elderly. As more is learned about the microbiome and nutrition, studies are needed to determine their effects on the risk of listeriosis. Finally, the amount of risk contributed by each risk factor (e.g. aged ≥65 years, diabetes etc.) individually as well as in the presence of one or more additional risk factors (e.g. medication) needs to be determined to accurately identify high-risk individuals, prevent new listeriosis cases, and develop effective therapies.

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Infectious Dose and an Aging Population: Susceptibility of the Aged to Foodborne Pathogens

James L. Smith

Abstract The elderly (≥65 years of age) are a population of individuals highly susceptible to morbidity and mortality from infection by foodborne pathogens. The susceptibility to gastroenteritis in the older population is largely due to decreased immune function and age-associated bodily changes. Many of the elderly are also likely to have chronic diseases, which weaken the bodily defenses and increase the susceptibility to foodborne illness. World-wide demographic data indicate that the elderly population is increasing, and many elderly individuals will be institutionalized in long-term care centers. Therefore, personnel who handle food in long-term care facilities must be trained in food safety procedures, such as proper handling of food ingredients, preparation of cooked foods, and serving of food to prevent foodborne gastroenteritis. However, an increasing elderly population indicates that illness and death associated with foodborne pathogen gastroenteritis will increase in the future.

Keywords Long-term care facilities • *Clostridium difficile* • *Campylobacter* • *Salmonella* • *Listeria monocytogenes* • Norovirus • Reactive arthritis • Guillain-Barré syndrome • Irritable bowel syndrome • Hemolytic uremic syndrome • Meningitis

Introduction

The world is facing a great challenge—by the year 2020, there will be more elderly persons (≥65 years) than children <5 years of age. The number of people ≥65 years of age in the world is expected to increase from an estimated 524 million (ca. 8% of the world's population) in 2010 to an expected 1.5 billion (ca. 16% of world's population) by 2050 (Anon 2011). Between the years 2010 and 2050, the United States

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population will increase from ca. 310 million to an expected 439 million. In 2010, the United States population of individuals ≥65 was ca. 40 million (13% of US population), and it is expected to reach ca. 89 million (ca. 20% of the population) in 2050 (Vincent and Velkoff 2010). The elderly have a high susceptibility for morbidity and mortality from microbial-induced gastroenteritis. As a result, the United States and the rest of the world, will face extensive health concerns. These health concerns will create the necessity of caring for an increasing elderly population suffering from the consequences of gastroenteritis. More and more of the ≥65 aged individuals will need long-term care and will be living in long-term care facilities (LTCFs). For the year 2012, the number of nursing home residents in the United States was 1,409,749: 66.8% were female; 15.0% ranged in age from 0 to 64; 15.2% ranged from 65 to 74; 27.1% ranged from 75 to 84; 35.0% ranged from 85 to 94; and 7.7% were ≥95 in age (Nursing Home Data Compendium 2013). It is likely that the number of elderly in LTCFs will continue to increase in the United States and other developed countries.

Why Foodborne Gastroenteritis and Other Foodborne Illnesses Persist

One of the most common infections in humans is foodborne infection, and the elderly and other immunocompromised individuals are particularly susceptible. Scallan et al. (2011) have estimated that 9,388,075 (range 6,641,440–12,745,709) cases of domestically-acquired foodborne illnesses due to 31 pathogens (bacterial, parasitic, and viral agents) occur annually in the United States, leading to 1351 (range 712–2268) deaths. There are several reasons for the continual occurrence of foodborne illness. As the population has increased in the United States with its resultant increased food consumption, it has become necessary to develop large centralized production systems; those systems include large-scale farming practices, large and intensive animal production facilities, and large-scale food processing and food distribution systems. Therefore, if a microbial problem occurs anywhere in these areas, the problem may spread through multiple states or even other countries. There has been an increase in foods obtained from the global market place; imported foods may be problematic if they are obtained from countries with unsafe agricultural practices or from countries with less satisfactory food safety standards (as compared to the United States). Many present-day wage earners in the U.S. do not have time to cook and prepare meals so that there is more consumption of foods not prepared at home such as eating in full-service or fast-food restaurants, as well as purchasing refrigerated foods, frozen foods, or ready-to-eat foods from commercial sources. Traveling and tourism also contribute to eating outside the home. Eating foods not prepared at home means that the consumer has essentially no control over how the food was prepared. Foodborne illness is particularly prevalent in individuals with decreased immune function, such as young children, the elderly, pregnant women, and individuals immunocompromised due to disease or drug treatment. Thus, there is an

increase in the number of consumers at risk for foodborne illness. Other factors include the fact that pathogens can adapt to new environmental conditions and can acquire new virulence factors and antimicrobial drug resistance by horizontal gene transfer. The opening of new wilderness areas may lead to the introduction of new pathogens not previously encountered and changes in climate conditions may allow pathogens to invade new areas (Nyachuba 2010). Therefore, these factors ensure that foodborne illnesses will continue to occur.

Gastroenteritis in Long-Term Care Facilities

Sporadic cases of gastroenteritis occur in community-dwelling elderly; however, outbreaks are common in Long-Term Care Facilities (LTCFs) where elderly individuals are concentrated. In 2011, 3.6% of the 65+ population lived in institutional settings. Approximately 1% of persons 65–74 years, 3% of persons 75–84 years, and 11% of persons 85+ years lived in aged-care institutions (Anon 2012). In a survey of the literature, Grieg and Lee (2009) studied 75 enteric outbreaks that occurred in LTCFs during the period 1997–2007: 23 outbreaks (30.1%) were associated with bacterial agents and 52 (69.3%) were associated with viral agents. *Salmonella* spp. accounted for 11/75 (14.7%) of the outbreaks, and four other bacterial agents (*E. coli, Clostridium perfringens, Shigella* spp., and *C. difficile*) were responsible for 12/75 (16.0%) of outbreaks. Norovirus accounted for 43/75 (57.3%) of outbreaks and other viruses caused 9 (12.0%) of the outbreaks. Transmission of gastroenteritis was mainly foodborne (52%) for bacterial agents and person-to-person (71%) for viral agents. There were 60 deaths; 16 of the deaths were due to norovirus and 28 were due to *Salmonella* (Grieg and Lee 2009).

Kirk et al. (2010a) studied gastroenteritis outbreaks in Australian LTCFs in 2002–2008. There were a total of 6295 outbreaks in Australia with 3257 (51.7%) of the outbreaks occurring in LTCFs. Only 43 (1.3%) of the 3257 outbreaks were foodborne. Norovirus infections were responsible for 1136/3257 (35.5%) of the outbreaks. Most of the LTCF gastroenteritis outbreaks, 2042/3257 (62.7%), were of unknown etiology; therefore, the etiology was known for only 1215 outbreaks. Thus, 1136 norovirus outbreaks out of 1215 outbreaks with known etiology made up 93.5% of the LTCF outbreaks that occurred in Australia in the 2002–2008 period (Kirk et al. 2010a). For the year 2013 in Victoria, Australia, there were 206 outbreaks (≥4 individuals with gastroenteritis) in aged-care facilities and 65.0% of the outbreaks were due to norovirus infection (Bruggink et al. 2015). In the 19-month period of 2010–2012 in French LTCFs, there were 298 outbreaks of gastroenteritis (Barrett et al. 2014). Norovirus, *C. difficile*, and *Salmonella* accounted for 218, 4, and 2 outbreaks, respectively. Thus, norovirus accounted for 73.2% of the outbreaks, and the two bacterial species accounted for 2.0% of the outbreaks in France (Barrett et al. 2014). For the period of July 1999 through June 2007 in the United States, norovirus accounted for 7.7% of gastroenteritis deaths (death rate 20/1,000,000), whereas *C. difficile* was associated with 75.6% of gastroenteritis deaths (death rate 195/1,000,000) for individuals ≥65 years of age (Hall et al. 2012).

More than 90% of the community-associated gastroenteritis infections are sporadic, and the association of gastroenteritis with ingestion of a particular food product is seldom determined (Jones 2010). Studies to determine the incidence of gastroenteritis in the elderly in a community setting are limited. In a study in Australia for the period 2000–2009, community residents ≥65 years revealed a rate of 97.6 cases of *Campylobacter* gastroenteritis per 100,000 population, whereas the rate was 61.7 cases for LTCF (Kirk et al. 2012a). The rate for *Salmonella* serotype Typhimurium-associated gastroenteritis cases for the ≥65 individuals was 9.2 per 100,000 for community residents, as compared to 25.0 for patients in LTCFs. Similarly for non-Typhimurium *Salmonella* serotypes, the gastroenteritis rate was ca. 1.5-fold greater for LTCF elderly as compared to community elderly. The combined total rate of cases per 100,000 for the food-associated bacterial pathogens *Listeria monocytogenes, Shigella* sp., and Shiga toxin-producing *Escherichia coli* was ca. 2.0 for community-living elderly and 0.9 for elderly living in LTCFs (Kirk et al. 2012a). In a telephone survey conducted in Australia, the elderly (≥65 years) living in community settings reported 0.33 episodes of gastroenteritis per person each year, whereas community-associated adults ≤64 years of age experienced 0.95 episodes (Kirk et al. 2012b). The authors stated that the elderly were less likely to report episodes of gastroenteritis, suggesting that telephone surveys are not a reliable method for gaining information concerning community-associated gastroenteritis in elderly patients.

Keeping the Elderly's Food Safe

LTCFs, as institutions for elderly care, have a responsibility for serving safe foods to their clients. Nelson et al. (2008) conducted a survey of food practices in 865 LTCFs in the United States, which were certified to receive medicare or medicaid. Only three of the establishments followed national recommendations for preventing *L. monocytogenes* contamination. Soft cheeses made from unpasteurized milk were served in 9% of the LTCFs. Most of the LTCFs served ready-to-eat meat products; however, only a few (<20%) reported reheating such meat products to 165 °F (74 °C) before serving. Pasteurized egg products were utilized by 92% of the LTCFs, but only 36% reported using pasteurized whole shell eggs. Regular whole shell eggs were used by 62% of the facilities. Irradiated meat products were underutilized; only 7% served irradiated ground beef, and only 6% served irradiated poultry meats. Pasteurized fruit juices were served in 63% of the LTCFs. Nelson et al. (2008) concluded that pasteurized and irradiated foods were underutilized, and many facilities did not observe national recommendations concerning avoidance of high risk foods. Moreover, the authors recommended a greater emphasis on educating LTCF-care personnel on food safety practices.

Kendall et al. (2006) have suggested that the avoidance of certain foods will aid in providing a safe food environment for community-dwelling elderly populations. These unsafe foods are listed in Table 1. The elderly eating at home can easily avoid

Table 1 Foods that community-dwelling elderly should avoid

1. Raw or undercooked seafood and shellfish
2. Raw or undercooked fresh meat (beef, pork, poultry)
3. Soft cheeses made from unpasteurized milk (brie, camembert, queso fresco), cold smoked fish, and cold deli salads
4. Raw sprouts, unwashed vegetables and fruit
5. Unpasteurized fruit juices
6. Hot dogs and luncheon meats not reheated to 165 °F (74 °C)
7. Cheese and yogurt made from unpasteurized milk
8. Raw milk
9. Raw or undercooked eggs
10. Unrefrigerated eggs

Adapted from Kendall et al. (2006)

these unsafe foods. However, when eating away from home, the elderly should observe caution in their food choices. It is advisable for the public at-large (regardless of age or health status) to avoid the foods listed in Table 1.

Specific Microbial Pathogens That May Cause Gastroenteritis in the Elderly

The increase in an aging population in the United States indicates that there is a group of individuals with compromised immune systems who are increasingly more susceptible to microbially-induced gastroenteritis, which could possibly lead to death For the period 1996–2012 in the United States, individuals aged ≥65 living in the FoodNet surveillance area, 4 bacterial pathogens accounted for 21,405 laboratory-confirmed infections: 49.3% were hospitalizedand 2.6% died. The average annual rate of infection per 100,000 population for *Salmonella* was 12.8; for *Campylobacter,* 12.1; for *Listeria*, 1.4; and for *E. coli* O157, the rate was 1.1. The estimated annual deaths for *Salmonella* was 220; *Listeria*, 180; Campylobacter, 60; and *E. coli* O157, 20 (Scallan et al. 2015). In addition, Scallan et al. (2015) estimated that these four pathogens caused ca. 226,000 illnesses, ca. 9700 hospitalizations, and ca. 500 deaths annually among adults aged ≥65.

Jagai et al. (2014) have studied the trend of gastroenteritis-induced death for the period 1985–2005 in the United States. The total number of gastroenteritis-associated deaths in 1985, 1990, 1995, 2000, and 2005 were 7265, 6978, 7813, 10,477, and 19,589, respectively. Thus, over the 21-year period of 1985–2005, the deaths due to gastroenteritis have increased 3.7-fold (Jagai et al. 2014). Most of the deaths occurred in the ≥65 age group. The adjusted relative risk for death due to gastroenteritis is 5.17 in the 55–64 age group, 14.44 in the 65–74 age group, and 61.72 in the 75+ age group (Jagai et al. 2014). Thus, as the individual ages, the risk of death due to gastroenteritis increases dramatically.

Clostridium difficile It appears that *C. difficile* infections are increasing: mortality data for the United States indicate that the cause of death due to *C. difficile* increased from 793 in 1999 to 7284 in 2010 (Murphy et al. 2012); ca. 91% of *C. difficile* deaths occurred in the ≥65 aged population. Hall et al. (2012) estimated that for the period July 1999 through June 2007 in the United States, 1.0% (rate 0.3/1,000,000) of gastroenteritis cases due to *C. difficile* ended in death for individuals of ages 1–4 years; 63.4% (rate 3.6/1,000,000) of cases due to *C. difficile* ended in death for individuals of ages 5–64 years; and 75.6% (rate 195/1,000,000) of *C. difficile* infected individuals died in the ≥65 age group. The infectious dose is unknown but is considered to be low (Yakob et al. 2013).

C. difficile infections have long been associated with health care institutions; however, community-associated infections make up at least one-third of *C. difficile* cases (Lessa 2013). The community-associated cases may be underestimated since younger individuals, who are more likely to develop community-associated *C. difficile* infections, do not report mild diarrheal illnesses. The presence of *C. difficile* has been demonstrated in chicken, beef, and pork meats and in ready-to-eat salads (Eckert et al. 2013; Harvey et al. 2011a, b). It is likely that the source of *C. difficile* in meat products is from the animal itself; the ingredients in salads may have been contaminated by the environment in which salad ingredients were grown or contaminated by a food handler. Lund and Peck (2015) postulated that foods, particularly meats, may be a source of *C. difficile* to consumers who become ill. However, food products have not been confirmed as a vehicle of *C. difficile* illness (Hensgens et al. 2012).

Norovirus Scallan et al. (2011) estimated that there are 5,461,731 (range 3,227,078–8,309,480) cases of norovirus infections annually in the United States, making the virus the most common cause of food-associated gastroenteritis. The National Outbreak Reporting System for the United States reported that for the period of 2009–2010, 67.7% (1908/2819) of single etiology agent gastroenteritis outbreaks were due to norovirus, resulting in 77.7% (69,145/88,958) of the outbreak illnesses and 85.6% (125/146) of the outbreak deaths (Hall et al. 2013). Person-to-person transmission of the virus accounted for 66.1% of the illnesses, and 25.9% were foodborne. Health-care facilities were associated with 48.8% of the norovirus outbreaks, and 15.0% of norovirus outbreaks originated in restaurants (Hall et al. 2013). Norovirus isthe major agent causing food-associated gastroenteritis in the United States. *Salmonella* species were second to norovirus as a cause of gastroenteritis (Hall et al. 2013). *Salmonella* species were responsible for 12.6% (355/2819) of gastroenteritis outbreaks resulting in 9.7% (8590/88,958) of outbreak-related illnesses and 4.1% (6/146) of outbreak-related deaths. Most of the *Salmonella* outbreaks were foodborne (71.5%), and only 4.8% were estimated to be transmitted person-to-person. Health-care facilities were associated with 1.4% of *Salmonella* gastroenteritis outbreaks; however, 19.4% were associated with restaurants (Hall et al. 2013). Thus, 80.3% of the outbreaks, 87.4% of illnesses, and 89.7% of the deaths were due to norovirus and *Salmonella* gastroenteritis in the United States during 2009–2010. For the period of July 1999 through June 2007 in the United States, individuals of ages 0–4 years accounted for 4.5% of gastroenteritis deaths (rate 1.3/1,000,000 population) from norovirus; individuals of ages 5–64 years

accounted for 3.9% of gastroenteritis deaths (a rate of 3.9 per 1,000,000) from norovirus, and the virus was responsible for 7.7% of gastroenteritis deaths (a death rate 20 per 1,000,000) in individuals ≥65 years of age (Hall et al. 2012).

Scallan et al. (2011) estimated that 26% of norovirus illness in the United States is foodborne and Verhoef et al. (2015) have estimated that ca. 14% of global norovirus outbreaks are foodborne. Person-to-person transmission appears to be the major mode of infection, since humans are the only known reservoir of the virus (CDC 2011). The low infectious dose (≥18 viral particles), coupled with copious viral shedding makes norovirus a very infectious agent (Hall 2012). The presence of norovirus in food is generally due to contamination by an infected food handler.

Non-typhoidal Salmonella spp. The percentage of cases of *Salmonella* infections in the United States, according to the 2012 FoodNet Surveilliance Network, for the 60–80+ age group was 18.5% (1446 per 7837). Interestingly, the percentage of *Salmonella* infections in children <5 years of age was 24.7% (1938 per 7837) (CDC 2014). The age-specific mortality rate (per 100,000 population) for children <1 year was 0.086 and that for children of ages <1–4 years was 0.006; the age-specific death rate for individuals of ages 65–74 years was 0.073, the rate for 75–84 years was 0.160, and the rate for the age group >85 years was 0.314 (Cummings et al. 2012). Non-typhoidal *Salmonella* organisms originate in feces. Therefore any foods contaminated by fecal matter from a *Salmonella*-infected animal or human can be a source of the organism. However, it should be recognized that *Salmonella* growth niches can be established in the production environment; they may be a significant source of food contamination in comparison to direct contact with fecal matter (Kornacki 2010, 2014). Such food products include meat and milk and other dairy products. Contamination of the environment by feces can lead to contaminated water or contaminated produce, fruit, or root vegetables (Cummings et al. 2012). Scallan et al. (2011) estimated that 94% of salmonellosis cases are foodborne. The infectious dose is low, probably in the range of 10–25 organisms (Schmid-Hampel and Frank 2007).

Campylobacter species *Campylobacter* follows non-typhoidal *Salmonella* species and *Clostridium perfringens* as a cause of bacterial-induced gastroenteritis in the United States. There is an estimated 845,024 (range 337,031–1,611,083) cases of campylobacteriosis annually in the United States; 80% of cases are estimated to be foodborne (Scallan et al. 2011). Scallan et al. (2011) estimated that 21 bacterial pathogens caused 3,645,773 (range 2,321,468–5,581,290) cases of gastroenteritis; therefore, *Campylobacter* caused 23.2% of the cases. Using CDC FoodNet Surveillance data for 2012, the number of cases of *Campylobacter* infections was 5914; 1379 (23.3%) cases were reported for the 60–80+ age group (CDC 2014). The percentage of cases in the <5 age group was 12.3% (728/5914). Death is not common in *Campylobacter* infections; for the period of 1996–2005, 0.6% (23 of 4033) of the ≥65 age group died, whereas 0.02% (2 of 6080) of the ≤4 age group died (Behravesh et al. 2011). The most common source of *Campylobacter* is chicken and poultry, but the pathogen is also found in other meat animals, contaminated shellfish, untreated water, and raw milk (Silva et al. 2011). The infectious

dose of *C. jejuni* varies with the strain used. In a challenge study of 111 adult volunteers, the infective dose range of *C. jejuni* strains ranged from 800 to 2×10^9 (i.e., two billion) cells (Adedayo and Kirkpatrick 2008).

Listeria monocytogenes There were 1651 cases of invasive *Listeria* infections in the United States for the period 2009–2011; 950 cases, 57.5% (950 of 1651), occurred in the ≥65 age group. The total number of deaths from listeriosis during that period was 291; 193 deaths (66.3%) occurred in the ≥65 age group (CDC 2013). The number of listeriosis cases in the United States for the 2012 FoodNet Surveilliance Network was 123; 70 (56.9%) of the cases involved the age groups 60–80+ years. Deaths among the elderly were few, with 3 deaths in the 40–49 group as well as in the 50–59 age group, 1 death in the 60–69 and 1 death in the 70–79 age groups, and 6 deaths in the 80+ age group (CDC 2014). However, for the period 1996–2005 in the United States, 23.9% (122/511) of the ≥65 age group died from listeriosis (Behravesh et al. 2011). For the period of 2001–2010, there were 0.5 cases per 100,000 population of listeriosis in Australians of age 60–79, and 1.6 cases per 100,000 population in the 80+ age group (Popovic et al. 2014). Scallan et al. (2011) have estimated that 99% of the listeriosis cases are foodborne. A variety of foods are associated with listeriosis; however, consumption of contaminated ready-to-eat foods such as deli meats and salads, hotdogs, raw milk, soft cheeses prepared from unpasteurized milk, and raw vegetables are the major cause of listeriosis (Smith et al. 2013). According to Schmid-Hempel and Frank (2007), the infectious dose is fewer than 1000 organisms.

Shiga toxin-producing Escherichia coli (STEC) The 2012 FoodNet Surveilliance Network for the United States revealed that 11.6% (58 of 500) of O157 STEC cases were among the 60–80+ age group, whereas 12.2% (68 of 557) of non-O157 STEC cases was for that age group. Children <5 years of age accounted for 28.8% (144 of 500) of cases of O157 STEC and 26.2% (146 of 557) of non-O157 STEC cases (CDC 2014). There was one death from O157 STEC in the 60–80+ age group and no deaths from non-O157 STEC infections (CDC 2014). For the period 1996–2005 in the United States, mortality due to O157 STEC in the ≥65 age group was 5.4% (23 of 428) (Behravesh et al. 2011). O157:H7 STEC have been associated with nursing home outbreaks, resulting in cases of hemolytic uremic syndrome (HUS) as well as deaths (Reiss et al. 2006). STEC are zoonotic organisms harbored in the intestines of ruminants and other animals; namely, raw meats, dairy products made from raw milk, and fruit and vegetables irrigated with water contaminated by animal manure are sources of both O157 and non-O157 STEC. In addition, foods that are consumed raw or undercooked can be implicated as contaminated (Caprioli et al. 2005; Grant et al. 2011). Scallan et al. (2011) estimated that 68% of O157 STEC and 82% of non-O157 STEC illnesses are foodborne. The infectious dose of STEC is believed to be <100 cells (Caprioli et al. 2005).

Shigella spp. The 2012 FoodNet Surveilliance Network for the United States revealed that 5.8% of *Shigella* infections was among the 60–80+ age group (125/2141). Most cases of shigellosis occurred in the ≤9 age group, 45.2% (967 of

2141) (CDC 2014). Only 1 death was reported for the 70–80+ age group (CDC 2014). Mortality due to shigellosis in the ≥65 age group in the United States for the period 1996–2005 was 1.0% (4/413) (Behravesh et al. 2011). Raw and undercooked foods are vehicles of *Shigella*. Humans are the only known reservoir of *Shigella* spp., and infected food handlers with poor personal hygiene can contaminate food. Irrigation water contaminated with human feces can lead to vegetables and fruit containing *Shigella* (Haley et al. 2010). Scallan et al. (2011) estimated that 31% of shigellosis cases are foodborne. The infectious dose is estimated to be 10–200 organisms (Haley et al. 2010).

Yersinia The percentage of *Yersinia enterocolitica* infections was 23.0% (37 of 159) in the 60–80+ age group, according to the 2012 FoodNet Surveilliance Network for the United States; the percentage in the <5 age group was 26.0% (41 of 159) (CDC 2014). No deaths were reported for the 60–80+ age group. For the period, 1996–2005, death from yersiniosis in the ≥65 age group was 5.1% (9 of 177) (Behravesh et al. 2011). Swine are a major reservoir of *Y. enterocolitica*, and raw and undercooked pork products are vehicles of infection (Huovinen et al. 2010; Rahman et al. 2011). Other raw or undercooked meat products, raw milk and dairy products, eggs, and vegetables can also be sources of *Y. enterocolitica* (Huovinen et al. 2010; Rahman et al. 2011). A total of 90% of the *Y. enterocolitica* infections are estimated to be foodborne (Scallan et al. 2011). The infective dose is not known but is believed to be in the range of 10^4–10^6 viable cells (FDA 2014).

Cryptosporidium The 2012 FoodNet Surveillance Network report for the United States revealed that the rate of *Cryptosporidium* infections among the elderly (60–80+) was 19.2% (241 of 1258 infections) (CDC 2014). Deaths are rare from *Cryptosporidium* infections (FDA 2014). Animals, especially cattle, are a reservoir of *Cryptosporidium*, and its presence in farm animals indicates that manure can be a source of C*ryptosporidium*. Contaminated water is a major source of infection (Rossle and Latif 2013), and only 8% of cryptosporidiosis cases are considered to be foodborne (Scallan et al. 2011). The infectious dose is 10–100 oocysts (FDA 2014).

Vibrio The rate of *Vibrio* infections (species not identified) for the 60–80+ age groups was 34.7% (68 of 196) for the 2012 US FoodNet Surveillance Network report (CDC 2014). Most cases of vibriosis are associated with the consumption of raw or undercooked seafood (Iwamoto et al. 2010). The ten-state Foodborne Diseases Active Surveillance Network revealed that the rate of vibriosis in the United States has increased from 0.15 of 100,000 population in 1996 to 0.42 of 100,000 in 2010. For the period 1996–2010, *V. parahaemolyticus* accounted for 54% (820 of 1519) of the cases, and 0.5% the cases (4 of 820) died. However, *V. vulnificus* only caused 12.7% (193 of 1519 of the *Vibrio* cases, but 30.1% (58 of 193) of these patients died. Therefore, *V. vulnificus* causes a more serious infection (Newton et al. 2012). For the period 1996–2005, the FoodNet system reported that 7.4% (12 of 163) of the deaths from vibriosis occurred in the ≥65 age group (Behravesh et al. 2011). Except for *V. vulnificus*, which has a low infectious dose of ca. 10^3 (i.e. 1000 viable cells) for predisposed individuals, the infectious dose for *Vibrio* species is estimated to be ≥10^6 (i.e., one million viable cells) (FDA 2014).

Long-Term Sequelae Resulting From Foodborne Illness

The major manifestation of foodborne illness is an acute illness, which can include diarrhea, cramps, fever, and vomiting; however, for the immunocompromised, elderly, infants, and pregnant women, there is also a risk of death. A small percentage of patients, including the elderly, suffer from long-term sequelae induced by the pathogen. These sequelae include reactive arthritis, Guillain-Barré syndrome, hemolytic uremic syndrome, meningitis, and irritable bowel syndrome depending upon the infecting organism (Ford et al. 2014; Minor et al. 2015). Ford et al. (2014) estimated that there were 4.1 million episodes of foodborne gastroenteritis in Australia in 2010; there were an estimated 35,840 (0.9%) cases of illness due to gastroenteritis-associated sequelae with an estimated 1080 hospitalizations, and an estimated ten deaths. Interestingly, *Campylobacter* infections were responsible for ca. 80% of the long-term sequelae.

Reactive arthritis (ReA) ReA is not a well-defined disease, but is rather a concept that describes an arthritis that may occur after a *Salmonella, Shigella, Campylobacter,* or *Yersinia* gastrointestinal infection. The arthritis is a reaction to an infection of the gastrointestinal tract, but the infecting organism is not found in the joint and, therefore, ReA is a sterile arthritis (Townes 2010). The mean duration of arthritic symptoms has been estimated to be ca. 200 days (Minor et al. 2015). Ajene et al. (2013) conducted a systematic research of the literature on diarrhea-associated ReA and concluded that the mean incidence of ReA was 9, 12, and 12 cases per 1000 cases of *Campylobacter, Salmonella,* and *Shigella* infections, respectively. There are studies that reveal that human leukocyte antigen-B27 (HLA-B27) positive individuals are more susceptible to ReA, but in general, most studies have not determined the presence of HLA-B27 in affected patients. So the association of HLA-B27 with ReA is not clear (Ajene et al. 2013).

Guillain-Barré syndrome (GBS) GBS has replaced polio as an acute flaccid paralysis. The syndrome is an autoimmune derangement of the peripheral nervous system characterized by weakness of the limbs and respiratory muscles, paralysis, and loss of reflexes. Patients may require assisted ventilation and many patients are bedridden (Desai et al. 2010; Nyati and Nyati 2013). World-wide, approximately 25–40% of GBS patients have had a gastroenteritis attack induced by *Campylobacter* prior to showing GBS symptoms; ca. 1 of 1000 patients with campylobacteriosis develop GBS (Desai et al. 2010). The molecular mimicry between sialylated lipooligosaccharides of the *Campylobacter* bacterial cell envelope, and the gangliosides on human nerves give a cross-reactive response leading to autoimmune nerve damage (Nyati and Nyati 2013). The mean duration of illness for *Campylobacter*-induced GBS is estimated to be 103 days; however, for patients with more severe disease, the duration of illness may be several years (Minor et al. 2015). The incidence of GBS increases with age (Nyati and Nyati 2013). Baker et al. (2012) in New Zealand conducted an interesting study showing that interventions to reduce the presence of *Campylobacter* in poultry led to a decline of campylobacteriosis by 52%. There was

a resultant decrease in hospitalizations for GBS of 13%. Therefore, preventing foodborne campylobacteriosis had a positive health effect of decreasing GBS. It is probable that the incidence of *Campylobacter*-induced ReA was reduced, also.

Postinfectious Irritable bowel syndrome (PI-IBS) IBS is characterized by lower abdominal pain, feeling of abdominal distension, and altered defecation in the absence of a structural change in the gastrointestinal tract. The syndrome is a common morbidity and is estimated to affect 10–20% of the population resulting in a high socioeconomic impact (Smith and Bayles 2007; Thabane et al. 2007). The symptoms of postinfectious-IBS (PI-IBS) are similar to that of IBS; however, the onset of illness follows a gastrointestinal infection; generally, after the diarrheal symptoms subside. PI-IBS develops in 4–32% of microbial-induced gastroenteritis cases and symptoms of PI-IBS may persist for several years (Collins et al. 2012; DuPont 2008). Gastroenteritis induced by *Salmonella, Shigella, Campylobacter,* and *Escherichia coli* O157:H7 have been associated with PI-IBS (Collins et al. 2012; Smith and Bayles 2007; Thabane et al. 2007).

Diarrhea-associated hemolytic uremic syndrome (D+ HUS) HUS is a thrombotic microangiopathy characterized by: microangiopathic hemolytic anemia (narrowing or obstruction of small blood vessels resulting in distortion and fragmentation of erythrocytes, hemolysis, and anemia), thrombocytopenia (abnormal low amount of platelets), and acute renal failure; D+ indicates that there was diarrhea before development of HUS (Cheung and Trachtman 2014; Ray and Liu 2001). An infection by Shiga-toxin producing *E. coli* (STEC) is the major cause of over 90% of D+ HUS cases in the United States (Mayer et al. 2012). D + HUS is the major cause of acute kidney failure in children from the United States and children <5 years of age are likely to develop complications requiring hospitalization and kidney dialysis. However, individuals ≥60 years of age are more likely to die from D + HUS (Gould et al. 2009; Mayer et al. 2012). The serotypes of STEC that induce D+ HUS include *E. coli* O157:H7 as well as the non-O157:H7 serogroups of O26, O45, O103, O111, O121, and O145 (Cheung and Trachtman 2014; Kaper and O'Brien 2014). More recently, infections by Shiga toxin-producing enteroaggregative *E. coli* serotype O104:H4 were shown to induce D+ HUS (Kaper and O'Brien 2014). Some patients (<10%) with D+ HUS progress to end-stage renal failure with a glomerular filtration rate of less than 15%, and these patients require dialysis or a kidney transplant (Foley and Collins 2007).

Many reports concerning D+ HUS state that thrombotic thrombocytopenic purpura (TTP; a blood disorder that causes blood clots to form in small blood vessels throughout the body as well as the brain with resultant low platelet count) is part of the definition of D+ HUS. However, TTP is a separate syndrome and is not part of the description of D+ HUS (Tarr 2009; Trachtman 2013).

Meningitis *Listeria monocytogenes* infections of adults can result in acute bacterial meningitis which may proceed to long-term neurological deficits. In a Spanish study, *L. monocytogenes* was the third most frequent cause of community-acquired bacterial meningitis (following *Streptococcus pneumoniae* and *Neisseria menigitidis*)

during a 39-month period (Amaya-Villar et al. 2010). There were 43 cases of *Listeria*-induced meningitis; 24 of the 43 (55.8%) patients were ≥60 years of age, 16/24 (66.7%) were immunocompromised, and 7 of 24 (29.2%) died (Amaya-Villar et al. 2010). Over a 16-year period (1997–2005), a Czech study revealed that 31 of 440 (7.0%) of hospitalized adult patients were diagnosed with acute bacterial meningitis due to *L. monocytogenes* and 15 of 31 (48.4%) of the patients were ≥65 years of age. A *Listeria* infection was the third most common etiologic agent for meningitis in elderly Czech individuals (Dzupova et al. 2013). These reports reveal that elderly patients are more susceptible to acute listerial meningitis than other adults.

Prevention of Foodborne-Induced Gastroenteritis in the Elderly

The elderly (≥65 years of age) population is highly susceptible to morbidity and mortality from microbial-induced gastroenteritis due to factors such as age-associated decrease in humoral and cellular immunity (a recent review on the aging immune system is presented in Montecino-Rodriguet et al. (2013)). Research has also demonstrated other factors such as malnutrition, lack of exercise, chronic disease, age-related changes in the gastrointestinal tract, including decreased intestinal motility and gastric acid production, entry into nursing homes, and excessive use of antibiotics (Kendall et al. 2006; Smith 1998). The elderly must be guarded against exposure to conditions and environments that may result in gastroenteritis.

Prevention of foodborne microbial-induced gastroenteritis is difficult, and steps should be taken to prevent its spread to others. Some advice on preventing gastroenteritis in individuals living in elderly care centers and in community-dwelling elderly is provided in Table 2. The most important protective mechanisms are thorough hand washing, cleanliness, and maintenance of a high degree of hygiene in food handling and preparation. In addition, person-to-person spread of infection must be prevented. Lund and O'Brien (2011) suggest the that the elderly should consume foods with low microbial loads such as meat and seafoods that have been cooked to a safe internal temperature, pasteurized milk and eggs, cooked sprouts, cheeses made from pasteurized milk, washed fresh vegetables and salad ingredients, and hot dogs (as well as luncheon and deli meats) reheated to 74 °C. The consumption of a low-microbial diet should aid in reducing microbial-induced gastroenteritis in the elderly living in elder care centers and in the community (Lund and O'Brien 2011); however, consumptions of fermented foods such as yogurt with high pro-biotic microbial load should be of benefit in the diet. The elderly with a chronic disease or diseases are probably more vulnerable to foodborne gastroenteritis, and irradiated foods would be a more proper diet for those individuals. It is important to emphasize that under normal food preparation conditions, it is not possible to achieve bacteria-free food. Some countries have passed laws requiring "zero tolerance" for certain pathogens in food products. However, in actual practice,

Table 2 Prevention of food-borne microbial-induced gastroenteritis in the elderly

Elderly care facilities (LTCFs and nursing homes)
1. The basic requirement for prevention of gastroenteritis is proper, adequate, and frequent hand washing by all individuals (including the elderly) of the facility.
2. Reduce human traffic between the community or hospital and elderly care facilities in order to reduce transmission of pathogens by outsiders who are ill.
3. Health care facility personnel (nurses, doctors, care takers, food preparers) who are ill should not enter the facility. Paid sick care leave for work personnel is a must.
4. Food handling errors must be recognized and prevented. Facility administrators, care personnel, and personnel who handle foods must be trained in food safety procedures such as proper handling of food ingredients, proper preparation of cooked foods, and proper serving of food. Food and food ingredients must be obtained from reliable, food-safety conscious suppliers.
5. Foods must be cooked to the proper temperature, avoid temperature abuse of stored foods, avoid cross-contamination between raw and ready-to-eat foods.
6. Facility personnel must maintain a high level of person hygiene and maintain a high level of cleanliness throughout the facility.
7. The personal hygiene of the elderly population must be monitored and maintained at a high level; ill elderly should be isolated to prevent transmission of disease to others.
8. Pets should not be allowed in the elderly care facility.
9. The elderly health care facility must follow the regulations promulgated by the appropriate health agency.
Community-dwelling elderly
1. The community-dwelling elderly must maintain a clean and hygienic home. All occupants of the home must practice adequate and frequent hand washing.
2. The elderly must practice safe food handling and preparation. Food ingredients must be obtained from a source that practices safe food policies. 'Risky eating behavior' (consumption of raw or undercooked foods of animal origin) must be avoided. Foods must be stored at the proper temperatures (hot foods should be maintained hot and cold foods maintained cold), foods should be cooked thoroughly and promptly served, cross-contamination must be avoided, and an infected food handler should not prepare nor serve foods. Pets should not be allowed in the kitchen.
3. An ill occupant should avoid contact with other occupants and ill visitors should not enter the home.
4. The period of time between packing and last delivery of home-delivered meals to the elderly should be short (<2 h). Home-delivered foods should be nutritious and hot and cold foods should be maintained at the appropriate temperatures. The elderly should not keep home-delivered meals at room temperature for more than 2 h; ideally, such meals should be eaten quickly as possible after delivery.
5. While it may be difficult to implement, all elderly persons should be educated in the proper handling, preparation, and serving of foods.

Adapted from Greig and Lee (2009), Kirk et al. (2010b), Namkung et al. (2007), Scott (2003)

application of "zero tolerance" is problematic (Wilson and Worosz 2014). Sampling design and statistics, as well as microbiological methodology play important roles in the determination of zero tolerance (NACMCF 2010).

Implementation of the Food Safety Modernization Act (FSMA; Public Law 111-353, 1/4/2011) should aid in the reduction of foodborne gastroenteritis in the elderly. The Act states that the food industry must apply, from farm-to-table, science- and risk-based preventive measures to ensure the safety of foods and decreased burden

of microbial foodborne illness (Doyle et al. 2015). The act has three important parts: (a) improving the capacity to prevent food safety problems; (b) improving the capacity to detect and respond to food safety problems; and (c) improving the safety of imported food (Public Law 2011). Full implementation of the FSMA has the potential to greatly improve food safety in the food industry of the United States. However, in order to reduce contamination of food by pathogens and to curtail the exposure of consumers to foodborne pathogens, close cooperation will be necessary among food industry personnel, clinicians, educators, government, and consumers.

Education of the elderly about food safety should be an important part of the prevention of foodborne microbial gastroenteritis. The U.S. Department of Agriculture and the Food and Drug Administration have issued a short booklet entitled "Food Safety for Older Adults" (FSIS/FDA 2011). It is described as a need-to-know guide to those 65 years of age or older. The guidelines are stated clearly and simply. The booklet should be helpful to both the elderly and their caretakers by giving important information about personal hygiene, and proper handling and preparation of foods.

Concluding Comments

Foodborne gastroenteritis in the United States and other developed countries is a major health concern in elderly populations, and considerable effort has been undertaken to combat these illnesses. The food chain has increased in length and complexity and various demographic, cultural, economic, and environmental factors are creating conditions that can lead to increased foodborne health problems. Unfortunately, a food safety failure at a link in the food chain due to pathogen contamination during primary production, processing, transport, retail sale, or home can lead to significant health and economic consequences. Among the individuals who are at risk when there is a food safety failure are the elderly. The elderly are particularly susceptible to foodborne pathogen-induced gastroenteritis due to physical, physiological, and immunological changes produced by the aging process. There is every indication that there is an increasing elderly population, and it is probable that morbidity and mortality related to foodborne gastroenteritis will increase in that population in the coming years.

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The Effects of Food Composition on Foodborne Illness Infectious Dose and Host Susceptibility

Monica A. Ponder

Abstract The ability to cause disease in humans is chiefly determined genetically, through the presence of virulence-associated genes, but is also influenced by the effectiveness of the host defenses to destroy the pathogen before the infection process begins. The ability of pathogens to evade the body's first line of defense, gastric acid, is affected by the food composition and the gastric emptying rate associated with the components. Surviving pathogens may be better able to resist digestive chemical and competitive microbiota of the small intestine based on prior exposure to stressors within the food product, including sub-lethal exposures to organic acids, preservatives and thermal processing. This chapter addresses the factors that influence the infectious dose of the bacterial pathogens and describes resistance associated with food components both *in vitro* and *in vivo.*

Keywords Foodborne pathogens • Virulence • Infectious dose • Food composition • Host susceptibility

Infectious Dose and Vehicle

Each year over 2.2 million people die globally from diarrheal diseases, the majority due to consumption of foods and water contaminated with pathogenic bacteria, parasites and viruses (Kuchenmuller et al. 2013). Traditionally, foodborne diseases have been associated with acute, rather mild and self-limiting gastrointestinal symptoms (nausea, vomiting and diarrhea). Risk analysis frameworks must consider not only the potential for contamination of a product by a pathogen, but also if the agent is present in adequate numbers to cause disease (infectious dose, ID) and the extent to which people exposed are likely to develop illness (attack rate). As may be expected the larger the number of pathogens consumed, the greater the attack rates for a variety of bacterial pathogens. However, the minimum infectious doses based on volunteer studies range from very small doses (1 cyst for *Entamoeba*, 10

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Cryptosporidium parvum oocytes, 10–500 colony forming units (CFU) for *Shigella*, 500 CFU *Campylobacter jejuni*), to moderate doses (10^3–10^4 CFU *V. cholerae*) and much higher doses for *Salmonella* (10^5–10^9 CFU) and selected pathogenic *E. coli* (10^6–10^8 CFU) (Kothary and Babu 2001). For pathogens such as enterohemorrhagic *E. coli* and *Listeria monocytogenes*, where the risk of severe complications and mortality is too great for human volunteer studies infectious doses are estimated based on the numbers of cells recovered from outbreak-associated foods and the reported or average serving size of that food. Estimated IDs for enterohemorrhagic *E. coli* are comparable to those determined for *Shigella,* reflective of the very high similarity of the genomes. Infectivity of *Listeria monocytogenes* is highly dependent on the health status of the host (Farber et al. 1996). In pregnant and immunecompromised individuals, infectious doses of 10^5–10^8 CFU have been estimated; in contrast, the ID ranges from 10^8–10^9 CFU in healthy hosts. Estimated infectious doses of *Salmonella* from outbreaks where the number of cells in the food was quantified reveals a much broader range of infectious doses for *Salmonella* than reported from human dose experiments, ranging from 10^2–10^{11} cells depending on the serovar and vehicle (Teunis et al. 2010). In the human volunteer studies, *Salmonella* were typically delivered in milk, sodium bicarbonate, or a similar buffering solution but were not consumed as part of a meal. Human-volunteer-determined infectious doses likely do not reflect the infective dose for the entire population because young, elderly and immunocompromised, who are known to be more susceptible to illness, are excluded from the studies.

Epidemiologic data suggest that the infectivity and virulence of foodborne bacteria, particularly serovars of *Salmonella* and *Listeria* may be associated with a number of variables including the health status and age of the host, but also the vehicle. Outbreaks with higher attack rates were associated with larger numbers of *Salmonella* isolated from the food vehicles, leading to larger calculated infectious doses. Yet, a smaller number of outbreaks had infectious doses calculated at <100 cells suggesting a role of the food matrix (Teunis et al. 2010). In one outbreak, the consumption of a single sundae cone, with an estimated 28 cells per cone, resulted in severe salmonellosis in an 8-year-old boy and only mild illness in the adult parents (Vought and Tatini 1998). In this outbreak, the attack rate (number becoming ill vs. number exposed) was low and may reflect unequal distribution of the pathogen in the product. In Japan, a ministry of health directive requiring restaurants and caterers to freeze food for 2 weeks has been useful for understanding the *Salmonella* dose response from different types of foods. In outbreaks with low attack rates, low infectious doses (49–8000 CFU) based on numbers of cells recovered from the frozen food and a typical serving size were common, especially for products such as dressings and confections (Kasuga et al. 2004), suggesting that exposure may be more variable due to unequal distribution of the pathogen in the product. Low infectious doses have also been reported for well-mixed products such as chocolate balls. In one outbreak, *Salmonella* Eastbourne could be recovered from multiple chocolate balls at concentrations of 1–2.5 CFU/g (Blaser and Newman 1982). While unequal distribution is less likely in chocolate, the low infectious dose may reflect challenges in the recovery of sub-lethally stressed cells, especially on selective

media. Presence of viable but non culturable (VBNC) cells may be associated with a decreased number of cells per serving, especially for foods that have been exposed to prolonged periods of freezing and low water activity, and thus a lower calculated infectious dose. Induction of a VBNC state in *Salmonella enterica* Tennessee by prolonged exposure to desiccation (a_w 0.5) in dried milk powder stored for 28 days was recently described (Aviles et al. 2013a). Culturability decreased, however molecular analysis indicated no changes to the log copies of 16S rDNA and stress response genes indicating the cells were still metabolically active. Moreover, the culturability of the cells returned after challenge with simulated gastric fluid and log CFU/g increased within simulated intestinal fluid, indicating these cells remained infectious. Infectivity of VBNC cells of *Listeria monocytogenes* and *Vibrio* spp. have been demonstrated within animal model systems (Oliver 2010). While it seems likely, that some of these discrepancies in infectious dose are due to challenges in recovery from foods, there is also intriguing evidence that *Salmonella*, and other food-borne bacteria are constantly responding to the environment within the food-matrix. It is likely that some of these changes offer cross-protection to gastrointestinal stresses such as surviving gastric acid and digestive enzymes, and altering disease-causing ability (virulence).

Factors Affecting Bactericidal Activity in the Stomach

In order to cause disease, foodborne pathogens must survive passage through the stomach, attach to and then invade intestinal epithelial cells, or in the case of toxigenic bacteria persist within the lumen. Within the stomach, bacterial pathogens are subjected to high levels of hydrochloric acid (150–160 mEq/liter HCl, pH 1) secreted in the organ by gastric glands. This gastric fluid is typically regarded as the first defense against infection. It is chiefly the acidity of the gastric fluid that is recognized as having the greatest bactericidal activity (Giannella et al. 1972). Gastric fluid aspirated from patients with pernicious anemia, a condition resulting in achlorhyria (lacking HCl, intragastric pH of 6–8) had little to no bactericidal activity. However bactericidal activity could be restored by adjusting with HCl to pH 2.0, killing >99.9% of *Salmonella* in 30 min, but little to no kill was accomplished at pH 4.0 for 120 min (Giannella et al. 1972). Reducing the acidity of the stomach or increasing the gastric emptying time may allow foodborne pathogens to evade this important first line of defense.

Reduction of Gastric Acid

Reduction (hypochlorhydria) or complete disruption (achlorhyria) of hydrochloric acid production in the stomach can be caused by a variety of factors including: malnutrition, some disease processes such as gastritis, and with increasing frequency of

medicines. Individuals with hypochlorhydria due to autoimmune destruction of acid-producing parietal cells, or due to gastric by-pass surgery are more susceptible to infections such as *Vibrio vulnificus* and more likely to display symptoms of small intestinal overgrowth syndrome (Naylor and Axon 2003). Patients with acute stage *Helicobacter pylori* gastritis, which is associated with reduced gastric acidity, are more likely to develop gastric giardiasis, a rare disease where the acid-sensitive parasite *Giardia lamblia* invades the stomach mucosa (Doglioni et al. 1992; McColl et al. 1998). Increased associations of chronic diarrhea and bacterial overgrowth syndrome have also been observed for bottle-fed infants with increased gastric pH (Maffei and Nóbrega 1975). Age is another factor associated with hypochlorhydria. Infants less than 2 months of age produce little hydrochloric acid, and incidence of hypochlorhydria is greater for the elderly populations. A reduction in gastric acidity may explain increased incidence of salmonellosis described in these two populations (Blaser and Newman 1982).

Hypochlorhydria is more likely to be associated with oral administration of antacids, H2 antagonists or proton pump inhibitors. Antacids, such as sodium bicarbonate, calcium carbonate, aluminum hydroxide and magnesium hydroxide are also effective in neutralizing stomach acid (Russell et al. 1993). H2 receptor antagonists block the action of histamine on parietal cells, decreasing production of HCl by the cells. Proton pump inhibitors are a class of drugs that block the action of the gastric proton pump of gastric parietal cells, which are responsible for secreting the H+ ions into the gastric lumen. By suppressing these activities, the intragastric pH of individuals on H2 antagonists and proton pump inhibitors routinely increases to above 3.0. During fasting the pH may further increase, with reports of gastric pH 5.0 common in some patients (Tolman et al. 1997; Wang et al. 2004). These levels of gastric acidity may be inadequate to inactivate *Salmonella* (Giannella et al. 1972) or other acid-tolerant bacteria. Increased asymptomatic fecal carriage of *Listeria monocytogenes* has been reported for individuals on long-term treatment with H2 antagonists compared to untreated controls (Cobb et al. 1996). Administration of cimetidine, a H2 antagonist, lowered the oral infectious dose of *L. monocytogenes* resulting in invasive disease in rats from 10^6 CFU to 10^2 CFU. Sodium bicarbonate, which acts to buffer gastric acidity, has also been shown to reduce the infectious dose of *V. cholerae* from 10^8 to 10^4 cells in volunteers presumably due to increased survival and passage into the small intestine. Current use of H2 antagonists and proton pump inhibitors have both been described as risk factors in recent outbreaks of *Campylobacter, E.coli* O157*, Listeria monocytogenes, Salmonella, Shigella* and *Vibrio cholerae* (Doorduyn et al. 2006; Neal et al. 1994; Leonard et al. 2007).

Effect of Food Composition on Gastric Acidity and Emptying

In the fasting state, the gastric pH is typically below 2 (range from 1.5 to 5.5), but during and after consumption of a meal the pH increases to 4–7 in a very short time due to the buffering effect of the foodstuff (Takumi et al. 2000). Several food

components such as fats, proteins, or ionic substances may also enhance the meal buffer capacity. It has been hypothesized that buffering capacity may have an important role in improving survival of enteric pathogens and may partially explain differences in the infectious doses calculated from outbreaks compared to human volunteer studies under defined conditions (Waterman and Small 1998).

As we age, the rate at which our parietal cells synthesizes HCl declines, either increasing the overall fasting pH or increasing the time necessary for the pH to stabilize. In studies of elderly (n = 79; age range 65–83) and young individuals (n = 24, 21–35 years) eating a standard meal of hamburger, bread, potatoes and milk, the postprandial rise in pH were comparable for the two groups (6.2 and 6.6, respectively) (Russell et al. 1993; Dressman et al. 1990). The most striking difference between the two populations was the much longer time needed for the gastric pH to return to the germicidal pH of 2.0 in elderly (150 min) compared to the young population (100 min) (Russell et al. 1993; Dressman et al. 1990). In infants, the postprandial pH also increases to pH 5–7, but may take as long as 3 h to return to the typical fasting pH of 3 due to decreased parietal cell activity until 1 yr. of age (Armand et al. 1996). Young children may also have more rapid emptying of the gastric contents compared to adults, a potential factor allowing pathogens to more quickly escape the gastric pH (Blaser and Newman 1982). The combination of this increased post-prandial gastric pH and increased emptying rate may allow enteric pathogens to escape into the duodenum, which may help explain the increased incidence of many enteric pathogens in small children.

The composition and volume of the meal will have an important role on not only the pH but also on the gastric emptying time, or the amount of time before the food leaves the stomach. A recent review by Kong and Singh (2008) describe the large number of factors influencing the rate of gastric emptying including: food volume, fluid viscosity, caloric content, acidity, and food physical properties such as texture and density (Kong and Singh 2008). Overall, the rate of gastric emptying is dependent on the caloric content, on average delivering 2–4 kcal/min to the duodenum (Kong and Singh 2008). Liquids are emptied most rapidly, with rates 10–40 mL/min (Dressman et al. 1998). As the caloric content increases, the time required for half of the stomach contents to be emptied ($t_{1/2}$) increases; the $t_{1/2}$ of saline is 8 min; but increases to 40 min when 25% dextrose is added (Houghton et al. 1988). Small amounts of liquid (<50 ml) can pass rapidly through the stomach because the pyloric sphincter fails to constrict when challenged with such a small bolus (Mossel and Oei 1975). It is possible that enteric pathogens transmitted in liquids with little to no solutes may quickly pass through the stomach evading inactivation.

As solids are masticated, their particle size is reduced creating the bolus. As the texture and density of the bolus increases with the addition of solid foods the gastric emptying rate of the liquid components significantly increases (Fisher et al. 1982). Contrary to the rapid emptying of liquids, a period of approximately 3–4 h is typical for complete emptying of solids (Kong and Singh 2008). Chemical composition and caloric content of the meal plays an important role in regulating the emptying rate. Meals of larger weights and caloric contents are associated with longer emptying times (Christian et al. 1980). It's possible that human pathogens may quickly escape

the stomach when consumed as part of meals or snacks with low caloric content (lettuce, salads) or in foods consumed in small quantities. This may correspond with higher attack rates (number of individuals ill/number of individuals exposed) in outbreaks or lower infectious doses.

In addition to the chemical composition, the particle size, viscosity and hardness of the solids may influence the gastric emptying rate. Softer foods empty faster than harder foods with observed $t_{½}$ of 52 min for cooked noodles and 82 min for liver (Weiner et al. 1981). Gastric emptying rates were significantly longer for chicken livers incorporated into beef stew ($t_{1/2}$ 117 min) or noodles ($t_{1/2}$ 82 min). The composition, hardness and viscosity will also influence the lag phase, or the time after swallowing before gastric emptying begins because this is the period needed for mechanical breakdown of the solid particles. The length of the lag phase and half-emptying time increases with solid food density (31 ± 8 min and $t_{1/2}$ 77.6 ± 11.2 min for egg and 62 ± 16 min and $t_{1/2}$ 94.1 ± 14.2 min for chicken liver, respectively) (Siegel et al. 1988). In vitro experiments indicated that the egg meal disintegrated much more rapidly than the chicken liver under mechanical agitation in gastric juice, lending further support to the hypothesis that the initial lag in emptying of solid food is due to the processing of food into particles small enough to pass the pylorus (Siegel et al. 1988). Once the food is broken into smaller particles its progression into the duodenum can be further affected by it composition, chiefly due to structure, density and osmolarity. Inclusion of acid-instable emulsions within meals accelerates gastric emptying, whereas ingestion of acid-stable emulsions delays gastric emptying (Marciani et al. 2009). Fats are emptied more slowly than carbohydrates or proteins not only due to increased kcal/g but also the increased density of fat results in its layering upon water (Kong and Singh 2008). An increase in the osmolality of the stomach contents decreases gastric emptying rate. Lemon- water beverages of comparable volume and calorie content, but sweetened with sucrose or maltose, which differ in the resulting osmolality of the solution, result in different t ½ values, 86 and 115 min respectively (Lavin et al. 2002). Fluids ingested at body temperature are emptied faster than colder or warmer fluids (Sun et al. 1988). Further research is needed to determine the rate of transfer of microbes in different ingredient formulations.

Survival and Virulence of Enteric Pathogens on Foods in Model Systems

The need to better integrate dose response into quantitative microbial risk frameworks has prompted the development of key events associated with development of foodborne infections for future characterization including: (i) survival of enteric pathogens in the stomach, (ii) establishment within the intestine and attachment and invasion of epithelial cells, (iii) survival and escape from the phagosome, (iv) transfer of pathogens outside of gut, including across placenta

(*Listeria monocytogenes*), (v) growth leading to fetal morbidity and mortality (Buchanan et al. 2009). The influence of the food microenvironment on these key events is poorly characterized. While some of these key events, e.g., transfer out of the gut and fetal growth, may only be accomplished in animal model systems, a number of model systems for upper gastrointestinal survival and invasion of epithelial cells have been developed (Stopforth et al. 2005; Koseki et al. 2011; Guerra et al. 2012; Tamplin 2005).

Although there is a large body of literature describing how environmental conditions affect the pathogens physiological and virulence characteristics, within model systems relatively little is known about how presence in the food micro-environment affects the pathogens ability to survive gastric acid challenge (Table 1, and Peterson et al. (2007)) and to colonize the intestine (Barmpalia-Davis et al. 2008; Baron et al. 2004; Larsen et al. 2010) and in few cases result in disease. This section will review relevant literature addressing in animal and *in vitro* model systems.

Table 1 The estimated infectious dose for various foodborne pathogens

Organism	Estimated infectious dose	
	Volunteer studies (compiled from Kothary and Babu (2001))	Food (compiled from Teunis et al. (2010)
Campylobacter jejuni	500	
Cryptosporidium parvum	10	
E.coli		
EPEC	10^6	
ETEC	10^7	
EaggEC	10^8	
Entamoeba coli	1	
Salmonella serovars		
Sal. Anatum	5.9×10^5	
Sal. Bareilly	1.3×10^5	Octopus sauce 1.4×10^7
Sal. Derby	1.5×10^7	
Sal. Meleagridis	7.6×10^6	
Sal. Newport	1.5×10^5	
Sal. Pullorum	1.3×10^9	
Sal. Typhimurium	3.0×10^9	Water 10^2, Ice cream 10^8
Shigella		
S. dysenteriae	10	
S. flexneri	100	
S. sonnei	500	
Vibrio		
Classical	10^4	
El Tor	10^3	
O139	10^4	
Non-O1	10^6	

Effect of Food Composition on Enteric Pathogen Survival: Animal Studies

The composition of the food matrix, especially antimicrobial compounds, fat and protein content, is hypothesized to influence the colonization of the host. An epidemiologic survey identifying risk factors associated with diarrheal illness in children under the age of 1 identified consumption of high fat milk as a protective factor (Koopman et al. 1984). This protective effect was hypothesized to result from antimicrobial activity of milk lipids, specifically fatty acids and monoglycerides, which has been described for Gram-positive bacteria (Sprong et al. 1999). To test this hypothesis, Sprong et al. (1999) fed rats either high-fat milk diets (40% energy from milk fat, 19.6 g/100 g) or low-fat milk diets (10% energy from milk fat, 4.2 g/100 g diet) then challenged the rats with clinical isolates of *L. monocytogenes* 4B or *Salmonella* Enteritidis phage type 1. Consumption of the high-milk fat diet was associated with decreased fecal shedding of *Listeria* (~1 log CFU/g reduction after day 1, 0.8 log CFU/g after day 3) compared to rats consuming the low-milk fat diet. No statistical difference was observed in fecal shedding of *Salmonella* but there was a trend toward increased fecal shedding in the high fat diet fed rats. The majority of the listericidal activity occurred in the stomach; however, the fat content did not significantly affect the gastric pH, indicating this activity was not due to gastric acidity but due to bactericidal activity of phospholipids. Concentrations of C10:0, C14:0 and C16:0 monoglycerides increased in the stomach of high-fat fed rats compared to rats fed the low-milk fat diet (Sprong et al. 1999). These monoglycerides have been described as listericidal within *in vitro* challenge studies (Kabara et al. 1972). Since the phosphoplipids in milk fat reduced listeriae invasion of rats, (Sprong et al. 1999) examined the effectiveness of milk fat globule membrane (MFGM) from different sources in the rat model. Rats fed diets with skimmed milk powder (low MFGM) or sweet buttermilk powder (high MFGM) were again not protected from colonization by *S*. Enteritidis, but lower translocation and colonization of *L. monocytogenes* were observed in the rats fed high MFGM (Sprong et al. 2012). MFGM is rich in sphingolipids, however, no listericidal activity was observed in rats fed purified sphinogomyelin or phosphatidylcholine, indicating that other phospholipids, or another component obtained from the whole food, was needed for colonization inhibition (Sprong et al. 2012). In a mouse model, the affect of milk fat on dosages of *L. monocytogenes* serotype 1/2a on infection was less clear, perhaps due to the lower percentage of fat in the diets compared to the above study (Mytle et al. 2006). The percentage of mice feces positive for *L. monocytogenes* was significantly reduced for mice on a high-fat diet (whipping cream, 30% milk fat) compared to those administered in skim milk (0.25% milk fat) or half & half (11% milk fat); however, this was only true for an administered dosage of 10^6 CFU. Despite the differences in fecal shedding, there was no significant difference in the percentage of mouse livers or spleens positive for *L. monocytogenes* in each dose group when administered in skim milk, half & half or whipping cream, indicating that the overall infectivity of the organism was not influenced by fat content of the vehicle in a mouse model. In a non-human

primate model, stillbirths occurred at lower numbers among a small number of pregnant animals receiving *L. monocytogenes* in whipping cream compared to skim milk or half- & half (Smith et al. 2008). The fecal shedding of *L. monocytogenes* was larger when administered in high-fat whipping cream compared to half & half; however, too few animals were enrolled in the study to provide enough power for statistical analysis. As the nonhuman primate model is most similar to the gastrointestinal system of humans, these results support that fat within the diet may have an important role in enhancing survival of enteric bacteria leading to increased disease (Table 2).

Table 2 Bacterial survival and virulence in simulated gastric fluid and/or challenge trials

Bacteria	Food	Outcome	Reference
Simulated gastric fluid (SGF) and/or simulated intestinal fluid (SIF) challenge			
Escherichia coli O157:H7	Romaine lettuce, modified atmosphere packaged	Survival % after simulated gastric challenge increased in MAP lettuce samples compared to culture grown control	Chua et al. (2008)
Escherichia coli O157:H7 and *E. coli* O26:H11	Raw cow milk cheese	Overall reductions in gastric compartment but survival of *E. coli* O26:H11 greater compared to *E. coli* O157:H7	Miszczycha et al. (2014)
Listeria monocytogenes	Bologna and salami, vacuum packaged	Increased resistance to gastric challenge of salami-inoculated *L. monocytogenes* compared to bologna during gastric challenge	Barmpalia-Davis et al. (2008)
	Deli Turkey Meat	Listeria grown on turkey meat more resistant to low pH and SGF compared to brain heart infusion grown cells	Peterson et al. (2007)
Salmonella enterica ser. Agona, Gaminara, Michigan, Montevideo and Poona	Apple, orange and tomato juices	Juice adapted cells had greater survival in SGF compared to non-adapted cells	Yuk and Schneider (2006)
Salmonella enterica ser. Tennessee	Dry Milk Powder	No difference in number of *Salmonella* genomes detected after simulated gastric phase compared to non-digested control, significant increase after simulated intestinal phase	Aviles et al. (2013a)
	Peanut butters with different fat contents and aw	Improved survival in high fat and low a_w formulations after *in vitro* digestion compared to control and low fat formulations	Aviles et al. (2013b)

(continued)

Table 2 (continued)

Bacteria	Food	Outcome	Reference
Invasion			
Listeria monocytogenes	Cured cooked ham and fermented sausages, MAP, 10°C	Increase in invasiveness of CaCo-2 and INT-407 intestinal epithelial cell line of ham incubated for 2 or 4 weeks compared to BHI control. No difference in invasiveness between control and fermented sausage	Larsen et al. (2010)
Salmonella enterica ser. Enteritidis	Egg White	No significant difference between LD50 of egg white incubated cells or control cells in mouse or chicken virulence models	Baron et al. (2004)
Salmonella enterica ser. Typhimurium	Soil, transferred to lettuce leaves, cut and modified atmosphere packaged	Slight increase in survival in SGF, increased growth in SIF, but decreased attachment and invasion of Caco-2 cells compared to cultured control.	Oliveira et al. (2011)

Eggs are a food matrix that is considered an unfavorable environment due to a high pH (pH 9) and high levels of the bactericidal proteins lysozyme, ovotransferrin and avian B-defensins. Acid-resistant *Salmonella* Enteritidis isolates are able to withstand these harsh conditions and maintain invasiveness of the liver and spleen of chickens at comparable levels to culture grown cells (Shah et al. 2012). In another study, prolonged incubation in an egg white matrix did not affect colonization of the liver, spleen or caeca compared to culture-grown *S*. Enteritidis in a mouse model of infection or within a chicken model of infection (Baron et al. 2004).

The desire for identifying alternatives to antibiotic growth promoters in food animal production systems provides a wealth of information about the effect of food components, especially non-digestible oligosaccharides and probiotic bacteria, on fecal shedding and infection of *Salmonella* in swine and poultry. Inclusion of medium chain fatty acids such as caproic (C6:0), caprylic (c8:0) and capric acid (C10:0) in feed decreased epithelial invasion by *Salmonella* in chicks (Van Immerseel et al. 2004), but were ineffective in reducing fecal shedding in pigs (Boyen et al. 2008). Similarly, milk fats that were rich in these fatty acids, also failed to reduce invasiveness of *Salmonella* in a mouse model (Mytle et al. 2006). Short chain fatty acids (SCFAs), specifically coated butyric acid decreased fecal shedding of *Salmonella* Typhimurium strain 112910a by 4.5 log CFU/g in pigs, but did not reduce colonization of the tonsils, spleen and liver compared to pigs fed the control diet (Boyen et al. 2008). Inclusion of other organic acids (such as formic, lactic, or benzoic), limonene or lactulose in the feed did not influence the number of *Salmonella* in the ileum and caecum of experimentally challenged pigs (Martin-Pelaez et al. 2010; Michiels et al. 2012; Farrow et al. 2012).

Particle size and grain type can influence*Salmonella* colonization in poultry and incidence disease of pigs (Berge and Wierup 2012). In broilers, feeding whole or coarsely ground grains compared to a fine ground grain decreased caecal populations of *Salmonella* potentially due to improved gastric motility (Santos et al. 2008). In pigs, there are no studies directly comparing the effects of different grain types on enteric pathogen-inoculated animals. Instead, epidemiologic studies have revealed that different forms of feed are associated with different *Salmonella* prevalence in herds. Liquid feed containing fermented by-products was associated with a decreased risk of *Salmonella* infection in Dutch farms (van der Wolf et al. 2001). A French prospective study of 105 farrow-to-finish farms revealed that the risk of *Salmonella* shedding in feces at the end of the fattening period was increased when dry feed (vs. wet feed) was provided during the fattening period (Beloeil et al. 2004). In these cases, the liquid feed was fermented or supplemented with whey to reduce the pH below 4.0. The gastric emptying rate in swine of fermented liquid feeds is reduced compared to liquid feed (Missotten et al. 2015) and therefore a longer exposure of *Salmonella* to the gastric contents would be expected. Pigs fed non-pelleted feed, or fed whey, had 2.5 and 2.6 times lower odds, respectively, of being sero-positive, compared with pigs fed pelleted feed (Lo Fo Wong et al. 2004). These results suggest that the gastric emptying, texture and composition of food may play an important role in the survival of *Salmonella* in the stomach and developing disease.

Inclusion of probiotic bacteria within the food matrix or taken within a capsule shows some promise for reducing enteric disease. Weanling pigs fed a mixture of probiotic bacteria (3 species of *Lactobacillus* and *Pediococcus* sp.), and subsequently challenged with *Salmonella* Typhimurium, had reduced incidence, shedding and duration of diarrhea (Casey et al. 2007). Probiotic bacteria can also reduce symptoms in toxin-borne disease by occupying the binding sites for bacterial toxins, including *Vibrio cholerae* toxin (Ctx) and *Escherichia coli* heat labile toxin (HLT) (Lanne et al. 1999). In a murine typhoid fever model system, *Saccharomyces boulardii* Biocodex, significantly decreased translocation of *Salmonella* Typhimurium to the spleen and liver of mice (Pontier-Bres et al. 2014) by modifying *Salmonella* movement (Pontier-Bres et al. 2012). Probiotic bacteria, including several strains of *Lactobacillus* naturally occur in fermented foods, where they are an integral part of the food matrix, and may naturally reduce the virulence of enteric pathogens (Eom et al. 2015).

Effect of Food Composition on Enteric Pathogen Survival: Simulated Gastrointestinal Systems

Types of *In Vitro* Model Systems

Model systems using simulated gastrointestinal fluids provide cost-effective information about the survival of *Salmonella* and other foodborne pathogens outside of an animal model (Stopforth et al. 2005). The simplest models for pathogen survival are static; including only simulated gastric fluid and incubated for different time

periods. These models allow for rapid tests on survival when influenced by a minimal number of parameters. However, to simulate the complex physiological and physicochemical events occurring within the upper human digestive tract, it is crucial to expose a meal to each step of digestion with realistic transit time, pH, and enzymatic conditions. A few dynamic bi-compartmental or multi-compartmental models (recently reviewed in Guerra et al. (2012)) have been developed for nutrition and pharmaceutical applications, but relatively few studies have used these models to evaluate human pathogen survival. These model systems still remain simplistic compared to actual *in vivo* situations: they do not include feedback mechanisms, resident microbiota, immune system, or specific hormonal controls. Feedback and hormonal control systems determine gastric emptying rates; therefore, influencing time of exposure of enteric pathogens to gastric acidity. The resident microbiota and the immune system play very important roles in the colonization and infection of gastrointestinal epithelial cells (Neish 2009), and represent the major limitation to the use of *in vitro* models.

Mono-compartmental models have been used most widely to study inactivation of enteric pathogens. In these studies, physical breakdown of food is first accomplished using a blender or stomacher device to reduce particle size to more closely resemble the food bolus as it passes from the mouth into the stomach. The gastric phase is reproduced by a pepsin hydrolysis of homogenized food, under fixed pH and temperature, for a set period of time (pH 1–2, 37 °C, 1–3 h). This step may be followed, in the same bioreactor, by an intestinal phase involving pancreatic enzymes with or without bile (pH 6–7) (Guerra et al. 2012). Other static mono-compartmental models include additional parameters: hand-mixing or stirring to simulate stomach mechanical forces (Aviles et al. 2013b), removal of digestion end products (Naim et al. 2004) to better approximate the volume changes due to gastric emptying, and pumping in simulated gastric fluid at biologically relevant flow rates to better approximate continuous changes in pH.

Dynamic, bi-compartmental models have been developed to simulate the luminal conditions of the stomach and proximal small intestine. Two-compartments connected by a peristaltic pump are used to transfer fluid from the gastric compartment into the intestinal compartments, while titrating additional simulated gastric as well as intestinal fluids into the appropriate compartment. Flow rate can be changed to simulate the gastric emptying rate, and the appropriate digestive enzyme input can be made to correspond to the food composition and caloric content (Barmpalia-Davis et al. 2008). The closest simulation of the *in vivo* digestive system is approximated by the TIM-1 (TNO gastro-Intestinal Model 1) (Guerra et al. 2012). This is a multi-compartmental system composed of the stomach and the three parts of the small intestine: the duodenum, the jejunum, and the ileum. The model integrates key parameters of human digestion: temperature, kinetics of gastric and intestinal pH, gastric and ileal deliveries, transit time, peristaltic mixing and transport, sequential addition of digestive secretions, and passive absorption of water and small molecules through a dialysis system.

Application of *In Vitro* Model Systems to Determine the Fate of Enteric Pathogens Incorporated in a Food Matrix

Fat

Epidemiologic data reveals that the fat content of a food may be a factor affecting dose response (Buchanan et al. 2000). It is hypothesized that organisms become trapped in hydrophobic lipid moieties, where they are protected from the acidic conditions of the stomach, and pass into the intestinal tract. The precise role played by fat in protecting bacteria from low-pH inactivation has yet to be determined and may be important in the protection (Waterman and Small 1998).

Incorporation of *Listeria monocytogenes* in high-fat foods increases fecal shedding and the invasiveness of the bacteria in animal models (section "Effect of Food Composition on Enteric Pathogen Survival: Animal Studies"). However, these studies provided little understanding of the inactivation kinetics in the gastric and intestinal compartments. Barmpalia-Davis et al. investigated the survival of *Listeria monocytogenes* in meat products with varying fat contents during simulated digestion using a dynamic bi-compartmental model system (Barmpalia-Davis et al. 2008, 2009). In these studies, a multiple strain cocktail of *L. monocytogenes* was inoculated into bologna (18% fat), salami (29% fat) or frankfurters (4.5% or 32.5%) and the survival of listeriae during simulated digestion was determined throughout the shelf life of the product. The pH of the gastric fluid significantly affected the rate of *L. monocytogenes* inactivation. Reductions were small (0.2–1.5 log CFU/g) during the first 60 min of gastric challenge when the pH of the gastric contents was >3. When the acidity was restored, by 120 min, the effects of product type and storage duration on gastric survival became evident. In frankfurters, higher fat content was associated with increased survival in the gastric compartment compared to lower fat frankfurters. The decrease in gastric inactivation rates associated with fat content were more pronounced after product storage for 55 days, suggesting an important role of adaptation (Barmpalia-Davis et al. 2009). Slower gastric inactivation rates (GIR) were also observed for the higher fat content salami compared to bologna (Barmpalia-Davis et al. 2008). Other chemical and physical properties differences between bologna and salami may have influenced survival of *L. monocytogenes,* including pH (5.24, 4.49) and moisture content (61% vs. 36.5%), respectively. Overall the GIR was lower for high-fat foods, indicating a protective role of fat from gastric acidity, yet the time of storage also influenced the GIR, perhaps due to extended periods of stress-adaptation. The use of this dynamic gastrointestinal system verified the protective effects of gastric emptying against the gastric destruction of *Listeria monocytogenes.* Populations of *L. monocytogenes* could be detected within the intestinal compartment within 30 min of challenge. This indicates that *L. monocytogenes* was somewhat dissociated from the meat matrix into the gastric fluid during the first 30 min of the process, when the pH was greater than 3, allowing the bacteria to escape to the small intestine before gastric acidity becomes lethal.

D'Aoust described that *Salmonella* outbreaks with a low ID are often associated with a food source with a high fat content such as chocolate and cheese (D'Aoust and Pivnick 1976). The protective effect of fat content was recently examined for a peanut butter outbreak–associated strain of *Salmonella enterica* serotype Tennessee within peanut butters with different fat contents (19 or 65%) and varying a_w (0.96 or 0.24) that were challenged in a static mono-compartment gastrointestinal model (Aviles et al. 2013b). Exposures to increased fat content and decreased a_w were both associated with a protective effect on the survival of *Salmonella* Tennessee in the simulated gastric fluid, compared with control cells. In the peanut butter matrix, *Salmonella* Tennessee was protected from inactivation in the simulated gastric fluid for 2 h, with only small reductions of *Salmonella* Tennessee in low-fat treatments and small, statistically insignificant, increases for high-fat formulations. After exposure to bile and digestive enzymes in a simulated intestinal phase, the populations of *Salmonella* Tennessee in the control and low-fat formulations were not significantly different; however, a 2-log CFU/g increase occurred in high-fat formulations, suggesting increased virulence in the high-fat environment. It is important to note the protective effect of fat is not universal but does vary with bacteria and the food composition. Exposure to increased fat content in ground beef was not protective for *Campylobacter jejuni* or *Vibrio cholerae* in media at pH 2.5 (Waterman and Small 1998). Survival of *Salmonella* Typhimurium SL1344 in acidified lactose broth (LB) was greater in high-fat ground beef (28% fat) than in rice (0% fat). Increased survival was also observed for boiled egg white (33%) with low-fat content, suggesting that protein content may be concomitantly protective for *Salmonella*.

Protein

High-protein foods have frequently been implicated in outbreaks of foodborne disease. *Listeria monocytogenes*, a pathogen notorious for causing outbreaks associated with ready-to-eat meats, displays enhanced resistance to inactivation by simulated gastric fluid at pH 7.0, 5.0 and 3.5 when grown on turkey meat compared to cells grown using brain heart infusion broth (Peterson et al. 2007). Reduction of BHI-grown listerial cells was greater by 1 log CFU/ml compared to turkey-associated cells at pH 7.0. As the pH decreased, the inactivation rate of the BHI-grown cells was more pronounced, with differences in reduction of 3 log CFU/ml at pH 3.5. Dynamic, single-compartment systems were recently used to describe pathogen behavior in high protein foods (e.g., tuna, scrambled egg and ground beef) in the stomach (Koseki et al. 2011). The pH of the simulated gastric fluid increased to between 5 and 5.5 immediately after addition of the meat, this pH gradually decreased over 120 min to reach pH 3.5, 3 and 2.75 for the tuna roll, hamburger and scrambled egg, respectively. There was a direct correlation with the final pH and protein content of the food. This indicates that differences in food composition had an important role in the buffering capacity of the food. During the 180-min incubation time, there was minimal inactivation of *E. coli* O157:H7 in ground beef (0.5 log

CFU/ml), *L. monocytogenes* in tuna and *Salmonella* in scrambled egg (~1 log CFU/ml) (Koseki et al. 2011). This low rate of reduction of *E.coli* O157:H7 during exposure to simulated gastric fluid was also observed for contaminated ground beef that had been temperature abused, especially when shifting from vacuum packaging to aerobic retail-style packaging (Stopforth et al. 2006). Reductions of *Salmonella* Typhimurium in ground beef challenged in acidified LB (pH 2.5) were not affected by inoculum size (Waterman and Small 1998; Stopforth et al. 2006). Survival in gastric challenge is also improved for *Salmonella* Tennessee in peanut butter (25% protein w/w) and dry milk powder (36% protein) compared to control cells, indicating that non-animal sources of protein are also protective (Aviles et al. 2013a, b). Recently, some types of proteins (e.g., albumin and turkey meat) were described to have superior protection to *Salmonella* during simulated gastric digestion, compared to bovine serum albumin, indicating that protection may be protein-specific and depend on buffering capacity or pKa (Birk et al. 2012). The high rates of survival of *Salmonella* in this simple *in vitro* digestion model indicate that the presence of protein, low water activity and high fat content serve to protect the cells from damage due to low pH and enzymatic activity.

Vegetables

Ready-to-eat produce is increasingly associated with foodborne disease (Sivapalasingam et al. 2004). It is hypothesized that exposure to a low nutrients, with increased presence of organic acids and oxidative stress associated with modified atmosphere packaging increases the adaptation of enteric pathogens to the gastrointestinal environment. The ability of enterohemorrhagic *Escherichia coli* to induce acid resistance on modified atmosphere- packaged lettuce is strain dependent. Survival after gastric acid challenge (viz., pH 2.0) on lettuce was increased after 7 days of modified atmosphere package storage (Chua et al. 2008). Survival in simulated gastric fluid for 180 min was improved for multi-strain mixtures of *E. coli* O157:H7 and *L. monocytogenes* inoculated onto cut lettuce, with only minor reductions of 1.5 log CFU/g (Koseki et al. 2011). Larger reductions of a mixture of *Salmonella* Enteritidis and *S*. Typhimurium on lettuce occurred after 120 min when the pH was above 3. However *Salmonella* was no longer detected by 180 min when the pH decreased to 2.0. Only small reductions (8%) of *Salmonella* Typhimurium DT104 in simulated gastric fluid at pH 3.5 were observed for cells that were sequentially incubated in soil and on lettuce (Oliveira et al. 2011). In addition, these cells had an increased the ability to grow in simulated intestinal fluid compared to control cells, but a decreased ability to attach to epithelial cells and invade Caco-2 cells, suggesting the overall survival probability in the gastrointestinal tract system was decreased for soil-lettuce passaged isolates. Decreased attachment and invasion of *S*. Typhimurium ATCC 14028 to Caco-2 cells was reported after exposure to the water-soluble fraction of cherry tomatoes (Koh et al. 2013).

Organic Acids/Fermentation Products

The ability of enteric pathogens to withstand acidic conditions in various foods is well-documented. Exposure to organic acid in juices and used as preservatives is associated with enhanced resistance to gastric stress. Adaptation of *Salmonella* spp. to the acidic juice environment increased acid resistance in SGF and varied by serovar, juice type and adaptation temperature (Yuk and Schneider 2006). Adaptation at 7 °C in apple juice for 24 h was associated with increased survival to SGF for 100 s in 4 of 5 *Salmonella* strains tested compared to adaptation at 20 °C (Yuk and Schneider 2006). Acid resistance of the serovars in SGF was influenced by types of organic acids such as malic and citric acids, which are predominately present in apple and tomato juice and in orange and tomato juice, respectively. Differences in undissociated ions in these different acids had been previously shown to result in differences in resistance of *E.coli* O157:H7 to SGF (Yuk and Marshall 2005). Prior adaptation to organic acid salts at concentrations that would typically be associated with ready-to-eat meats, is associated with significant differences in survival of *Salmonella* Typhimurium during incubation in SGF (Yuan et al. 2012). For example, preadaptation in sodium acetate (5%) resulted in a 3-log CFU/ml increase in survival after exposure to simulated gastric fluid compared to control cells, whereas a 2-log reduction in survival was observed for cells pre-adapted in sodium lactate (5%).

Adaptation of *L. monocytogenes* and *E.coli* O157:H7 to acidic and osmotic stresses encountered during the fermentation and drying of sausages (pH 5.0, a_w 0.91) may enhance resistance to the acidity of the stomach and the action of bile. Populations of *E. coli* O157:H7 within contaminated fermented, dry sausages were not affected by exposure to the harsh environment of mucus and SGF and grew significantly better in the presence of simulated intestinal fluid compared to non-stressed cells (Naim et al. 2004). Acid-adapted cells within the fermented sausage were also associated with increased Shiga-toxin production within the simulated intestinal compartment, and increased death of Vero cells (Naim et al. 2006). Gastrointestinal inactivation rates following exposure to simulated gastrointestinal fluids were reduced for *L. monocytogenes* in salami, compared to non-fermented meats (Barmpalia-Davis et al. 2008). Unlike fermented meats, the survival dynamics of *E. coli* O157:H7 in fermented olives was very low in simulated gastric fluid for comparable periods of time. After 150 min, no *E. coli* O157:H7 could be detected in the stomach component (Arroyo-Lopez et al. 2014). However, this study was performed in a TIM TNO multicomponent gastrointestinal system, which models the stomach and three sections of the small intestine, and fluid is transferred from each of the sections every 30 minutes. This transfer rate, which simulates the gastric emptying rate for a small meal, resulted in the transfer of large populations of *E. coli* O157:H7 (7-log CFU) from the gastric component to the duodenal compartment before gastric acidity could influence inactivation. These populations experienced no reduction for the 30 min of exposure to high concentrations of digestive enzymes before they were transferred to the jejunal compartment. In the jejunum and ileum compartments, *E. coli* populations increased to 7.9 log CFU after 300 min. If a static

model in simulated gastric fluid exposed to the average time when solids are emptied from the body (180 min, see above) is considered, then the dose-response and associated risk of *E. coli* O157:H7 illness associated with olives would be greatly underestimated.

Application of Epithelial Cell Line Models to Investigate Pathogen Adhesion and Invasion

Foodborne bacteria must constantly adapt to the conditions encountered in the environment, i.e., food, water, surfaces, etc. A growing body of evidence supports the concept that resistance to stress alters the virulence of many foodborne pathogens, thereby increasing the likelihood of infection (Wesche et al. 2009). The use of simulated gastrointestinal fluids provides cost-effective information about the survival of enteric pathogens within simulated gastric and intestinal fluids, but models systems are needed to examine the invasiveness of gut cells.

For most foodborne pathogens, pathogenesis requires their adhesion to host cell surfaces and the ability to invade and survive within intestinal epithelial cells. The human intestinal epithelium is comprised of absorptive enterocytes and mucin-producing goblet cells. The human intestinal cell lines, CaCo-2 and H29, are examples of enterocytes widely used for examining host-pathogen interactions *in vitro*, especially addressing the pathogenesis of several human pathogens including *Salmonella, Listeria,* and *Campylobacter* (Bhunia 2008; Gagnon et al. 2013).

While several studies have examined the ability of pathogens to survive within simulated gastric and intestinal fluids, few studies have examined the effect of a food matrix to influence the attachment and invasion of model cell lines, which is an essential final step for disease manifestation. Cell lines, particularly Caco-2 enterocytes are useful tools to elucidate mechanisms associated with decreased disease potential. Protein hydrolates from high-fat milk diets, previously shown to reduce fecal shedding of *L. monocytogenes* in rats, were recently determined to reduce adhesion and invasiveness of *L. monocytogenes* of human Caco-2 enterocytes (Kuda et al. 2012). Reduced Caco-2 cell adhesion was also observed for *L. monocytogenes* and *S.* Typhimurium in the presence of soybean protein hydrolysates, and may explain the small number of these illnesses reported from soy-dense meals (Yang et al. 2008). The ability to grow on other high-protein foods, including raw salmon, was associated with increased infective potential of *L. monocytogenes* compared to BHI-grown cells, especially when subjected to temperature abuse (Duodu et al. 2010). Growth on retail deli meat was associated with increased virulence as determined by increased invasiveness of Caco-2 cells (Larsen et al. 2010). Prolonged incubation of *L. monocytogenes* on chilled, cooked ham was associated with a slight increase in invasiveness to Caco-2 cells compared to cells incubated in BHI broth or on sausage. While growth continued on ham for 4 weeks during storage, no growth was seen in the fermented sausage (low pH and a_w), indicating that both actively growing, and sub-lethally stressed cells maintain their ability to cause disease.

Supplementation with probiotics, micronutrients and proanthocyanidins in food may influence invasive potential of foodborne pathogens. Phenolic compounds from tea, black bean extracts and pomegranate rinds significantly decreased adherence of *L. monocytogenes* to enterocytes (Xu et al. 2015; Zhao and Shah 2015). Oral supplementation with iron results in excess luminal iron concentrations resulting in increased adherence and invasion by *S*. Typhimurium (Dostal et al. 2014). Competitive binding of mucin by *Lactobacillus plantarum* CIF17AN2 inhibits *S*. Typhimurium SA2093 adhesion to HT-29 cell lines (Uraipan et al. 2014). In addition, lactic acid bacteria secrete bacterocins further reducing the ability of pathogens to adhere to epithelial cells.

Cross-Protection and Virulence of Pathogens in Food

Foodborne pathogenic bacteria must for long-term survival continually adapt to conditions encountered during the processing, storage and cooking of foods, and within the gastrointestinal tract. These stresses include: starvation; physical/mechanical stress due to heat, pressure, or osmotic shock; chemical treatments such as acids or detergents; and antimicrobial agents. Exposure within the food environment may improve the resistance of the pathogen to *in vivo* stresses, including the low pH encountered in the stomach and macrophages, increased osmolarity of the intestine, and detergent-like activities of bile salts in the intestine. The phenomenon that exposure to one stress may also confer protection against a different type of stress is referred to as cross-protection or cross-adaptation (Wesche et al. 2009). The similarity of the stress encountered and the types of protein that stress induces will determine the degree of cross-protection. RpoS is the key sigma factor controlling stress responses in Gram-negative bacteria such as *E. coli*, *Salmonella*, and *Vibrio,* whereas the alternative sigma factor SigB, modulates the stress response in Gram-positive bacteria such as *L. monocytogenes*, *Bacillus subtilis*, and *Staphylococcus aureus*. These transcriptional regulators coordinate the cells to tolerance of high acid conditions, elevated osmolarity and temperature, bacteriocins, antibiotics, ethanol, and starvation, and play a role in biofilm formation (Schellhorn 2014). Mechanistic responses to these stresses were recently reviewed by Begley and Hill (2015). In this chapter, only stress response and virulence-associated gene expression characterized within a food matrix was addressed.

Exposure to stress conditions within a food-matrix is associated with different responses compared to laboratory studies, likely due to the increased complexity of the food environment compared to growth media. Typical laboratory growth media, (e.g. BHI, TSB, and LB), are rich in nutrients that support rapid replication of human pathogens. In contrast, bacteria are typically surface associated in the majority of relevant food agro-ecosystems including within environment, on foods or in the GI tract, where they may experience low nutrient availability or encounter antimicrobial compounds. A study comparing *E. coli* protein expression when grown in whole bovine milk or laboratory media identified hundreds of differences in the

levels of proteins between the two substrates (Lippolis 2009). Growth in milk was associated with up-regulation of proteins involved in the metabolism of lactose and various amino acids as well as the LuxS protein, a quorum-sensing associated protein linked to the expression of flagellar components and virulence genes. *L. monocytogenes* grown on ready-to-eat sliced turkey uniquely expresses 34 proteins compared to growth on BHI agar at 15 °C. These meat-specific proteins were involved in energy metabolism and cellular processes, including several proteins associated with stress responses such as elongation factors and chaperone proteins (Mujahid et al. 2008). Increased numbers of stress-related proteins were also produced in *L. monocytogenes* cells exposed to an artificial cheese-based medium [pH 5.5, 3.5% NaCl weight/volume (w/v)] compared to non-adapted cells (Melo et al. 2013). However, the presence of the proteins failed to result in enhanced survival within a simulated gastrointestinal environment compared to non-adapted cells.

Adaptation to certain food environments, including plant surfaces, may be associated with increased virulence of *S.* Typhimurium and *E. coli* O157:H7, and may explain the large number of outbreaks associated with leafy green vehicles. Growth in lettuce or cilantro slurries is associated with increased transcription of genes involved in carbohydrate transport, including carbon sources also present in the intestinal tract, as well as genes important for withstanding oxidative stress and antimicrobials (Wesche et al. 2009; Goudeau et al. 2013; Kyle et al. 2010). These genes may enable improved survival in the gastrointestinal system and prime cells for attachment and invasion. *S*. Typhimurium within soft-rot lesions on lettuce or cilantro increased expression of genes associated with propanediol utilization and cobalamin synthesis, which are both required for replication in macrophages and are involved in the colonization of the chicken lumen (Goudeau et al. 2013). *E. coli* O157:H7 on the surfaces of lettuce and spinach leaves up-regulate the expression of attachment-associated genes, which are integral for the pathogenesis of *E. coli* O157:H7 (Kyle et al. 2010; Lopez-Velasco et al. 2010). Additionally, Shiga toxin genes are expressed by *E. coli* O157:H7 under storage at 4 °C on romaine lettuce (Carey et al. 2009), and at 4 and 10 °C on modified atmosphere-packaged spinach (Lopez-Velasco et al. 2010), indicating that adaptation to the environment of ready-to-eat produce may increase virulence.

Adaptation to low water activity stress is hypothesized to contribute to increased virulence of foodborne pathogens, chiefly through up-regulation of stress-response genes that improve the pathogen's survival within the gastrointestinal tract. Despite failure to grow within the low-nutrient, low-water activity (a_w 0.3) environment of peanut oil, *S*. Enteritidis maintains metabolic activity, albeit with substantially fewer genes actively transcribed (5%) compared to LB broth (78% of the transcriptome) (Deng et al. 2012). Transcripts detected in peanut oil included genes important for heat shock and starvation, such as chaperone proteins, alternate sigma factor *rpoE* and heat/cold shock proteins. Metabolic activity also continued for *S*. Tennessee stored in dry milk powder (a_w 0.3) for 120 days. Exposure of *S*. Tennessee to desiccation resulted in increased expression of stress-response (*rpoS, ots)* and invasion-associated genes (*hilA, invA* and *sipC),* and was associated with improved survival

in a static, simulated gastrointestinal model (Aviles et al. 2013a). Future studies should incorporate the use of dynamic, bi-compartment gastrointestinal digestion models and tissue culture adhesion and invasion assays to determine if cross-protection to low-water activity stress is associated with an increased ability to cause disease.

Conclusions and Future Directions

A growing body of evidence supports that the component of a food vehicle and its processing history alters the infectivity of enteric bacterial pathogens. Future research is needed on the effect of product formulations and the occurrence of sub-lethally damaged bacteria. Method development to improve recovery of sub-lethally damaged pathogens is integral to our understanding of these interactions, and will further contribute to improve the performance of risk models by reducing under estimation of survival and infectious agents. Expanding trends in product formulation designed to decrease the gastric transit time as well as increased proportions of the public who produce less gastric acid will lead to a greater portion of individuals with increased susceptibility to enteric pathogen infection. Research that seeks to elucidate the virulence-food matrix interactions will allow both regulators and industry to tailor acceptable levels of bacterial pathogens in specific product types and identify product types where more stringent control is needed to result in meaningful changes to the attack rate and incidence of foodborne illness.

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Foodborne Pathogens and Host Predilection

David C. Bean and Laurie S. Post

Abstract During food manufacturing, the potential exists for contamination of products with pathogenic microorganisms. While the ingestion of a bacterial pathogen will typically result in illness in a susceptible host, it is not the case for each strain within a given species. Pathogenic bacteria display various levels of host specificity: some infect a wide range of hosts, while others have strict host selectivity and are obligate pathogens. Host specificity of bacterial pathogens is determined by multiple molecular interactions between both the pathogens and their hosts. Understanding these interactions in detail will allow risk-based decisions to be made on affected foods, informed by knowledge of specific strains or pathotypes. This has the potential to avoid costly and unnecessary recalls with classical pathogens that can be proved to have a low potential for causing illness.

Keywords *Escherichia coli* • *Salmonella* • *Listeria monocytogenes* • Host specificity • Virulence

Introduction

Food products may be considered adulterated based on the presence of certain microorganisms. While, in many cases, the ingestion of these pathogens may cause disease; it is not the case for each strain within a given species. The tendency of regulatory agencies to view all pathogenic microorganisms of a given genus (e.g. *Salmonella*) or species (e.g. *Listeria monocytogenes*) as being equally virulent is a robust, yet conservative approach to ensuring food safety. This is important, as in the absence of knowledge; public health cannot be unnecessarily compromised. However, the FSIS ruling

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that six *E. coli* serovars (in addition to *E. coli* O157:H7) are now classified as adulterants creates an interesting precedent (USDA-FSIS (United States Department of Agriculture Food Safety and Inspection Service) 2012). The FSIS ruling might cause costly, yet unnecessary recalls. In an age of molecular biology and whole genome sequencing, it may be prudent to screen for pathotypes (a bacterial strain or set of strains with the same or similar virulence characteristics) rather than serotypes. What, then, are the most appropriate targets for detecting bacterial adulteration of foodstuffs? Possibly virulence genes of individual pathogens; however, it is not the purpose of this chapter to discuss the virulence genes of individual bacterial pathogens – this is covered elsewhere in this book. Instead, this chapter aims to define the disparate host specificities of foodborne pathogens.

Defining host specificity is in itself a difficult task. Kirzinger and Stravrinides described host specificity as "*partiality to infecting one or a group of defined hosts*" (Kirzinger and Stavrinides 2012). This definition captures the notion that some pathogens are very specific in their host preference (e.g., *Salmonella* Typhi), whereas others are generalists and may infect a number of different host species (e.g., *Salmonella* Typhimurium) (Baumler and Fang 2013). Other bacterial pathogens (e.g., *Listeria monocytogenes*) may have a certain predilection for a defined subset of a host species (e.g., pregnant, elderly and people with weakened immune systems). Each pathogen-host interaction, then, must be considered separately to understand the particular predilection for each microorganism. This chapter considers three groups of pathogens: *Salmonella* species, *Escherichia coli*, and *Listeria* species. The host-specificity of each is explored, and the following areas will be examined: (1) epidemiology and surveillance, (2) human/animal studies, (3) *in vitro* studies. The last section will, unavoidably, overlap some of the other chapters in this book, but this will be minimized when possible.

The review of Pan et al. (2014) describes three key principles defining host specificity of bacterial pathogens. These are:

1. The specific recognition of host specific receptor molecules by pathogens to facilitate colonization and/or dissemination.
2. The ability to evade or overcome the host immune system.
3. The ability of the pathogen to acquire essential nutrients for growth *in vivo*.

When considering the molecular mechanisms defining host specificity, these three principles will be addressed.

Salmonella Species

Non-typhoidal *Salmonella* is estimated to cause more than one million illnesses, 19,000 hospitalizations and 370 deaths each year in the United States (Scallan et al. 2011). Non-typhoidal *Salmonella* is also a leading cause of foodborne illness in other

countries: 92,000 illnesses and 1300 hospitalizations in Australia (Hall et al. 2005) and 73,000 illnesses and 2666 hospitalizations in England and Wales (Adak et al. 2005).

The genus *Salmonella* consists of two species: *S. enterica* and *S. bongori*. The former species is further subdivided into six subspecies. Of these, *Salmonella enterica* subspecies I has a very broad host range that has adapted to warm-blooded vertebrates. It is the most important in terms of human disease (Uzzau et al. 2000). *Salmonella bongori* and the remaining *Salmonella enterica* subspecies II, IIIa, IIIb, IV, VI and VII tend to be associated with cold-blooded vertebrates (Baumler et al. 1998). *S. bongori* was formerly classified as *S. enterica* subspecies V. This chapter addresses only subspecies I: *Salmonella enterica* subspecies *enterica* which can be further differentiated into 1586 serovars based upon lipopolysaccharide (O), flagellar (H) and capsular (Vi) antigens (Issenhuth-Jeanjean et al. 2014; Grimont and Weill 2007).

Salmonella serovars vary widely in their capacity to cause disease in different host species (Baumler and Fang 2013). Some are almost exclusively associated with one particular host species (for example, *S*. Typhi in humans), whereas others are capable of causing gastroenteritis in a broad range of unrelated host species. Following the nomenclature proposed by Uzzau et al. (2000), three broad groups of *Salmonella* host adaptation can be defined:

1. Host unrestricted (HU) – Ubiquitous: these serovars can cause limited enteric infections in a number of unrelated hosts (e.g., *Salmonella* Typhimurium).
2. Host restricted (HR) – Serovars, which are almost exclusively associated with a certain host species and can cause serious systemic infection within that host (e.g., *Salmonella* Typhi in humans).
3. Host adapted (HA) – Serovars which are prevalent in one particular host species but which can also cause disease in other host species (e.g., *Salmonella* Dublin in cattle).

Specific examples of these three groups of host adaptation are in Table 1. Host-restricted serovars have followed distinct evolutionary pathways based upon their specific pathogen-host interaction. This provides an opportunity to investigate these particular serovars to determine the specific mechanisms causing host specificity. However, these pathogens also tend to cause severe systemic (typhoidal) disease in their specific host. While this is a massive global burden in terms of human infections (Parry et al. 2002), typhoidal *Salmonella* infections tend to be considerably less frequent in developed countries: causing an estimated 1821 illnesses in the United States (Scallan et al. 2011) and 177 illnesses in England and Wales (Adak et al. 2005). Non-typhoidal *Salmonella* are responsible for most cases of salmonellosis in developed countries. Within this group, there are certain serovars (or strains within them) which have become host adapted. Investigating the subtle evolutionary divergence within serovars to have certain host predilections also allows an understanding of host adaptation. Both of these situations (HR and HA) will be addressed in this chapter, with an emphasis on the latter.

Table 1 Examples of host-restricted and host-adapted serovars of *Salmonella*

Serovar	Primary host	Other hosts	Reference
Host restricted (HR)			
Salmonella Abortusequi	Equine	–	Uzzau et al. (2000), Baumler et al. (1998), Madić et al. (1997)
Salmonella Abortusovis	Ovine	–	Uzzau et al. (2000), Baumler et al. (1998)
Salmonella Gallinarum/Pullorum	Avian	–	Barrow and Freitas Neto (2011)
Salmonella Paratyphi A/B/C	Human	–	Uzzau et al. (2000), Baumler et al. (1998)
Salmonella Sendai	Human	–	Uzzau et al. (2000), Baumler et al. (1998)
Salmonella Typhi	Human	–	Parry et al. (2002)
Salmonella Typhisuis	Porcine	–	Uzzau et al. (2000), Baumler et al. (1998); Barnes and Bergeland (1968)
Host adapted (HA)			
Salmonella Cholerasuis	Porcine	Human	Uzzau et al. (2000)
Salmonella Dublin	Bovine	Human Ovine	Uzzau et al. (2000), Rice et al. (1997)
Salmonella Typhimurium	Mice	Human	Edwards and Bruner (1943)
Salmonella Typhimurium DT2, DT 99	Pigeons		Rabsch et al. (2002), Pasmans et al. (2003)

Salmonella Epidemiology and Surveillance

As a crude measure of *Salmonella* host preference, it is possible to interrogate epidemiologic databases to identify which serovars are most frequently implicated in human disease. Many countries have national surveillance programs for *Salmonella*: in England and Wales these records date back to 1941 (McCoy 1975); Table 2 illustrates a snapshot of this data. The data reveals that in any given region, only a very small number of serovars are responsible for most cases of human illness. This suggests that these pathogens, by virtue of sheer numbers, are better adapted to causing disease in humans (or better at colonizing human food sources). In most countries, *S.* Typhimurium and *S.* Enteritidis are the most common serovars (Herikstad et al. 2002).

The alternative hypothesis, i.e., that the human prevalence of certain serovars in human disease is due to their ability to colonize the intestinal tract of animals used for human food; this hypothesis can also be tested by examining veterinary surveillance data. Data from England and Wales reveal that animal-adapted strains accounted for 71% of animal infections between 1958 and 1967, but only 1% of human infections (McCoy 1975). A summary of these data is shown in Table 3.

Table 2 Most frequently recovered serovars causing human salmonellosis by geography and years

	Australia			Canada	England/Wales			
Rank	1991–2000	2001–10	2011–15	2000–04	1941–48	1949–55	1956–63	1999–2000
1	Typhimurium (42.3%)	Typhimurim (41.1%)	Typhimurium (48.2%)	Typhimurium (20.6%)	Typhimurium (67.8%)	Typhimurium (82.7%)	Typhimurium (74.8%)	Enteritidis (59.4%)
2	Virchow (6.1%)	Virchow (5.5%)	Enteritidis (6.4%)	Enteritidis (17.4%)	Enteritidis (7.3%)	Enteritidis (3.8%)	Heidelberg (5.4%)	Typhimurium (15.7%)
3	Enteritidis (4.9%)	Enteritidis (5.2%)	Virchow (4.7%)	Heidelberg (16.0%)	Thompson (5.9%)	Thompson (3.6%)	Enteritidis (3.3%)	Hadar (2.7%)
4	Saintpaul (4.0%)	Saintpaul (4.6%)	Saintpaul (3.2%)	Hadar (3.9%)	Newport (5.8%)	Newport (2.1%)	Newport (2.9%)	Virchow (2.6%)
5	Birkenhead (3.2%)	Birkenhead (2.7%)	Paratyphi B Bv Java (2.2%)	Thompson (3.4%)	Oranienburg (3.5%)	Bovismorbificans (1.1%)	Thompson (2.6%)	Newport (1.0%)
6	Infantis (2.1%)	Infantis (2.1%)	Infantis (1.9%)	Newport (2.6%)	Montevideo (3.0%)	Dublin (0.9%)	Saintpaul (1.4%)	Infantis (0.9%)
7	Bovismorbificans (1.9%)	Chester (2.1%)	Birkenhead (1.4%)	Agona (2.0%)	Dublin (1.7%)	Anatum (0.8%)	Bredeney (1.2%)	Montevideo (0.8%)
8	Chester (1.9%)	Paratyphi B Bv Java (1.7%)	Stanley (1.3%)	Typhi (1.9%)	Bovismorbificans (1.2%)	Stanley (0.8%)	Anatum (1.2%)	Agona (0.8%)
9	Muenchen (1.8%)	Muenchen (1.6%)	Chester (1.2%)	I 4, (Scallan et al. 2011),12:i:- (1.8%)	Anatum (1.0%)	Heidelberg (0.6%)	Stanley (1.0%)	Bredeney (0.6%)
10	–	–	–	Infantis (1.5%)	Meleagridis (0.8%)	Montevideo (0.6%)	Bovismorbificans (0.8%)	Oranienburg (0.4%)
Total	68.1%	66.6%	70.6%	71.1%	98.2%	97.0%	94.6%	84.9%
Ref	OzFoodNet Working Group (n.d.)	OzFoodNet Working Group (n.d.)	OzFoodNet Working Group (n.d.)	Public Health Agency of Canada (2009)	McCoy (1975)	McCoy (1975)	McCoy (1975)	BgVV-FAO/WHO Collaborating Centre for Research and Training in Food Hygiene and Zoonoses (2003)

(continued)

Table 2 (continued)

	Thailand	United States			Global
Rank	1993–2002	2005	2010	2012	1995
1	Weltevreden (12.5%)	Typhimurium (19.3%)	Enteritidis (19.2%)	Enteritidis (14.5%)	Enteritidis (66.7%)
2	Enteritidis (11.4%)	Enteritidis (18.6%)	Typhimurium[a] (13.2%)	Typhimurium[a] (11.6%)	Typhimurium (15.5%)
3	Anatum (7.4%)	Newport (9.1%)	Newport (10.9%)	Newport (10.4%)	Hadar (1.8%)
4	Derby (6.6%)	Heidelberg (5.3%)	Javiana (6.5%)	Javiana (5.9%)	Infantis (1.8%)
5	1, 4, 5, 12:i:-ssp.I (6.4%)	Javiana (3.7%)	I 4, (Scallan et al. 2011),12:i:- (2.6%)	I 4, (Scallan et al. 2011),12:i:- (3.6%)	Newport (1.4%)
6	Typhimurium (5.3%)	I 4, (Scallan et al. 2011),12:i:- (2.3%)	Heidelberg (2.4%)	Montevideo (2.5%)	Typhi (1.4%)
7	Rissen (5.3%)	Montevideo (2.2%)	Montevideo (2.3%)	Infantis(2.3%)	Agona (1.2%)
8	Stanley (3.8%)	Muenchen (2.0%)	Saintpaul (1.9%)	Muenchen (2.1%)	Virchow (1.2%)
9	Panama (3.3%)	Saintpaul (1.9%)	Muenchen (1.8%)	Heidelberg (2.0%)	Heidelberg (1.2%)
10	Agona (2.7%)	Braenderup (1.7%)	Infantis (1.8%)	Bareilly (1.8%)	Derby (0.7%)
Total	64.7%	66.1%	62.6%	56.7%	92.9%
Ref	Bangtrakulnonth et al. (2004)	Centers for Disease Control and Prevention (CDC) (2007)	Centers for Disease Control and Prevention (CDC) (2013)	Centers for Disease Control and Prevention (CDC) (2014)	Herikstad et al. (2002)

[a]Including Typhimurium var. 5-

Table 3 Most frequently recovered *Salmonella* serovars recovered from animals by geography and year

	Canada[a]			Great Britain (England, Scotland and Wales)						United States
	Bovine	Porcine	Chicken	Bovine		Porcine		Chicken		Chicken
Rank	2000–04	2000–04	2000–04	2009	2013	2009	2013	2009	2013	1985
1	Typhimurium var Copenhagen (29.2%)	Typhimurium var Copenhagen (32.4%)	Heidelberg (50.4%)	Dublin (67.5%)	Dublin (72.5%)	Typhimurium (71.6%)	Typhimurium (33.1%)	Senftenberg (22.7%)	Mbandaka (18.9%)	Kentucky (39.0%)
2	Typhimurium (29.0%)	Typhimurium (26.8%)	Kentucky (20.6%)	Typhimurium (8.5%)	Mbandaka (7.5%)	4,5,12:i:- (6.4%)	4,5,12:i:- (24.4%)	Livingstone (18.0%)	Senftenberg (16.3%)	Heidelberg (13.2%)
3	Kentucky (15.2%)	Derby (15.8%)	Typhimurium (5.9%)	Mbandaka (7.9%)	Typhimurium (5.0%)	Derby (5.4%)	4,12:i:- (18.9%)	Kedougou (14.1%)	Montevideo (15.5%)	Typhimurium (7.9%)
4	Muenster (7.2%)	Infantis (8.0%)	Hadar (4.5%)	Montevideo (3.9%)	Montevideo (3.3%)	Rissen (3.9%)	Derby (7.1%)	Mbandaka (12.3%)	Kedougou (12.6%)	Typhimurium var. 5- (3.9%)
5	Newport (6.7%)	Brandenburg (5.2%)	*S.* ssp. I:ROUGH-O:r:1,2 (3.7%)	Anatum (3.6%)	4,5,12:i:- (2.8%)	Kedougou (2.9%)	Kedougou (3.9%)	Typhimurium (5.2%)	13,23:i:- (10.9%)	4,5,12:i:2 (3.8%)
6	Heidelberg (3.1%)	Heidelberg (3.3%)	Thompson (3.7%)	4,5,12:i:- (2.7%)	Agama (1.7%)	Reading (2.0%)	Reading (3.9%)	Enteritidis (4.4%)	Ohio (5.0%)	Schwarzengrund (3.7%)
7	Thompson (2.9%)	Agona (2.7%)	Brandenburg (3.1%)	Newport (0.9%)	Anatum (1.2%)	Bovismor bificans (1.5%)	Bovismor bificans (1.6%)	Ohio (3.0%)	Orion (3.3%)	Montevideo (3.4%)
8	Montevideo (2.5%)	Mbandaka (2.5%)	Schwarzen grund (2.7%)	Ohio (0.7%)	Bovismorbificans (1.0%)	London (1.5%)	London (1.6%)	Montevideo (2.7%)	Livingstone (2.9%)	Ohio (3.3%)

Table 3 (continued)

	Canada[a]			Great Britain (England, Scotland and Wales)						United States
	Bovine	Porcine	Chicken	Bovine		Porcine		Chicken		Chicken
Rank	2000–04	2000–04	2000–04	2009	2013	2009	2013	2009	2013	1985
9	S. ssp. I:18:-:- (2.3%)	London (1.8%)	Enteritidis (2.7%)	Agama (0.4%)	4,12:i:- (0.7%)	–	–	–	–	Kiambu (3.2%)
10	Cerro (2.2%)	Bovismor bificans (1.7%)	Mbandaka (2.6%)	–	–	–	–	–	–	Betha (2.8%)
Total	100%	100%	100%	96.1%	95.7%	95.2%	94.5%	82.4%	85.4%	84.2%
Ref	Public Health Agency of Canada (2009)	Public Health Agency of Canada (2009)	Public Health Agency of Canada (2009)	Animal Health and Veterinary Laboratories Agency (2014)	Animal Health and Veterinary Laboratories Agency (2014)	Animal Health and Veterinary Laboratories Agency (2014)	Animal Health and Veterinary Laboratories Agency (2014)	Animal Health and Veterinary Laboratories Agency (2014)	Animal Health and Veterinary Laboratories Agency (2014)	Berrang et al. (2009)

[a]Canadian isolates reported as percentage of the top 10 most frequently occurring serovars, not all recorded serovars

Salmonella Human and Animal Studies

The question of whether all *Salmonella* serovars are equally pathogenic to humans has been asked since at least 1950. Human volunteer studies offer a unique opportunity to access the relative virulence of various serovars (Oscar 2004; Coleman and Marks 1998). Volunteer studies differ from retrospective analyses of food-borne outbreaks as matrix effects are considered. Food matrix can have a huge effect on the *Salmonella* infectious dose, particularly in high-fat foods like chocolate (Bean and Post 2014), which might mask differences in infectious dose between different serovars. Blaser and Newman comprehensively reviewed both outbreaks and human studies with respect to determining *Salmonella* infective dose (Blaser and Newman 1982). A summary of these volunteer trials is shown in Table 4.

Volunteer studies have been performed on *S.* Meleagridis and *S.* Anatum; serovars which were recovered frequently from eggs and egg products (McCullough and Eisele 1951a). Three strains of *S.* Meleagridis and *S.* Anatum were administered to 118 and 115 prison inmates, respectively. The minimum dose required to cause illness varied between 7.7×10^6 and 2.4×10^7 cells for *S.* Meleagridis and between 5.9×10^5 and 4.5×10^7 cells for *S.* Anatum. The level of infectivity appeared to be strain, rather than serovar, dependent. Similar studies have been performed on *S.* Bareilly, *S.* Derby and *S.* Newport: the minimum dose required to cause illness was 1.3×10^5, 1.5×10^7 and 1.5×10^5 cells, respectively (McCullough and Eisele 1951b). Therefore, for many host-unrestricted strains of *Salmonella*, the infectious doses appear similar in an infection model: between 5 and 7 log cells should be sufficient to cause disease. This contrasts markedly with an avian host-restricted serovar. The minimum dose of *S.* Pullorum causing illness in humans varied between 1.3×10^9 and 1.0×10^{10} cells (McCullough and Eisele 1951c). It has been speculated that the reduced infectivity of *S.* Pullorum in the human trial is due to human volunteers having received typhoid vaccination and there may be some protection due to antigenic similarity (McCullough 1951). Regardless, the minimum infective dose of *S.* Pullorum was 2–5 log values greater than any of the other serovars tested. *S.* Pullorum was less effective at causing disease in mice than any of the other five serovars tested (McCullough 1951).

Animal models are also used to study *Salmonella* infection. While primates provide the best models (Edsall et al. 1960), the cost and lack of availability of these animals precludes their use. Instead, the mouse and the calf are the two most successfully employed animal models (Santos et al. 2001). The murine model is used to investigate typhoid fever, but utilizes *S.* Typhimurium which produces in mice an illness similar to typhoid fever in humans. The bovine model is used to investigate *Salmonella*-induced enteritis which mimics human enteritis (Costa et al. 2012).

Salmonella In Vitro Studies

Salmonella pathogenesis has been described in Chap. 7 of this book and elsewhere (Salyers and Whitt 2002; Foley et al. 2013); hence, this section will focus on the molecular mechanisms of *Salmonella* pathogenesis that mediate host preference.

The genus *Salmonella* includes pathogens with a spectrum of host preferences (Uzzau et al. 2000). Here we consider the genetic basis of HA and HR pathogens separately, as both are particularly well-suited for studying the basis of host specificity. They are considered in isolation on the assumption that HR pathogens have undergone wholesale genome rearrangements, which have increased their virulence, while narrowing their host range (called antagonistic pleiotropy). HA pathogens, by contrast, may infect numerous hosts while showing a preference for one. This may represent the evolution of new emerging HR strains of *Salmonella.*

The disease process has been well-studied in *Salmonella* (Ohl and Miller 2001). After ingestion, the bacterium moves through the alimentary canal until it reaches the small intestine where it binds and invades intestinal epithelial cells. Two type-III secretion systems (T3SS) mediate cell invasion and survival in macrophages. In an HA or HU serovar such as *S.* Typhimurium, this elicits acute intestinal inflammation and the onset of gastroenteritis within 24 h. The ability of a given serovar to cause disease appears to be related to its ability to evade the host's mononuclear phagocytes, which are important barriers to host restriction (Baumler et al. 1998). In the HR serovar, *S.* Typhi, the innate immune response is evaded by virulence factors, including the production of a polysaccharide capsule (Haneda et al. 2009). Furthermore, *S.* Typhi survives chronically in the human macrophage allowing for dissemination in the host (Schwan et al. 2000). By evading the primary host defenses, *S.* Typhi can invade the bloodstream and cause systemic infection. Studying the mechanistic basis of host specialism within the same bacterial species provides insights into co-evolution and microbial pathogenesis (Baumler and Fang 2013).

Molecular Mechanisms of Host Specificity in HR *Salmonella*

The pan-genome concept describes the full complement of genes in a bacterial species (Rouli et al. 2015). The pan-genome includes the "core genome" containing genes present in all strains of the species, an "accessory genome," containing genes present in two or more strains, and "unique genes," specific to single strains. The pan-genome of *Salmonella enterica* was described by Jacobsen et al. in 2011 (Jacobsen et al. 2011). This was based on 35 publicly-available genome sequences and compares the genomes of ubiquitous and host-restricted *Salmonella* serovars. They described a core genome comprised of 2811 genes, whereas the pan genome contained 10,015 genes. This study also revealed that genes were conserved within each serovar, which includes a given number of unique gene families. It is not unreasonable then to assume these serovar-specfic genes may shape a particular serovar's host predilection.

Table 4 Results of selected human *Salmonella* feeding trials

Serovar	*n*	Dosage (cfu)							Lowest dose illness	Lowest dose shedding	Ref.
		10^4	10^5	10^6	10^7	10^8	10^9	10^{10}			
S. Anatum strain I	47	0/23 (0%)	5/24 (21%)	–	–	–	–	–	5.9×10^5	1.2×10^4	McCullough and Eisele (1951a)
S. Anatum strain II	50	0/6 (0%)	0/6 (0%)	0/12 (0%)	5/26 (19%)	–	–	–	4.5×10^7	8.9×10^5	McCullough and Eisele (1951a)
S. Anatum strain III	18	–	0/6 (0%)	6/12 (50%)	–	–	–	–	1.3×10^6	1.6×10^5	McCullough and Eisele, 91951a)
S. Bareilly	18	–	3/12 (25%)	4/6 (67%)	–	–	–	–	1.3×10^5	1.3×10^5	McCullough and Eisele (1951d)
S. Derby	30	–	0/12 (0%)	0/12 (0%)	3/6 (50%)	–	–	–	1.5×10^7	1.4×10^5	McCullough and Eisele (1951d)
S. Meleagridis strain I	64	0/24 (0%)	0/18 (0%)	0/12 (0%)	5/12 (42%)	–	–	–	2.4×10^7	1.2×10^4	McCullough and Eisele (1951a)
S. Meleagridis strain II	30	–	–	0/12 (0%)	8/18 (44%)	–	–	–	1.0×10^7	1.0×10^6	McCullough and Eisele (1951a)
S. Meleagridis strain III	24	–	0/6 (0%)	1/12 (8%)	2/6 (33%)	–	–	–	7.7×10^6	1.6×10^6	McCullough and Eisele (1951a)
S. Newport	20	–	2/14 (14%)	3/6 (50%)	–	–	–	–	1.5×10^5	1.5×10^5	McCullough and Eisele (1951d)
S. Pullorum strain I	60	0/48 (0%)						12/12 (100%)	1.0×10^{10}	1.0×10^{10}	McCullough and Eisele (1951c)
S. Pullorum strain II	?	–	–	(0%)	–	(0%)	4/5 (80%)	–	6.8×10^9	6.8×10^9	McCullough and Eisele (1951c)
S. Pullorum strain III	?	–	–	(0%)	(0%)	–	6/6 (100%)	–	7.6×10^9	7.6×10^9	McCullough and Eisele (1951c)
S. Pullorum strain IV	?	–	–	(0%)	(0%)	(0%)	5/12 (42%)	–	1.3×10^9	1.3×10^9	McCullough and Eisele (1951c)

The acquisition of pathogenicity is typically accompanied by the accrual of virulence genes, such as the acquisition of the *Salmonella* Pathogenicity Islands (SPI), which differentiate *Salmonella* from other Enterobacteriaceae (Baumler 1997; Groisman and Ochman 1997). Subsequent host restriction of *Salmonella* serovars (such as the human-restricted Typhi and Paratyphi A serovars) involves the decay of functional genes to produce a genome specifically adapted for an individual host species (Baumler and Fang 2013; Baumler et al. 1998). In Typhi and Paratyphi A, for example, >4% of the genome-coding sequences are pseudogenes (inactivated coding sequences), compared to 0.9% in *S.* Typhimurium (Holt et al. 2009). In the chicken-restricted *S.* Gallinarum, the proportion of pseudogenes is >7% (Thomson et al. 2008). The loss of genes can allow a pathogen to adapt to survive better in a particular host (Maurelli 2007; Feng et al. 2013). This is a pattern also observed in other host-restricted pathogens, not only *Salmonella* (Andersson and Andersson 1999; Cole et al. 2001). The transition of a microorganism toward host restriction tends to be accompanied by a change in disease presentation. For example, the human-restricted serovars Typhi and Paratyphi cause only systemic disease, rather than gastroenteritis as caused by HU *Salmonella* (McClelland et al. 2004).

If, then, it is well-accepted that genome degradation leads to increased host specificity among many pathogens, what genes are losing functionality and leading to host restriction? *S. serotype* Gallinarum, which appears to have recently diverged from *S. serotype* Enteritidis (albeit not the "classic" *S.* Enteritidis clade (Langridge et al. 2015)), genes from several metabolic pathways have been lost (Thomson et al. 2008). These include genes for the catabolism of maltodextrins (*malS*), (*D*)-glucarate (*gudD*) and 1,2-propanediol (*ttr*, *cbi* and *pdu* operons). *S.* Gallinarum is also nonmotile, hence the mutations in five genes, required for chemotaxis and motility (*cheM*, *flhA*, *flhB*, *flgK* and *flgI*). *S.* Gallinarum has also lost a number of T3SS effector proteins, most notably *sopA,* which is implicated in intestinal inflammation.

Other host-restricted serovars have also lost T3SS effector proteins, i.e., GtgE, present in *S.* Typhimurium but absent in *S.* Typhi and *S.* Paratyphi A, is important for survival in the mouse macrophage (Spano and Galan 2012). *S.* Typhi can survive in human macrophages, but is unable to survive inside the mouse macrophage (Schwan et al. 2000). When *S.* Typhi was constructed to express the *S.* Typhimurium *gtgE,* survival increased in the murine macrophage; the target of the GtgE effector was further elucidated by SiRNA-mediated silencing of host genes. Depletion of Rab32 significantly increased the ability of *S.* Typhi to survive in the mouse macrophage. Host restriction in primary macrophages suggests that this observation is critical to the narrow host range of *S.* Typhi (Spano 2014).

While genome degradation is one mechanism of restricting host range, genes may also evolve differentially in response to different hosts. In particular, SPI-encoded virulence genes can drive host preference. Eswarappa et al. identified six genes which likely evolved in response to host adaptation (Eswarappa et al. 2008). For example, T3SS translocon protein components of both SPI-1 (SipD) and SPI-2 (SseC and SseD) have evolved differently in different serovars of *Salmonella*. These

proteins come in direct contact with host membranes and therefore drive the evolution of these molecules in response to specific host adaptation.

Molecular Mechanisms of Host Specificity in HA *Salmonella*

Whole genome sequencing has provided insights in bacterial evolution and host adaptation. A study that compared the genomes of 373 isolates of *Salmonella* Typhimurium DT104 revealed that within this one phage type, there were distinct lineages infecting either animal or human populations. Moreover, crossover between these lineages was infrequent, but when it occurred, it was in both directions (Mather et al. 2013).

Host predilection may be due to surface-exposed molecules on the bacterium's cell surface, as attachment to the host mucosa is an essential first step in *Salmonella* pathogenicity (Linke et al. 2011; Humphries et al. 2001). Fimbriae are proteinaceous molecules extending from the cell surface and promoting bacterial colonization of the susceptible host. The FimH subunit, which represents the tip of the fimbriae, is responsible for the varying binding properties on the host cell. FimH is important in promoting host specificity and hence considerable diversity has been identified. One study identified 67 different FimH alleles, resulting in amino acid substitutions among a collection of *Salmonella enterica* subsp. 1 (Yue et al. 2012). Another study of nonsynonymous single-nucleotide polymorphism in *Salmonella* Typhimurium examined the *fimH* gene from 580 strains and revealed 17 variants, including one with broad-host range, *fimH1*, and the second most common allele, *fimH7*, associated with bovine isolates (Yue et al. 2015). The two alleles differ from each other by a single amino acid substitution at position 223, substituting a valine for an alanine. The role of the alanine in bovine-specific adhesion is elucidated by mutagenesis of *S.* Typhi FimH with a V223A substitution. The mutants had decreased adhesion to human cells and increased adhesion to bovine cell lines (Yue et al. 2015). Further analysis of 1848 *fimH* genes from 76 different *Salmonella* serovars revealed 105 different FimH proteins, many with specific host predilections.

Listeria Species

Listeria is a genus comprised at least 15 species, of which five were recognized as recently as 2014 (den Bakker et al. 2014). Of these species, only two are recognized as mammalian pathogens: *Listeria monocytogenes and L. ivanovii*. *L. monocytogenes* is estimated to cause 1600 illnesses, 1400 hospitalizations and 250 deaths each year in the United States (Scallan et al. 2011). Although a relatively uncommon disease, it can cause particularly severe consequences. Invasive infections are often fatal and mortality associated with foodborne outbreaks can be high (Cosgrove et al. 2011; Linnan et al. 1988). Risk groups include pregnant women, infants and

the elderly (Schlech 2000). *L. ivanovii*, by contrast, are predominantly in ruminants, particularly sheep, but has caused documented cases of human disease on rare occasions (Snapir et al. 2006; Guillet et al. 2010).

Listeria Epidemiology and Surveillance

There are currently 13 described *L. monocytogenes* serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (Seeliger and Jones 1986). Hence, the value of *L. monocytogenes* serotyping is relatively low for epidemiologic purposes. However, of these 13 serotypes, 1/2a, 1/2b, and 4b comprise the majority of the strains associated with human listeriosis outbreaks, as shown in Table 5.

Phylogenetic analysis of *L. monocytogenes* isolates have divided the species into four evolutionary lineages (the history of these lineages has been reviewed by Orsi et al. (2011)). Most studies reveal a higher prevalence of lineage I isolates (mainly serotype 4b) among human listeriosis cases (Orsi et al. 2011; Jeffers et al. 2001). This increased burden of disease caused by lineage I does not correlate to a higher abundance of lineage I in food, which is more commonly associated with lineage II isolates (Gray et al. 2004; De Cesare et al. 2007). Therefore it seems likely that lineage I isolates, or at least those of serotype 4b, have a higher pathogenicity in comparison to isolates of lineage II (e.g. serotype 1/2a, 1/2c) (Pinner et al. 1992). Virulence genes appear to have evolved along two distinct pathways correlating to the phylogenetic lineage also (Orsi et al. 2008).

Listeria Human and Animal Studies

Human feeding trials of *L. monocytogenes* carry the potential for fatal outcomes among susceptible individuals and consequently the infectious dose has not been experimentally determined. The infectious dose is assumed to be high (Vazquez-Boland et al. 2001) and has been estimated to be 10–100 million cells in healthy hosts, and 0.1–10 million cells in individuals at high risk of infection (Farber et al. 1996). However, data from outbreak investigations suggest that contamination levels as low as 10^2–10^4 cells per gram of food might be sufficient to cause listeriosis (Farber and Peterkin 1991).

Animal surrogates are used for modeling *L. monocytogenes* pathogenicity and virulence. Indeed, the mouse model of *Listeria* infection has proved to be a paradigm for immunological studies (Pamer 2004), as well as the study of intracellular parasitism (Hamon et al. 2006; Cossart 2011). As well as mice (Takeuchi et al. 2006), other animal models of listeriosis have included gerbils (Roulo et al. 2014), guinea pigs (Williams et al. 2007), rabbits (Osebold and Inouye 1954a), sheep (Osebold and Inouye 1954b) goats (Miettinen et al. 1990) and non-human primates (Farber et al. 1991; Smith et al. 2003). Some species specific differences have been observed. For example, mice and rats tend to be less susceptible to *Listeria* infection

Table 5 Selected examples of *Listeria* outbreaks in which the serovar of the infecting strain was reported

Outbreak	Year	Vehicle	Infections	Deaths[a] (%)	Serovar	Ref
US	1983	Pasteurized milk	49	14 (29%)	4b	Fleming et al. (1985)
Switzerland	1983–84	Soft cheese	57	18 (32%)	4b	Bula et al. (1995)
US	1985	Soft cheese	142	48 (34%)	4b	Linnan et al. (1988)
France	1993	Rillettes	38	10 (26%)	4b	Goulet et al. (1998)
US	1999	Meat	108	14 (13%)	4b	Mead et al. (2006)
Italy	2000	Corn	1566	0	4b	Aureli et al. (2000)
US	2002	Turkey deli meat	54	11 (20%)	4b	Gottlieb et al. (2006)
Canada	2008	Deli meat	57	24 (42%)	–	Currie et al. (2015)
Germany, Austria	2009	Quargel	34	8 (24%)	1/2a	Fretz et al. (2010a, b)
US	2011	Cantaloupe	84	15 (18%)	1/2a and 1/2b	Cosgrove et al. (2011)

[a]Including fetal and neonatal deaths

than humans (possibly due to differences in E-cadherin, explained in Section "Listeria In Vitro studies", below). Guinea pigs are a good model for human infection (Williams et al. 2007), whereas Mongolian gerbils are less susceptible than both Guinea pigs and non-human primates (Roulo et al. 2014).

The primary motivation for conducting these studies is to determine the number of *L. monocytogenes* that must be ingested to cause illness. However, a study using multiple *L. monocytogenes* strains in infection model is used to infer data about the relative virulence of different isolates for their respective hosts. This revealed that in an immunocompromised mouse model, the LD_{50} did not vary significantly after 3 or 5 days after testing ten different *L. monocytogenes* strains (including multiple serovars) by intraperitoneal injection, (Takeuchi et al. 2006).

Listeria In Vitro Studies

The genome of *L. monocytogenes* is highly conserved. Comparison of 21 genomes revealed genome sizes ranging from 2.74 Mb to 3.14 Mb (den Bakker et al. 2013). Analysis of 10 completely-sequenced genomes revealed a core genome of 2438 genes present in all genomes. The accessory genome ranged from 323 to 753 genes depending on the strain.

Listeria pathogenesis is described in Chap. 4 of this book and in a number of excellent reviews elsewhere (Vazquez-Boland et al. 2001; Farber and Peterkin 1991; Kathariou 2002). At a molecular level, some of the details of *Listeria* host specificity are elucidated. The best-described determinants of host specificity are the internalin family of proteins, specifically InlA and InlB, which mediate *L. monocytogenes* entry into eukaryotic cells (Ireton and Cossart 1997). InlA is required for the crossing of the intestinal and placental barriers and is mediated by the interaction with the E-cadherin receptor. This interaction is species-specific and occurs between InlA and human E-cadherin or guinea pig E-cadherin, but not with mouse or rat E-cadherin (Lecuit et al. 1999). This specificity is mapped to a proline residue at position 16 in the human E-cadherin receptor. In mice, this residue is glutamic acid and prevents mouse E-cadherin from interacting with InlA (Lecuit et al. 1999). Transgenic mice have been created expressing human E-cadherin which are susceptible to orally administered *Listeria* (Lecuit et al. 2001; Lecuit and Cossart 2002). Moreover, an engineered form of the InlA protein has been created in *L. monocytogenes,* which interacts with murine E-cadherin and thus provides a useful murine model of human listeriosis (Wollert et al. 2007).

InlB recognizes three cell surface molecules: (1) Met, the hepatocyte growth factor (HGF) receptor; (2) gC1qR, a ubiquitous protein receptor with binds C1q (the first component of the complement cascade) and a number of other biologically important ligands; and (3) glycosaminoglycans. InlB is not involved in *Listeria* infection in guinea pig or rabbit models. However, it plays a role in liver and spleen colonization in the mouse model, although it is not involved in crossing the intestinal barrier (Khelef et al. 2006).

The other pathogenic *Listeria* species, *L. ivanovii*, also has internalin genes (Vazquez-Boland et al. 2001). The genome of *L. ivanovii* was sequenced in 2011 and includes paralogs of both InlA and InlB (Buchrieser et al. 2011). The sequenced strain, from an outbreak of abortion in sheep, contained genes encoding a capsule, which may be responsible for the host specificity of this strain. In addition to this, a *L. ivanovii* species-specific pathogenicity island has also been identified, i.e., LIPI-2 (Dominguez-Bernal et al. 2006). LIPI-2 contains a number of *L. ivanovii*-specific virulence factors, including the *smcL* gene encoding sphingomyelinase C, which is involved in disruption of the phagocytic vacuole (Gonzalez-Zorn et al. 1999). Another interesting feature of the *L. ivanovii* genome is that it encodes a large number of pseudogenes (86; ~3.1%) (Buchrieser et al. 2011). As in the host-restricted *Salmonella* serovars, this suggests genome decay leading to better host adaptation, which, in this case, is ruminants.

Escherichia coli

Escherichia coli is a common inhabitant of the intestines of warm-blooded animals and is a part of a healthy human intestinal tract (Tap et al. 2009). The pangenome of *E. coli* is estimated to contain a reservoir of up to 13,000 genes, of which 2200

genes are core (Ron 2006) resulting in a huge amount of genetic and pathogenic variability within the species. *E. coli* has long been considered a harmless commensal microorganism of humans, until 1945 when Bray discovered the pathogenic *E. coli* as the cause of infantile gastroenteritis (Finlay 1973; Bray 1945). Since then, several pathogenic variants (pathovars) of *E. coli* have been identified as shown in Table 6 (Kaper et al. 2004; Croxen and Finlay 2010). Seven pathovars are associated with diarrheal illnesses transmitted through water or food, or through contact with animals or persons. These are: Enteropathogenic *E. coli* (EPEC), Shiga toxin-producing *E. coli* (STEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC), Diffusely adherent *E. coli* (DAEC) and Adherent invasive *E. coli* (AIEC) (Croxen et al. 2013). These subtypes of *E. coli* have acquired genes that code for attachment, cell damage and/or toxin production, which differentiate them from the benign types commonly found in the intestine.

Host predilection is difficult to define in *Escherichia coli.* The various pathovars shown in Table 6 have each developed intricate mechanisms to subvert the cellular processes of the human cell, and consequently are defined by the diseases they cause in humans. In that sense, all the intestinal pathovars are considered human-adapted. An exception to this are the STECs, which have an animal reservoir (Lowe et al. 2009; Blanco et al. 2004). Given that STECs have this specific host predilection, they will be the focus of this section.

Shiga toxin-producing *E. coli* (STEC), also referred to synonymously as Verocytotoxin-producing *E. coli* (VTEC), are the most widely described *E. coli* pathotype causing foodborne illness (Coia 1998). Enterohemorrhagic *E. coli* (EHEC) is defined as the subset of STEC that are pathogenic to humans, typically carrying the *eae* (intimin) gene (Tarr et al. 2005). *E. coli* O157:H7 and other Shiga toxin-producing *E. coli* are major public health concerns in both North America and Europe, and increasingly in other areas of the world. Although absolute numbers of infections are low in comparison with other enteric pathogens such as *Salmonella* or *Campylobacter*, it is well-recognized that STEC can produce severe, potentially life-threatening illness (Coia 1998).

Escherichia coli Epidemiology and Surveillance

Initially, the STEC of primary concern and the focus of previous surveillance activities globally was *E. coli* O157:H7, first implicated in foodborne illness in the U.S. in 1982 (Riley et al. 1983; Wells et al. 1983). Humans do not usually carry this strain (although asymptomatic carriage has been documented (Hong et al. 2009)); its main reservoir is the intestines of healthy cattle in which it does not usually produce disease (Lowe et al. 2009; Blanco et al. 2004). STEC causes a diarrheal illness, often accompanied by bloody stools. Although most healthy adults can recover completely within a week, approximately 5–10% of infected people will develop hemolytic uremic syndrome (HUS) (Thorpe 2004). STEC-associated HUS is most likely

to occur in young children under 5 years of age. The condition can lead to serious kidney damage and, even with prompt treatment, has a fatality rate of 3–5% (Noris and Remuzzi 2005).

E. coli O157:H7 or other STEC strains infect humans through consumption of food or water contaminated by feces from infected animals. Major outbreaks of *E. coli* O157:H7 illness have been associated with ground beef (King et al. 2009, 2014; Greenland et al. 2009; Nowicki et al. 2010), leafy greens and vegetables (Launders et al. 2016, 2013; Wendel et al. 2009; Grant et al. 2008; Friesema et al. 2008), unpasteurized apple juice and cider (Hilborn et al. 2000), dairy products (McCollum et al. 2012), salami (Williams et al. 2000; MacDonald et al. 2004) and untreated water (Olsen et al. 2002; Licence et al. 2001). Meat is contaminated during slaughter or post-process from the hide or feces (Elder et al. 2000; McEvoy et al. 2003). Contamination of produce often occurs in the field through water run-off or contaminated water supplies used for washing the produce or irrigation (Jung et al. 2014). Produce can also be readily contaminated during preparation after handling raw meat (Wachtel et al. 2003).

While *E. coli* O157:H7 was the first described STEC, and certainly remains the most prevalent, over 400 different STEC serovars have been associated with human disease (Scheutz and Strockbine 2005). Non-O157 STEC infections are becoming increasingly more common worldwide. Table 7 shows some of the more frequently-recovered non-O157 STEC serogroups from different regions of the world.

Table 6 Described pathovars of *Escherichia coli*

E. coli pathovar	Condition/Disease	Ref.
Diarrhoeagenic pathovars		
Enteropathogenic *E. coli* (EPEC)	Profuse watery diarrhea	Croxen et al. (2013); Ochoa and Contreras (2011)
Shiga toxin-producing *E. coli* (STEC)	Watery diarrhea, hemorrhagic colitis, HUS	Croxen et al. (2013)
Enterotoxigenic *E. coli* (ETEC)	Watery diarrhea	
Enteroaggregative *E. coli* (EAEC)	Traveller's diarrhea, HUS (adults) Persistent diarrhea (children)	Croxen et al. (2013)
Enteroinvasive *E. coli* (EIEC)	Watery diarrhea, mild dysentery	Croxen et al. (2013)
Diffusely adherent *E. coli* (DAEC)	Persistent watery diarrhea (children) (?) Crohn's disease (adults)	Croxen et al. (2013)
Adherent invasive *E. coli* (AIEC)	Crohn's disease	Croxen et al. (2013)
Extraintestinal pathovars (ExPEC)		
Uropathogenic *E. coli* (UPEC)	Urinary tract infections	Croxen and Finlay (2010)
Neonatal meningitis *E. coli* (NMEC)	Meningitis (newborns)	Croxen and Finlay (2010)

The increasing incidence of non-O157 STEC infections is attributable, in part, to an increase in the number of laboratories testing for Shiga toxin-producing isolates and consequently an increased recognition of non-O157 STEC infections. The outcome of this increased surveillance is a large body of evidence that non-O157 STEC infections are on the increase. Between 1999 and 2000, 46 outbreaks of *E coli* O157:H7 disease were investigated in Canada. The National Laboratory for Enteric Pathogens (NLEP) identified more than 100 non-O157 STEC serotypes from cattle and meat products. At least 23 STEC serotypes from humans were identical to those found in cattle and meat products (Woodward et al. 2002). In the U.S. in 2013, 6% of all foodborne disease outbreaks were attributed to STECs, of which 26 (90%) were caused by serogroup O157, two (7%) by O26, and one (3%) by O111 (Centers for Disease Control and Prevention (CDC) 2015).

An increase in non-O157 STEC infections is coupled with a concomitant decrease in the incidence of O157 STEC infections. The Foodborne Diseases Active Surveillance Network (FoodNet), a collaboration between the CDC, ten state health departments, the US Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) and the US Food and Drug Administration (FDA) monitors the incidence of laboratory-confirmed infections caused by nine pathogens transmitted commonly through food in ten U.S. geographic areas. FoodNet reported that the incidence of STEC O157 declined in 2014 compared with infection rates in 2006–2008 and in 2011–2013. However, the incidence of STEC non-O157 was significantly higher in 2014 (Crim et al. 2015).

The USDA-FSIS designated *E. coli* O157 (including O157:H7) an adulterant in raw ground beef, raw, non-intact beef products and raw beef that will be made into raw, non-intact beef products (USDA-FSIS (United States Department of Agriculture Food Safety and Inspection Service) 1994). Until August 2010, no definitive outbreaks or recalls were associated with non-O157:H7 STEC-contaminated beef in the U.S. However, in August, 2010, approximately 8500 lbs of ground beef products contaminated with *E. coli* O26 were linked to illness (Robbins et al. 2014). Following on this occurrence, surveillance programs were broadened to include other serogroups of STECs, referred to as non-O157 STECs: O26, O45, O103, O111, O121, O145 ("the big six"). In 2012, USDA-FSIS began verification testing for these STEC's in addition to *E. coli* O157:H7. Raw, non-intact beef products or the components of these products contaminated with "the big six" or *E. coli* O157:H7 were considered to be adulterated (USDA-FSIS (United States Department of Agriculture Food Safety and Inspection Service) 2012).

Non-O157 serogroups of STEC are clearly significant in terms of worldwide public health, given the STEC O104:H4 outbreak in Germany and France in 2011 (King et al. 2012; Muniesa et al. 2012) and the multitude of studies revealing that the number of non-O157 STEC infections can nearly equal or sometimes surpass the number of O157 STEC infections (Wylie et al. 2012). It is equally clear there is a large ruminant reservoir of STECs and they can eventually enter the food chain. The question that remains is whether all STECs are equally pathogenic for humans (Acheson 2000).

Table 7 Most commonly reported non-O157 STEC serogroups among clinical isolates

Region	Year	No. STEC[a]	Most commonly reported non-O157 STEC serogroups (% total STEC)	Total % non-O157 STEC serogroups	Ref.
Europe					
E.U.	2007–10	9368	O26 (8.0%), O103 (3.6%), O91 (2.1%), O145 (2.0%), O111 (1.4%), O146 (1.3%)	29.6%	EFSA Panel on Biological Hazards (BIOHAZ) (2013)
E.U.	2011	4499	O104 (23.6%), O26 (6.4%), O103 (3.1%), O91 (2.6%), O145 (1.7%), O128 (1.2%)	51.4%	EFSA Panel on Biological Hazards (BIOHAZ) (2013)
Norway	1992–2012	333	O103 (15.0%), O26 (10.2%), O145 (7.2%), O91 (3.9%), O117 (3.3%), O121 (2.1%)	66.4%	Brandal et al. (2015)
North America					
British Columbia	2009–11	260	O26 (6.5%), O121 (3.7%), O103 (2.8%), O111 (2.8%), O118 (0.9%)	30.0%	Wang et al. (2013)
Canada	1998–2012	?	O26, O121, O103, O111, O145, O117	?	Catford et al. (2014)
Michigan	2001–12	1497	O45 (11.4%), O103 (5.5%), O26 (4.7%), O111 (2.6%), O145 (1.8%), O121 (0.9%)	30.2%	Tseng et al. (2016)
U.S.	2000–10	7694	O26 (6.8%), O103 (5.7%), O111 (5.0%), O121 (1.6%), O45 (1.3%), O145 (1.0%)	26.1%	Gould et al. (2013)

[a]Number of isolates typed (excludes unspecified serotypes)

Escherichia coli Human and Animal Studies

Cattle are a reservoir for both O157:H7 and non-O157 STEC, which can cause severe disease in humans. Shiga toxin-producing *E. coli* can cause a mild diarrheal illness in calves, but shedding decreases as the animal matures (Mir et al. 2016). Shedding of STEC by adult cattle is episodic and generally greater during the warmer months of the year (Hussein 2007; Hussein and Sakuma 2005).

Reports from around the world on the prevalence of O157:H7 STEC in beef cattle, as indicated by examination of fecal samples from cattle presented for slaughter, revealed rates ranging from 0.2% to 27.8% (Hussein and Bollinger 2005). The rates for non-O157 STEC prevalence ranged from 2.1% to 70.1% (Hussein and Bollinger 2005). Results obtained from studies conducted in the USA and Canada were comparable to those obtained in other regions of the world, with 0.3–27.8% of samples being positive for O157:H7 STEC in 13 studies, and 3.6–19.4% of samples being positive for non-O157 STEC in 4 studies (Hussein and Bollinger 2005). The wide ranges of reported prevalence's is very likely a consequence of not only differences in climate, ecology, and farming practices in different regions, but also of variation in the sampling and testing protocols used in different studies.

Studies have revealed that new STEC types are emerging from animal reservoirs. For example, O178 STEC (a recently described O-serogroup; not included in Table 7) is increasingly isolated from cattle in Europe and South America (Miko et al. 2014). The primary serovar is O178:H19, which, despite its prevalence in cattle, is relatively infrequently associated with human disease. Genetic analysis revealed that many of the STEC O178:H19 strains lacked virulence properties often associated with human outbreaks, including LEE encoded genes (Miko et al. 2014).

In addition to cattle and food animals, STECs have been recovered from a number of different domestic animals (e.g., cats (Beutin et al. 1993), dogs (Beutin et al. 1993) and rabbits (Garcia and Fox 2003)) as well as wild animal species (e.g., wild boar (Miko et al. 2009), wild deer (Miko et al. 2014; 2009), wild hare (Miko et al. 2009) and wild birds (Foster et al. 2006; Nielsen et al. 2004; Wallace et al. 1997)). Genetic analysis of STEC-related virulence genes in fecal samples have been performed on a variety of animal species (Chandran and Mazumder 2013). This reveals the ability of STECs to colonize a variety of animals and further investigation will undoubtedly reveal adaptions of these colonizing strains to their respective hosts.

Escherichia coli In Vitro Studies

Pathogenic *E. coli* strains can cause numerous intestinal or extraintestinal infections. They depend on complex molecular interactions with their natural hosts to determine host specificity; however, complete information on the molecular mechanisms of bacterial host specificity is lacking.

Phylogenetic analysis of *E. coli* has been well reviewed by Chaudhuri and Henderson (Chaudhuri and Henderson 2012) and provides a useful method to describe *E. coli* host predilection independent of pathogenicity functions. A standard culture collection (the *E. coli* Reference Collection or ECOR) of 72 *E. coli* strains representing maximum genotypic diversity from a collection of 2600 isolates was determined by multilocus enzyme electrophoresis (MLEE) (Ochman and Selander 1984). This collection has been extensively studied and analysed again by MLEE using 38 enzymes, which divided the collection into four phylogenetic groups, A, B1, B2 and D (Herzer et al. 1990). To simplify the identification of an

isolate's phylogenetic group, a simple PCR assay was developed based on three genetic markers (Clermont et al. 2000).

These phylogroups differ in their ecological niches (Gordon and Cowling 2003; Jang et al. 2014). Extraintestinal pathogenic strains usually belong to groups B2 and to a lesser extent D (Picard et al. 1999; Johnson and Stell 2000), whereas the avirulent commensal strains belong to groups A and B1 (Herzer et al. 1990; Picard et al. 1999), but the intestinal pathogenic strains belong to all phylogenetic groups (Pupo et al. 1997). The scheme has been updated several times to include seven (A_0, A_1, B1, $B2_2$, $B2_3$, D_1, D_2) (Carlos et al. 2010) and eight (A, B1, B2, C, D, E, F and D_2) phylogroups to increase the discrimination power of *E. coli* population analyses (Clermont et al. 2013). However, until now, only one putative human-specific strain (Clermont et al. 2008) and one putative animal-specific strain (B1 phylogroup) have been found (Escobar-Paramo et al. 2006).

STECs can be classified into phylogenetic groups B1, A and D (Ziebell et al. 2008). A survey of *E. coli* from animals in Minnesota and Wisconsin revealed that all STEC were of phylogenetic group B1 (although all were negative for the *eae* gene) (Ishii et al. 2007). Several studies have revealed that phylogenetic group B1 contains the highest proportion of STECs (Ziebell et al. 2008; Contreras et al. 2011). Other studies, however, have revealed that phylogenetic group D contains the greatest number of EHEC (Pupo et al. 1997) and STEC (Jang et al. 2014) strains. There is evidence to suggest that the different prevalences of the various *E. coli* phylogenetic groups is due to geographic and climatic differences from where the samples were obtained (Gordon and Cowling 2003; Unno et al. 2009).

Attachment of a pathogen is a first step in the disease process (Pan et al. 2014). The pili of pathogenic *E. coli* mediate bacterial adherence to mucosal epithelia in a host-specific manner. *E. coli* strains from human and domestic animals/birds often display host specificity (Ron 2006). The host specificity of pathogenic *E. coli* is influenced by the molecular interactions of bacterial surface-exposed proteins with host cellular receptors. Septicemic *E. coli* strains from chickens and lambs express host-specific adhesins, such as avian-specific AC/I pili and lamb-specific K99 fimbriae, respectively (Pan et al. 2014).

Certain surface-associated or secreted molecules contribute to host specificity by promoting bacterial colonization and/or dissemination (Fabich et al. 2008). However, STEC are noninvasive enteric pathogens of humans. To cause disease, STEC must be ingested, survive the acidic environment of the upper gastrointestinal tract, and colonize the lower GI tract. The mechanism by which colonization of the lower GI tract occurs is mediated by the locus of enterocyte effacement (LEE) pathogenicity island (Law 2000). Colonization by STEC that do not contain the LEE region is not well understood (Thorpe 2004). Most of the STEC associated with outbreaks, including *E. coli* serotypes O157, O26, and O111, harbor this pathogenicity island. The LEE region encodes genes involved in stimulating intestinal epithelial cells to build a pedestal on the cell surface to which the bacteria intimately adhere (Thorpe 2004).

STECs have many virulence factors, including adhesins (e.g., intimin), effector proteins (e.g., *espG*), and toxins which are necessary to cause disease and likely

contribute to the strain's extremely low infectious dose (Fabich et al. 2008). The principal virulence factors associated with severe STEC infection are the Shiga toxins (Tarr et al. 2005). Shiga toxins are AB5 toxins, consisting of an enzymatically active A subunit covalently associated with a pentameric B subunit that mediates binding to host cells. Shiga toxins bind to host cells that bear appropriate receptors: neutral glycolipids bearing a terminal galactose-a1, 4-galactose moiety (Obata and Obrig 2014). The principal receptor for both Shiga toxin 1 and Shiga toxin 2 is globotriaosylceramide, or Gb3, but there may also be other important receptors (Cooling et al. 1998).

Once established in the gut, pathogenic *E. coli* pathotypes must compete with the resident microbiota to acquire the nutrients needed to grow from low to high numbers in the GI tract. Carbon and energy source metabolism is considered to be essential during the early stages of many bacterial infections. In one study in which mice intestines were colonized by both an EHEC pathotype and a commensal *E. coli* strain, the pathogenic strain used sugars that were not used by a commensal *E. coli* strain (Fabich et al. 2008). The ability of the pathogenic strain to use less abundant nutrients suggested a strategy whereby the invading pathogen gained an advantage by simultaneously consuming several sugars that were available because they were not consumed by the commensal intestinal microbiota (Fabich et al. 2008).

Other Pathogens Which May Have Host Predilection

Cronobacter sakazakii **(formerly** ***Enterobacter sakazakii*****)** is a member of the Enterobacteriaceae family. *Cronobacter sakazakii* infections are rare but they are frequently lethal for infants, causing sepsis or severe meningitis. Some infants may experience seizures. Those with meningitis may develop brain abscesses or infarcts, hydrocephalus, or other serious complications that can cause long-term neurological problems. The mortality rate for *Cronobacter* meningitis may be as high as 40%. Does *C. sakazakii* have a predilection toward infants, or is this simply a consequence of the vehicle of infection (powdered milk) being consumed more frequently by infants?

Cronobacter infection is rare. CDC typically receives reports of 4–6 infections in infants per year, although reporting is not required except in one state, Minnesota. As a result, rates of *Cronobacter* infection in the United States are not well understood. However, a national FoodNet survey in 2002 estimated the annual incidence of invasive *Cronobacter* infection at one per 100,000 infants aged <1 year and at 8.7 per 100,000 low birth weight infants (<2500 g [5.5 lbs]). Additionally, more recent, unpublished FoodNet data suggest that infections among older adults are more common, although milder, than infections among infants.

Cronobacter infections have been reported in all age groups. However, infants, the elderly, and people with immunocompromising conditions appear to be at greatest risk of *Cronobacter* infection and can experience more severe symptoms. Meningitis due to *Cronobacter* occurs almost exclusively among infants in the first 2 months of life.

Most neonatal meningitis cases are caused by one gene-based sequence type of *C. sakazakii*. MLST analysis of isolates collected over 30 years and across six countries have been associated largely with one genetic lineage, Sequence Type 4 (Joseph and Forsythe 2011). Therefore, this clone appears to be of greatest concern with infant infections. There are no epidemiologic data revealing the infectious dose of *C. sakazakii*; however, Iverson and Forsythe in their reviewed article assumed that ca. 1000 cells are required to cause infection in infants (Iversen and Forsythe 2003).

Campylobacter is estimated to cause more than 1.3 million illnesses, 13,000 hospitalizations and 100 deaths each year in the United States (Scallan et al. 2011). *Campylobacter* is a leading cause of gastroenteritis in both developed and developing countries (Kaakoush et al. 2015).

C. jejuni volunteer ingestion studies have revealed that low numbers of cells (500–800 CFU) are sufficient to cause disease (Black et al. 1988; Robinson 1981). Ingestion of two strains of *C. jejuni* by 111 healthy adult volunteers resulted in short-term immunity to *C. jejuni*. While there were no host-specific differences noted, subjects that were re-challenged did not develop illness whereas the control group did (Black et al. 1988). This suggests that hosts that are chronically exposed to *C. jejuni*, such as in developing countries, may develop immunity toward circulating strains – rather than a difference in host specificity.

Conclusion

Pathogenic bacteria display various levels of host specificity, from so-called "generalists" (e.g., *Salmonella* Typhimurium) to "specialists" (e.g., *Salmonella* Typhi). The former can infect a wide range of hosts, whereas the latter have strict host selectivity and are obligate pathogens. It is clear that the host specificity of bacterial pathogens is determined by multiple molecular interactions between both the pathogens and their hosts – in most cases a complex interplay that is only beginning to be elucidated. Understanding the genetic and molecular basis of host specificity in bacteria is important for understanding pathogenic mechanisms, developing better animal models, and designing new strategies and therapeutics for the control of microbial diseases.

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The Influence of Virulence Factors on Dose Response of Food-Borne Pathogens

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Abstract Considerable effort has gone into generating mathematical models for use in the assessment of risk of specific foodborne pathogens present in various foodstuffs. The usefulness of such models, especially to the food industry and regulators, is subject to generalizations made and assumptions used, by modellers. In particular, accuracy of these models is highly dependent on the human dose response data incorporated. For many pathogens, these data are sparse and (when available) are widely accepted as often inaccurate. The sweeping assumption made in all such models, that all strains of the same pathogen species are equally virulent and able to cause disease in humans, is incorrect. In this review, we speculate that some of the major bacterial foodborne pathogens (e.g., *Salmonella*, *Campylobacter* and Shiga toxin-producing *Escherichia coli*) substantially vary between strains, in their ability to (1) survive in and respond to the environment from the point of detection of the pathogen load in food, (2) colonize the human gut and (3) generate a disease outcome which will be observed as a dose response. There is increasing evidence for these speculations, and we propose that this variation could have a major impact on the dose response curves currently used for microbiological risk assessments. We discuss the importance of mechanisms that facilitate the survival of pathogens as they transit from farm-to-fork and then into the host. We also comment on the

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impact of genetic polymorphisms and how they impact the widely differing infectious doses observed with many pathogens. In conclusion, we suggest that mathematical models should be modified to incorporate more realistic dose response data, based on known knowledge of strain variation in environmental survival, colonization and clinical impact. Such modification would more accurately inform the food industry and regulators.

Keywords Dose response • Pathogen • Virulence factors • *Campylobacter* • *Salmonella* • *E. coli* • *In vivo* • *In vitro* • Environmental stresses

Introduction

The established view of infection is that for each individual pathogen there is a direct relationship between the number of organisms to which the host is exposed and the risk of disease presentation in that host. This dose response relationship is usually presented as a sigmoidal curve indicating that there is a minimum number of organisms needed to cause disease and that the proportion of hosts with disease symptoms increases with increasing numbers of organisms until all susceptible hosts are ill (Buchanan et al. 2000; Haas 2015).

Because many raw foods can be contaminated with infectious microorganisms (including bacteria, viruses, molds or parasites) that are potentially pathogenic, this dose response curve is an essential component of any assessment of the microbiological risk to consumers (Teunis and Havelaar 2000). Thus, defining the dose response of any prospective pathogen is of primary importance to the food industry and its regulators. In some cases "nil tolerance" (zero tolerance) of a specific pathogen may be unfeasible and in such cases the minimum number of organisms that can be tolerated in a foodstuff has to be carefully defined, balancing the risk of illness with economic and practical considerations (see chapters "In Defense of the European 100 CFU of *Listeria monocytogenes*/g in Ready-to-Eat Foods" and "Science to Support the Prevention of *Listeria monocytogenes* in Ready-to-Eat Foods" on the difference in acceptable limits of *Listeria monocytogenes* in foods in the European Union vs. the United States).

It is now widely recognized that illness is not only affected by the quantity of a pathogen but also by its capacity to cause disease. This capacity not only differs between pathogenic species, but often between strains within a given species. Many bacterial species contain both benign and highly pathogenic members (*Escherichia coli* is one well-known example) (Chaudhuri and Henderson 2012). Clearly, variations in pathogenic properties will influence the dose response curve. Since it is impossible to measure the dose response for every strain of pathogen, surrogate models of pathogenicity are needed to enable minimum doses to be adjusted for variation in virulence. Unfortunately, this has proved very difficult to achieve for a number of reasons but largely because the mechanisms of pathogenesis are extremely complex.

Over the past few decades, considerable knowledge has accrued regarding pathogenic properties of disease-causing bacteria including food-borne pathogens and the genes and gene products responsible for human virulence. Nevertheless, at the practical level, models of microbial risk assessment rarely take virulence into account; the present microbiological "state of art" is that bacterial pathogen presence in foods is often based on detection of genes rather than a bacterial isolate. Unfortunately, the mere presence of certain virulence genes is not the only factor that determines virulence potential. For example, expression levels of virulence genes may vary between strains due to polymorphisms, which will impact virulence potential of a pathogen.

In this review we aim to describe the current understanding of relationships between dose response and virulence of foodborne pathogens as well as those factors that might influence this relationship. This review will focus on the major bacterial pathogens *Salmonella* spp., *Campylobacter jejuni/coli*, Shiga toxin-producing *E. coli* and *Listeria monocytogenes,* for which there is reasonable information and data.

Effects of Virulence on Dose Response

Traditionally, the term virulence has been used to describe the pathogenic characteristic of a microorganism and is expressed in terms of the relative capacity of microorganisms to cause disease (Casadevall and Pirofski 2001). In practical terms, virulence is often equated with the severity and duration of symptoms and the outcome of disease caused by the pathogen, as determined by epidemiologic and experimental observations.

Relationships between virulence and dose are unclear and often varies between pathogens. Theoretically, when comparing two strains of the same pathogenic species that differ in virulence capacity, a direct relationship would be expected; as a result, bacterial strains with higher virulence capacities would require a lower dose

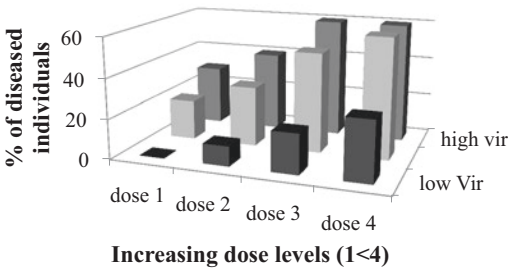

Fig. 1 Hypothetical relationships between virulence potential of a pathogen with strains expressing low, medium and high virulence, and dose required to produce a disease of defined severity and duration, expressed as percentage of diseased individuals

to induce the same degree of symptoms and duration of disease, as illustrated in Fig. 1. The relative outcome of disease will depend on the dose range and degree of virulence. In particular, a minimum dose level to detect illness might be observed for a strain of low virulence, but not in strains of higher virulence. Similarly, a maximum level of ill individuals might be seen in strains of high virulence, but not in low virulence strains.

Virulence capacity is genetically endowed and genes encoding virulence properties are often described as virulence factors. In 1988, Falkow proposed pathogen-based principles (Falkow 1988), based on the Koch Postulates (Robert Koch, 1843–1910), which defined virulence factors as:

1. The phenotype or property under investigation should be associated with pathogenic members of a genus or pathogenic strains of a species. Additionally, the gene in question should be found in all pathogenic strains of the genus or species but be absent from nonpathogenic strains.
2. Specific inactivation of gene(s) associated with the suspected virulence trait should lead to a measurable loss in pathogenicity or virulence. Virulence of the microorganism with the inactivated gene must be less than that of the unaltered microorganism in an appropriate animal model.
3. Reversion or allelic replacement of the mutated gene should lead to restoration of pathogenicity. In other words, reintroduction of the gene into the microorganism should restore virulence in the animal model.

However, research over the last 25 years questions this rather microbial-centric viewpoint. With a greater understanding of the pathogenic mechanisms by which organisms cause disease, it is now clear that the susceptibility of individual hosts is an extremely important determinant for the outcome of pathogen exposure. Host susceptibility is particularly important for foodborne pathogens, where large human populations may be exposed, some sectors of which may be much more susceptible than others (*e.g*. infants or elderly people). The role of host susceptibility in foodborne disease is covered in chapter "The Effects of Food Composition on Foodborne Illness Infectious Dose and Host Susceptibility" in this book.

Foodborne pathogens colonize the gastrointestinal (GI) tract. The human GI tract, extending from the mouth to the anus, provides a continuous tube ca. 9 m long, with multiple potential sites for microorganisms to lodge, colonize and multiply. Excreted material subsequently disseminates these organisms into the environment and directly or indirectly contaminates food thereby completing a cycle. Because many foodborne pathogens are zoonotic, this cycle is not restricted to the human host. Foodborne bacteria, such as members of the genera *Salmonella*, *Campylobacter* and *Escherichia,* enhance this distribution process by increasing intestinal flow (*e.g.* by initiating diarrhea or dysentery). Occasionally, such bacteria cause other symptoms, such as bacteraemia, septicaemia, meningitis and encephalitis by translocating across the gut endothelium or by colonizing extraintestinal organs.

For each pathogen, mechanisms by which disease symptoms are generated vary and are highly complex. Moreover, as stated above, microorganisms within a pathogen's genus or species can vary in their ability to cause disease. This variation

reflects experimental evidence indicating that disparate microbial properties are involved in the process of disease induction. Some of these properties can influence, and even reduce, the number of bacteria needed to induce illness. A number of "classical" bacterial virulence factors (as defined by Wassenaar and Gaastra (2001)), are considered essential to virulence when the microorganism fails to express these properties in a susceptible host and illness is not an outcome of exposure. However, identification and definition of such factors in pathogens is becoming increasingly complicated. For example, some *E. coli* strains designated as probiotic bacteria (e.g., accepted as nonpathogenic), express (admittedly, at low levels) well-recognized classical virulence genes such as HlyA hemolysin and others (Lodinová-Zádníková et al. 1998; Vejborg et al. 2010; Wassenaar et al. 2015; Wassenaar and Gunzer 2015). Observations of this kind oppose Falkow's first postulate, and they are not restricted to *E. coli*. Nevertheless, we consider these properties to be virulence factors, but only when present in a genome that supports the pathogenic phenotype.

Additionally, there are virulence factors that specifically enable pathogens to avoid or cope with environmental hazards experienced during the infection cycle. Evading such hazards is particularly important for foodborne pathogens. From proteolytic enzymes in saliva to gastric and bile acids, and innate and acquired immunity, each host response-related environment or response can affect the pathogen's ability to survive, colonize, multiply and induce disease.

Each of these barriers acts as a bottleneck, significantly reducing the microbial population, and thereby reducing the risk of illness. Pathogenic microorganisms deal with these barriers in similar ways to commensals: both have evolved complex mechanisms to enable their survival when confronting such barriers. Whereas these abilities to survive the GI tract increase fitness in the case of commensals, they also increase virulence in the case of pathogenic microorganisms. When present in the latter, inactivation of these survival mechanisms will reduce, if not eliminate, pathogenic effects in the host, which means they can be included in Falkow's postulates for virulence genes. Perhaps more accurately, they could be called accessory virulence genes (Wassenaar and Gaastra 2001). Thus, it is reasonable to describe such characteristics as virulence factors, but only when present in a microorganism that has the capacity to cause disease due to other factors.

Stress response mechanisms not only enable the pathogen to survive the gastrointestinal tract, compete with resident microflora and resist the host immune response, they may also enable survival in the environment outside the host prior to ingestion. These particularly include the capacity to survive in, and adapt to, adverse environments during food production and preparation, such as by generating biofilms. Such properties are also aided by certain genetic elements. As the measurement of pathogen burden in foodstuffs is often undertaken well before the point of ingestion, (e.g., in milk from bulk tankers or in chickens at the abattoir) the bacterial populations may experience many adverse environmental pressures post-sampling but prior to ingestion. Thus the pathogen burden, as measured according to the requirements of regulations, may not necessarily reflect the dose actually delivered to the host or to the site of colonization.

Because of the uncertainty around the identification of virulence factors for foodborne pathogens, we propose the adoption of a broader definition in order to encompass all those factors, which would influence the number of microorganisms required for ingestion to produce illness in humans. Thus, for the purpose of this review, we define virulence factors as all microbial characteristics which confer the capacity to enable a pathogen to survive food production and processing, ensure infectivity of a susceptible host and induce pathological outcomes in that host.

Dose Response Models

The combination of host resistance, adverse environmental pressures and modern sanitary conditions considerably reduces fecal-oral transmission and, thereby, the opportunity for a foodborne pathogen to reach a potential and susceptible host. As a consequence, it has been speculated that the minimum dose of a foodborne pathogen required to infect and cause disease is likely to be very low (Teunis et al. 2005). However, because of variation in host susceptibility, the risk to humans is usually described by the proportion of the exposed population with illness over a range of doses. A simple exponential dose-response model is given in Eq. (1)

$$P(d) = 1 - e^{-kd} \tag{1}$$

where *P (d)* is the probability of response at dose *d* and *k* is the probability that a single microorganism can survive and initiate infection. The more frequently used Beta-Poisson model (Teunis et al. 1999) is given by as Eq. (2)

$$P(d) = 1 - \left[1 + \left(d / N_{50}\right)\left(2^{1/\alpha} - 1\right)\right]^{-\alpha} \tag{2}$$

where N_{50} is the median infective dose and α is the slope parameter for the Beta-Poisson model. The Eq. (2) is derived from the exact Beta-Poisson equation (Teunis and Havelaar 2000) with certain assumptions (Haas et al. 1999).

Peleg and coworkers consider that such simplistic models are inadequate for food- and waterborne pathogens because the host has evolved many barriers to prevent microbial colonization of the gastrointestinal tract and/or translocation to other organs to initiate disease symptoms (Peleg et al. 2011). Thus, at each host barrier, the probability that all microorganisms ingested will remain viable and cause disease is reduced. Because such factors are difficult, if not impossible, to quantitate, estimates of this potential are introduced and the estimated final infective dose becomes a function of the sum of all such probabilities.

Obviously, virulence is among the many factors influencing this survival and disease potential. Using *L. monocytogenes* as an example, Buchanan et al. (2009) have proposed a logical series of key events which define a dose-response

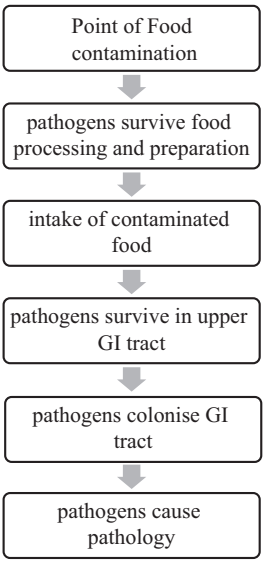

Fig. 2 Proposed key events that should be included in a dose-response framework. The *top three key events* were not included in the original model proposed by Buchanan et al. (2009)

framework for foodborne pathogens. Examples of key events are for pathogens to survive the upper gastrointestinal tract or to establish, attach and enter intestinal epithelial cells. At each key event along a pathway starting from food ingestion and ending in illness outcome, dose, type of pathogen and host factors should be considered to determine how many microorganisms need to be ingested to cause a disease outcome. In the case of *L. monocytogenes,* one disease outcome in pregnant women would be fetal death.

However, we propose that for foodborne pathogens this model is still too simplistic because, as explained previously, the measurement of pathogen load is performed prior to ingestion, often early in the food production process. In such circumstances, pathogens are subsequently exposed to multiple adverse environmental pressures which could substantially reduce the dose (bacterial population) before the point of ingestion. These environmental pressures should be included as key events because the ability of a pathogen to survive these exposures would crucially affect the risk when consumed. For example, assessment of levels of chicken skin contaminated with *Campylobacter* is currently undertaken at the slaughter plant, immediately after chilling of the carcass, but the survival of these microorganisms is generally poor (depending on storage conditions or freezing), and bacterial numbers may decline considerably prior to consumer handling. Food preparation by cooking, again, significantly reduces these bacterial numbers. Thus, we propose that it is logical to extend the framework of Buchanan et al. (2009) backwards to include in any dose response model, those barriers which might influence the numbers of microorganisms, from the point of measurement of food contamination to the point of ingestion (Fig. 2).

In Vivo and *In Vitro* Approaches to Defining Dose-Response in Relation to Virulence

A number of *in vivo* and *in vitro* approaches are available to compare the dose responses of strains with presumed differences in virulence. Human ingestion experiments are clearly the "Gold Standard" but are rarely feasible. Alternatives include the investigation of contaminated food that has caused outbreaks to determine the level of pathogen exposure, *in vivo* animal models of disease, and *in vitro* models as surrogates of disease.

Human Ingestion Experiments

To date, most of the available and used dose-response data come from human volunteer trials. However, for some microorganisms, (e.g., *E. coli* O157 and other STEC or EHEC), such studies have not been carried out for ethical reasons. Those volunteer studies that have been undertaken have rarely compared strains with defined differences in virulence.

The comparison of dose-response curves from volunteer studies using single strains of bacteria is fraught with difficulties. The comparative value of these studies is often limited by differences in the criteria chosen for measuring disease, the strain/dose administered and the subjects selected. In some studies, infection rather than illness is used and even the definition of illness can vary significantly. The selection of strain and dose is also important. Strains that are utilized have often been repeatedly sub-cultured and stored over variable time periods, which can affect their virulence. The culture conditions used to generate a challenge dose, and the dosing medium, often vary between studies. It is also well-recognized that the type of food (matrix and chemical composition) in which the pathogen enters the host can have a dramatic impact on the infectious dose (see chapter "The Effects of Food Composition on Foodborne Illness Infectious Dose and Host Susceptibility" on infectious dose as effected by food matrix composition) (e.g. Waterman and Small (1998), D'Aoust (1985)).

Thus, many microbial factors can affect infectivity as well as the ability to induce illness. Moreover, doses are often selected to greatly exceed realistic bacterial loads in consumed foods, so that infection is assured, which ignores low dose effects. The selection of volunteers can further affect the outcome of ingestion trials. With the development of immune markers, volunteer recruitment to target the immunologically naive can lead to a significant skew in a dose response curve.

A good example of the compounding issues encountered in determining dose-response with multiple confounding factors is suggested by the dose-response curve of *C. jejuni,* which is well accepted for the purposes of Quantitative Microbiological Risk Assessment (Enger 2013). This curve is based largely on an extensive trial

undertaken by Black et al. (1988). The issues and difficulties with this trial, for the purpose of delivering a usable dose response curve, have been well described by Coleman et al. (2004). In this trial, two strains of bacteria of different serovars were ingested in milk by young healthy volunteers. Strain A3249 (serotype not specified) was isolated from a 16-year old boy suffering from an infection during an outbreak in 1980, and strain 81-176 (serotype Penner 23/36) was isolated from a milk-borne outbreak first published in 1985 (Korlath et al. 1985). The overall results revealed that with both strains, infection (as defined by recovery of organisms from feces) increased with increasing dose, but illness did not, regardless of the indicators of illness. However, there were differences between strains, with strain 81-176 having a higher attack rate than A3249, and illness, when observed, was said to be more severe (Black et al. 1988). The dose response of strain 81-176 has been further investigated as part of a vaccine trial (Tribble et al. 2010). By using immunologically naive subjects and doses of 10^5–10^9 CFU in bicarbonate buffer, a dose responsive increase in incidence of campylobacteriosis, of any severity, was observed such that at the dose of 10^9 CFU 92% of the subjects were ill. The difference in results from the previous study by Black et al. (1988), apart from subject selection and dosing milieu, may be due to the active selection of flagellated campylobacters, which improves colonization (Caldwell et al. 1985) and increases virulence (Rossez et al. 2015). In addition, the infecting strain had been previously passaged through a human, as it had been isolated from a symptomatic individual from the previous Black study. Overall the observations suggest differences in virulence between *C. jejuni* strains, but also that dose response is affected by culture history, dose milieu and the expression of virulence factors such as motile flagella.

Few comparative volunteer studies have been undertaken with *Salmonella* strains. A very early trial challenged volunteers with *S. enterica* serotype Meleagridis, (isolated from market samples of high-moisture spray-dried whole egg powder) to male prison inmates in a glass of eggnog (McCullough and Eisele 1951). Although the aim of this study was to test the pathogenicity of serotypes of *Salmonella enterica* that had not previously been isolated from human cases, only infectivity was described. Interestingly, substantial strain to strain variability was observed, with the more "virulent" strains having an infectious dose of 7.6×10^6 CFU (Teunis et al. 1999). Other serotypes of *Salmonella* were also tested and found to have different ranges of infectivity, for example, *Salmonella* Bareilly had an infectious dose range from 1.3×10^5 to 1.7×10^6 compared to *Salmonella* Pullorum at 1.3×10^9 to 1.6×10^{10} CFU. The statistical significance of the *Salmonella* serotype variation in pathogenicity in this study has been further investigated by Coleman et al. (2004). The assumption that all strains investigated differed from each other in pathogenicity provided the best fit to the data. As the authors indicated, unfortunately, the study failed to investigate the two most common foodborne salmonellae (*S*. Typhimurium and *S*. Enteritidis), which leaves a significant question regarding what dose response curve should be used for *Salmonella* risk assessment.

Human Exposure Estimates from Epidemiologic Studies

An alternative to human experimental exposure data is to use information from epidemiologic studies of foodborne outbreaks, which are generally monitored for the purposes of source identification and public health control. Such reports usually include the causative agent, the number of human cases, hospitalizations and deaths, as well as any identified source. However, for dose-response investigations, more detailed information is required, especially the number of people exposed (to determine attack rate) and number of microorganisms per exposure. Using the level of food contamination as an indicator of infectious dose is fraught with difficulties, as described by others (*e.g.* Hara-Kudo and Takatori (2011)). For example, food matrices may or may not support growth of the microorganism over time. Therefore, depending on when the implicated food was sampled following an outbreak, the number of viable microorganisms may have decreased or even increased (Buchanan et al. 2009). Exposure to environmental stresses during food processing or storage may also have an impact on infectivity (Hara-Kudo and Takatori 2011). An additional complication is the degree of pathogen homogeneity within the food matrix, which results in variations in the number of microorganisms consumed by individuals. Thus, the usefulness of this approach to define dose response is limited, especially as some major foodborne pathogens, such as *Campylobacter*, rarely cause observed foodborne outbreaks. Nevertheless, calculations of dose response are possible where sufficient outbreak data are available, the source is well-characterized and stored, and the infectious agent is robust and evenly distributed. Because multiple outbreaks have been studied for some pathogens, the comparison of potential virulence between strains has been possible.

In 2004, Teunis and coworkers analyzed an outbreak of *E. coli* O157:H7 in a Japanese school for which extraordinarily detailed information was available (Teunis et al. 2004). Their analysis revealed that the average pathogen dose in this epidemic was 31 CFU. However, a later comparison of eight *E.coli* O157: H7 outbreaks revealed considerable variation in attack rate between epidemics (Teunis et al. 2008). Some of this variation was accountable by modelling heterogeneity on pathogen distribution in the food, causing differences in exposure dose. Nevertheless variation in infectivity (*i.e.,* virulence) between strains was also considered a plausible explanation.

Epidemiologic evidence reveals that some nontyphoidal *Salmonella* serovars (e.g., Typhimurium, Dublin, Choleraesuis, 9,12:l,v:−) have a greater potential to cause extra-intestinal infections in humans than other serovars (Humphrey 2000), suggesting substantial differences in virulence. Nontyphoidal salmonellosis is often associated with food-related outbreaks. In 1992, a meta-analysis was performed of 49 foodborne outbreaks caused by *Salmonella* serovars Typhimurium, Enteritidis, Infantis and Thompson (Glynn and Bradley 1992). In this study, attack rate and hospitalization were used as surrogates of dose and disease severity, respectively. Linear regression analysis determined a positive correlation between these two variables for all four serotypes, providing supportive evidence for a dose-response rela-

tionship in human *Salmonella* infections. Interestingly, some of the epidemics of *S.* Typhimurium, despite having similar vehicles and attack rates, had substantially higher hospitalization rates. This could be interpreted as an indication of differences in strain virulence affecting disease presentation.

Overall, this epidemiologic approach to defining dose-response has provided direct evidence regarding variations in pathogen strain virulence.

Animal In Vivo Models

Given the ethical issues associated with human experiments and the paucity of data from epidemiologic studies, *in vivo* animal models would seem a reasonable alternative to assess dose-response. However, such models rarely mimic the disease outcomes of the human infection. As a consequence, surrogate measures are often used as indicators of virulence, such as colonization, anorexia, fecal excretion, pyrexia, histopathology, bacteremia, septicemia or even death. There are many other difficult issues surrounding the use of animal models for dose-response determination, including availability/cost, route of administration, acquired immunity due to prior natural exposure, and species-specific receptors. In addition, there are the usual pathogen-related issues of strain selection, culture conditions and attenuation due to repeated laboratory subculture, which were previously observed in human experiments.

Although early observations revealed that nonhuman primates, such as Rhesus macaques (*Mucaca mulatta*), can present with foodborne illnesses similar to those seen in humans (Kent et al. 1966), the model was not used for comparisons of foodborne pathogen strains.

As the animal model departs further from the human host, the risk of anomalous data becomes greater. Despite the host manipulations, such as immune suppression, diarrhea is an infrequent outcome of animal models, even in the presence of massive colonization. Nevertheless, dose-response curves from some *in vivo* models have been important in the assessment of the role of virulence factors, especially using genetic mutants. For example, dose-response curves for *C. jejuni* in a one-day old chick model have been extensively investigated regarding factors involved in colonization as a surrogate of virulence. This model has revealed that there are strain-specific differences in colonization. Moreover, the model has revealed how strain handling and environment can influence these properties. For example, repeated subculturing and storage of *Campylobacter* strains reduces colonization (Cawthraw et al. 1996, Ridley et al. 2008). Nevertheless, this reduced capacity to colonize can be restored by passage through a chick. In addition, numerous genetic mutants, including flagella/motility mutants (Wassenaar et al. 1993), have been tested in this model, indicating that inactivated potential virulence factors can decrease, and occasionally increase, colonization in the chick. Although chickens are considered a natural host for *Campylobacter,* this model does not mimic the human disease because chicken colonization is asymptomatic. Nevertheless, the observation that

laboratory passage generally inhibits the ability of a strain to colonize *in vivo* (Chen et al. 2006) has implications for human dose-response curves. This was demonstrated by Black et al. (1988) with previously multiple-passaged strains.

Because foodborne salmonellae, such as *S*. Enteritidis, frequently come from poultry meat or products, chickens have often been used as *in vivo* models of colonization. However, the correlation of such models to human disease remains unclear. Variation in strain virulence would be particularly pertinent to human infection. For example, strains of *S*. Typhimurium and *S*. Enteritidis differ substantially in avian models of invasiveness, resulting in different degrees of facal shedding, bacteraemia and egg contamination. Such differences do not appear to be related to serotype alone but may also be strain-specific. Some of these strain-related differences in virulence are also observed in mammalian models. For example, strain D23580, a multidrug resistant clinical isolate of *S.* Typhimurium ST313, which causes fatal bacteremia in young children and HIV-infected adults, is invasive in chicken and mouse models of infection. This strain appears to be more virulent than *S*. Typhimurium strain SL1344 and exhibited enhanced resistance to acid stress relative to SL1344, which may explain its increased virulence (Yang et al. 2015).

There is ample evidence from animal *in vivo* models that different serotypes, and even strains, of the same species of various foodborne bacterial pathogens can vary in virulence and some of these data could be used to adjust the accuracy of existing human dose-response curves.

In Vitro Models

Cell and tissue culture *in vitro* models have become increasingly sophisticated in an attempt to mimic the natural host environment. The most recent complexity has been the development of 3-dimensional structures, sometimes incorporating several cell types. These models can be used to mimic regions of the gastrointestinal tract. The co-culture of bacteria and other pathogens in such models provides a cheap and readily available system to compare strain virulence. However, the relevance of such models to human illness is questionable. Criteria for assessment of virulence *in vitro* are varied and numerous: from toxin-induced cell distortion to intracellular invasion. However, without the complete host's anatomical structures and host-mediated defense mechanisms taken into account, it is difficult to translate any effects caused by a pathogen into human disease presentation or to dose-response. Nevertheless, pathogenic phenotypes, such as toxin expression, invasiveness or intracellular survival, can be used to compare strain differences in their potential for virulence as well as to extrapolate these data for evaluation of their potential effects on dose-response. For example, in the recent analysis of the dose response of *C. jejuni,* based on the human volunteer study by Black et al. (1988), Coleman et al. (2004) adjusted the dose-response to take into account the administration of equal doses of flagellated and nonflagellated microorganisms, because *in vitro* studies had revealed that active flagella were important factors in colonization (Wassenaar et al.

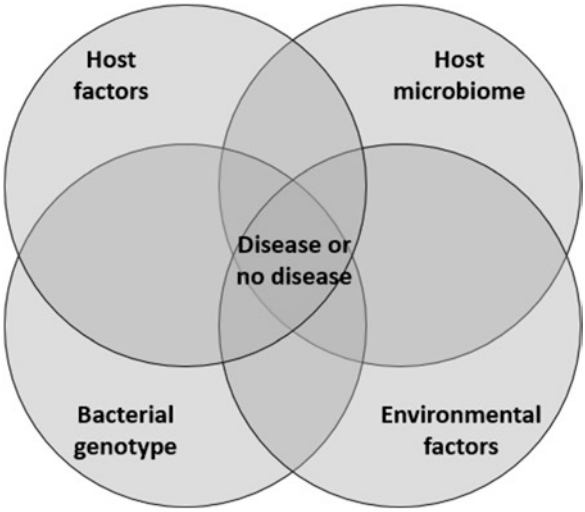

Fig. 3 Interconnecting factors influencing disease outcomes, following exposure to pathogens, categorized into four classes. While host and bacterial factors are well-recognized, the importance of environmental factors and the host microbiome are now being increasingly acknowledged

1993). Hence, *in vitro* models can inform human volunteer, and possibly animal model studies, to adjust for strain to strain differences even though such models do not provide direct data for human dose responses.

Classical Virulence and Accessory Factors Potentially Influence Infectious Dose

Mathematical models used for microbial risk assessment need to take into account, not only the quantitative dose required to induce disease, but also the virulence properties of the strain involved. The genetic basis of the virulence of many bacterial foodborne pathogens has been extensively investigated for more than 30 years. The genomic comparison of strains is now a routine tool and has revealed that the variation in genetic content between strains within a species is considerable, and includes virulence genes (Land et al. 2015). The presence or absence of potential virulence genes is now frequently used to indicate strain pathogenicity. However, such information has limited usefulness because gene expression can be affected by multiple additional factors.

The challenge to risk modellers and assessors is that the outcome of individual analyses depends upon several interconnecting sets of conditions. This is illustrated in Fig. 3, in which four classes of factors are given that can influence whether exposure to a pathogen may lead to disease. Changing any of these factors might influence the shape of the dose-response curve. Table 1 lists in more detail the content of these classes. Although not comprehensive, this table highlights the diversity of these factors. Traditionally, those factors involved in the host's status and the pathogen's virulence capability were considered essential to disease outcomes, but we

Table 1 Factors considered important in determining infectious dose

Host factors	Bacterial factors	Environmental factors	Host microbiome
Age	Virulence factors	pH	Commensals
Gender	Mutations	a_w	Probiotics
Underlying conditions, e.g.	Regulation	Temperature	Predators, e.g.
Cancer	Stress & adaptive	Redox potential	*Bdellovibrio*
HIV	responses e.g.,	Atmosphere	Bacteriophages
Gastric hypoacidity	RpoS regulon	Food processing	
Pregnancy	Acid tolerance	and preservation	
Diet	Heatshock	Heat-shock	
Medication	proteins	Cold-shock	
Immune modulators and	Oxidative	Osmotic shock	
immunosuppressive	stress response		
therapy	Starvation		
Antacids	strategies,		
Host genotype	e.g. Sporulation		
	Growth phase,		
	e.g. Stationary		
	phase regulon		

now also recognize the importance of stress responses to environmental factors both prior to and following ingestion. Most recently, a fourth factor affecting potential for infection following pathogen exposure has been added – the microbiome. It is becoming increasingly apparent that the resident microflora of the gut (the microbiome) can influence whether a pathogen can colonize or not. Inhibiting the colonization of pathogens is a critical function of probiotics and commensal microorganisms and from metagenomic studies, it has been revealed that the gut microbiome can vary considerably between individuals. This undoubtedly results in variation of disease resistance, but, so far, this effect has not been included in mathematical risk assessment models.

In summary, the relative capacity of a bacterial foodborne pathogen to cause disease can vary between and within bacterial species, between hosts and at the various stages of the food chain. This presents considerable challenges in determining dose-response to inform microbial risk assessment models.

Examples of Some Classical Bacterial Virulence Factors That May Affect Dose Response

Flagella and Motility

For most enteric microorganisms, accessing and maintaining residence at the appropriate site of infection is key to colonization and, in many cases, motility is essential to this process. Active flagella are, therefore, often defined as key virulence factors. As previously mentioned, *Campylobacter,* with its polar flagella and corkscrew-like

motility, is well-adapted to survive in the viscous mucous overlying the intestinal epithelium. The production of aflagellate cells is a natural and reversible event in campylobacters and the rate of their production varies between strains. Aflagellate or immobilized campylobacters have impaired virulence properties *in vivo* and *in vitro* (Newell et al. 1985; Wassenaar et al. 1993) which are theorized to have had an effect on human dose-response in volunteer studies.

Salmonella also uses motility to access the site of infection and motility is believed to be a key virulence factor for *Salmonella*. Recently, *S*. Typhimurium was determined to use flagella to carry out "near-surface swimming" as a means to scan the host cell surface to locate a suitable site of infection (Misselwitz et al. 2012) e.g. the Peyer's patches in the distal ileum (Jones 1997). However, variation in flagellar expression and competence exists within the natural populations of *Salmonella*. Nonmotile variants of *S.* Enteritidis have been identified; a proportion of the nonmotile variants have paralyzed flagella, which may impair the ability of these variants to cause disease in humans (Yim et al. 2011).

Adherence and Invasion

Once at the preferred site of colonization, the bacterium must establish itself within this niche. Some bacteria adhere to the surface of epithelial cells transiently, prior to entering the cells, whereas others are more persistent, (e.g., *E.coli* O157:H7). *Salmonella* requires intimate contact with host cells in order to pass effector proteins, such as toxins, via the needle-like structure of its two Type III Secretion System (TTSS) to host cells (recently reviewed by Moest and Méresse (2013)). These effector proteins are crucial virulence factors, being used by *Salmonella* to gain entry into, and survive within host cells (reviewed by Valdez et al. (2009)). Recently, it was revealed that the TTSS affects *Salmonella* fitness (Ali et al. 2014). The fitness of bacteria is generally considered its ability to adjust metabolism to suit environmental conditions, in order to survive and grow. Genes coding for TTSS, residing on *Salmonella* Pathogenicity Island 1, have been horizontally acquired. The horizontal uptake of such AT-rich, highly-expressed virulence-associated genes can potentially reduce the fitness of a recipient cell; for example, by accidental transcription activity initiated from such AT-rich promoter-like sequences. These negative effects on fitness can be ameliorated by histone-like protein HLP-II (H-NS), a DNA-binding protein implicated in transcriptional repression (silencing) as well as in bacterial chromosome organization. However, fitness-reducing artificial mutations can also be compensated by mutations in the TTSS (Ali et al. 2014). So, genetic mutations can affect *Salmonella* interaction with host cells at multiple levels.

For some invasive pathogens, such as *L. monocytogenes,* entry into the host cell is a vital step in its pathogenesis and subsequent onset of symptoms. The first step in the *Listeria* infection process, which allows the pathogen to cross the intestinal barrier, is an interaction between the bacterial virulence factor internalin (InlA) and

certain isoforms of E-cadherin on the host cell surface. Presence of this virulence gene, *inl*A, would be expected to indicate whether or not the strain is pathogenic. However, the presence of naturally occurring mutations in *inl*A, which result in a premature stop codon (PMSC), has confused the issue. Studies using a guinea pig model of infection revealed a significant increase in the infectious dose of PMSC-containing strains of *L. monocytogenes* compared with strains carrying the intact gene (van Stelten et al. 2011). These findings support the notion that the infectious dose relationship in *L. monocytogenes* is strain-specific. Interestingly, these naturally occurring mutations are present primarily among those strains of *L. monocytogenes* found more often within ready-to-eat foods. However, they are less likely to be associated with human disease, unlike the epidemic clones belonging to serotype 4b which possess intact *inl*A (van Stelten et al. 2011). This could mean that humans are exposed regularly to potentially nonpathogenic *L. monocytogenes,* which would significantly impact risk assessment for this bacterium in foods.

For *Campylobacter*, another potentially invasive pathogen, it is known that some strains are able to invade epithelial cells in tissue culture more readily than others (Fearnley et al. 2008). Unfortunately, the genetic basis for this variation is still unclear and, as yet, there is no direct link between invasion *in vitro* and what happens in the human host. (See chapter "Varying Pathogenicity of *Campylobacter jejuni* Isolates" on *Campylobacter* pathogenicity).

It remains widely assumed, for the purposes of dose-response models, that all strains of the same species are equally pathogenic despite variation in classical virulence factors of enteric bacterial pathogens, such as *Salmonella, Campylobacter* and *E. coli*.

Some Examples of Environmental Factors and Subsequent Bacterial Responses That Could Affect Infectious Dose

In terms of determining the potential of virulence to affect dose-response, certain host and classical bacterial factors have received the most attention to date. However, we propose that stress responses also contribute to virulence. Foodborne bacteria in particular, are continually exposed to stressful situations, for example in the farm environment, within an animal host, during food processing, storage and preservation or within the human host. At each point, different stresses will be encountered and a successful pathogen must be able to respond rapidly or die. At the farm, bacteria may be exposed to sunlight, extreme temperature fluctuations and desiccation, whereas during food manufacturing, high temperature, low pH, low water activity (a_w) and treatment with preservatives or other antimicrobials will all provide significant challenges to survival. Following ingestion, host defenses will provide other environmental challenges.

One of the most effective barriers to prevent colonization and invasion of the host by foodborne pathogens is the gastric acid of the stomach. When pH is <4.0, gastric

acid is a powerful bactericide, but increases above pH 4.0 may allow bacteria to survive. Acid environments can also be encountered during food production, for example, in cheese production or post-harvest treatment of chicken carcasses with organic acids.

Several studies have determined that raising the stomach pH can significantly reduce the infectious dose (Tennant et al. 2008; Smith 2003). The pH of the stomach is dependent on a number of factors, such as whether an individual has eaten, the time of day, a person's age, and whether proton pump inhibitors (PPIs) or other antacids are consumed (Birk et al. 2012; Bavishi and DuPont 2011). All of these factors can affect the risk of an individual becoming infected with an enteric pathogen.

There is considerable variation in the ability of bacterial foodborne pathogens to respond to low pH. This variation is apparent at the species and subspecies levels. Enterohemorrhagic *E. coli* O157:H7 (an STEC strain), is an acid-tolerant bacterium, and has a low infectious dose of 10–100 cells (Tuttle et al. 1999), whereas other *E. coli* pathovars, such as enteropathogenic (EPEC), enterotoxigenic (ETEC) and enteroaggregative *E. coli* (EAggEC) are reported to have infectious doses of 10^6, 10^7 and 10^8 cells, respectively (Kothary and Babu 2001). However, non-O157 STEC strains such as O26:H11 and O111:H8, are reported to have higher infectious doses than *E. coli* O157 (Bergholz and Whittam 2007), so the association between infectious dose and acid tolerance may not be directly correlated.

Mildly acidic environments induce an acid tolerance response in many bacteria, including EHEC, through the induction of the RpoS regulon. Several studies have revealed that the chromosomal locus encoding *rpoS* is highly polymorphic in wild-type strains of pathogenic bacteria, such as EHEC and *S. enterica* serovar Typhi (see, for example, Waterman and Small (1996), Robbe-Saule et al. (2006)). Moreover, strains with defective *rpoS* alleles, when transformed with plasmids expressing functional alleles, demonstrate significant increases in acid tolerance. These polymorphisms result in highly variable levels of acid tolerance and are thought to have an important role in determining infectious dose.

The RpoS regulon is not the only genetic component involved in acid tolerance. Glutamate decarboxylase enzymes such as *GadA* and *GadB,* as well as OmpR and RcsB, also play important roles (Bergholz and Whittam 2007; Johnson et al. 2011).

The type and composition of food ingested with a pathogen is another important factor that can affect the dose response. Some spices such as garlic, onion and oregano, added to food have antimicrobial activity (Billing and Sherman 1998). Protein-rich foods (e.g., ground beef or turkey and cheese and even simple proteins, such as ovalbumin and pepsin), can have a protective effect in gastric models (Waterman and Small 1998; D'Aoust 1985; Birk et al. 2012). Fat-rich foods also have a protective effect on pathogens. The low a_w of fat-rich foods, such as chocolate and peanut butter, can cause osmotic stress on the bacteria, which, like low pH, also results in the induction of the RpoS regulon. This may lead to more robust bacteria that are better able to transit the stomach (O'Byrne and Booth 2002). In 2006, the UK chocolate manufacturer Cadbury-Schweppes experienced the catastrophic consequences of *Salmonella* contamination in chocolate. For several years, the company had been

detecting contamination of *S. enterica* serovar Montevideo in their products. Although there were only small numbers of salmonellae, the fact that the bacteria were within a very low a_w environment resulted in induction of stress cross-protection systems, making them much more resilient and able to survive stomach acid. As a consequence, 37 people became infected after consuming *Salmonella*-contaminated chocolate, costing the company more than £20 million (28.8 million USD, March, 2016) (Manning 2007).

Stress responses may have indirect as well as direct effects on dose-response. Some important foodborne pathogens respond to different stresses by upregulating virulence factor expression. For example, expression of the prophage-encoded Shiga toxin genes of *E. coli* O157 can be induced by the SOS-response system following exposure to DNA-damaging agents, such as DNA gyrase-inhibiting antibiotics, i.e., fluoroquinolones, and oxygen species (Kimmitt et al. 2000). Thus, treatment of some foodborne bacterial infections with these inappropriate antibiotics could inadvertently result in a much more serious clinical outcome.

Nontyphoidal strains of *Salmonella enterica* possess the *spvRABCD* operon, which is usually located in the virulence plasmid and is upregulated by RpoS (Guiney and Fierer 2011). As a consequence, any stress, such as low pH, starvation or osmotic shock, which induce RpoS expression, could also result in expression of this operon. While no clear function has been ascribed to SpvA and SpvD, it is apparent that SpvB and SpvC are involved in cytoskeleton disruption and MAP-kinase inhibition, respectively. Molecular epidemiology of different nontyphoidal *Salmonella* strains from humans has revealed a strong association between the *spv* genes and bacteremic isolates rather than gastroenteritis, suggesting that these genes are required for entry and/or survival within the bloodstream (Guiney and Fierer 2011). Thus, once again, the clinical outcome of infection may be altered by environmental stress.

Conclusions

Many mathematical models have been developed for use in the assessment of risk of specific foodborne pathogens present in various foodstuffs. The accuracy of these models is highly dependent on the human dose-response data used. For many pathogens, these data are sparse and even when available, the data are widely accepted as inaccurate. Much of the analytical work to date can be described as attempting to modify the "Poisson-like" response curves, in order to collate the disparity and scarcity of data. Such mathematical ingenuity sheds little light on the reality underlying the processes leading to infection. The too often ignored truism that *"statistical correlation does not necessarily imply causality"* is increasingly applicable in this approach. The sweeping assumption that all such models make, e.g., that all strains of the same pathogen species are equally virulent, and that all potential pathogens detected in foods are able to cause disease to the same extent in humans is both incorrect and potentially dangerous.

In this review, we speculate that some of the major bacterial foodborne pathogens, (*i.e. Salmonella*, *Campylobacter* and Shiga toxin-producing *E. coli*), exhibit substantial variation between strains in their ability to: (1) survive and remain pathogenic in the food environment from the point of detection of pathogen load, (2) survive in and colonize the human gut, and (3) produce a disease outcome which will be observed as a dose-response. There is increasing evidence to support these speculations and we suggest that this variation has a major impact on the dose-response curves currently being used for microbiological risk assessments.

Buchanan et al. (2009), by introducing key events in the processes towards infection and disease, have shown the way towards an approach to risk assessment more closely linked to underlying causality. In this chapter, we have proposed that this approach should be extended backwards so as to embrace earlier stages in the food chain. We further suggest that modelling studies (based on such subdivisions, and recognizing the variation inherent within bacterial species at each stage in the process) are likely to lead to a clearer understanding of the role of those factors which lead to a determination of risk. Simple dose-response curves are unlikely to be adequate.

As ethical and financial considerations will likely preclude further human volunteer studies with multiple strain comparisons, alternative approaches to assess dose-response more accurately must be developed. Greater attention paid to the analysis of outbreak data, and relating this information to strain genotypic and patho-physiological characteristics using *in vitro* models would be helpful in providing credibility to these surrogates of virulence. Awareness of the type and effects of environmental stresses to which foodborne pathogens are exposed will also become important, especially when the pathogen load in foodstuffs is determined in an extended time and distance prior to consumption. Most importantly, microbiological risk assessment models must incorporate a more realistic appreciation of the variability in virulence, and consequently dose-response, among foodborne pathogen strains.

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Clostridium botulinum and the Most Poisonous Poison

Eric A. Johnson

Abstract Botulinum neurotoxin (BoNT) is the most poisonous toxin known to humankind and is the cause of the neuroparalytic disease botulism in humans and animals. Due to the extraordinary toxicity of BoNT, control of botulism is a perennial concern of the food industry, medicine, and regulatory agencies. BoNT also poses a serious concern as a bioterrorism agent with the potential of causing mass casualties, and BoNT is designated as a Category A Select Agent, the most severe group comprising six agents, which also includes anthrax toxins and hemorrhagic viruses. There are seven serotypes of BoNT (designated A–G), which are defined by their neutralization against death in mice by homologous antibodies. BoNTs are produced by neurotoxigenic clostridia, particularly the diverse bacterial group *Clostridium botulinum*, and by sporadic strains of *Clostridium argentinense*, *Clostridium butyricum*, *Clostridium baratii*, and *Clostridium sporogenes*. These clostridia produce heat and chemical resistant endospores, which are widespread in soils, freshwater and marine sediments, and the gastrointestinal tract of certain animals (but not humans). Surveys have revealed that *C. botulinum* spores are present in the USA in about 35% of soil samples examined. BoNTs are ~150 kDa proteins comprised of a Heavy Chain (HC) and a Light Chain (LC). During the intoxication process, the HC binds selectively and with high affinity to peripheral nerve terminals, and the LC enters into the cytosol and inhibits the presynaptic release of acetylcholine, causing postsynaptic denervation of muscle and a characteristic flaccid paralysis that can last for several weeks to months in humans. BoNTs also cause disturbances in the sensory and autonomic nervous systems. The recognition of diversity of BoNTs has expanded in recent years mainly through DNA sequencing efforts, and within the seven serotypes (A-G) there are more than 40 sequence variants (subtypes) of BoNTs currently recognized (Hill et al. 2013. BoNTs are produced naturally in protein complexes, in which nontoxic associated proteins protect the heat-labile BoNT component in the digestive tract of humans and animals and likely in the environment. Six categories of botulism are recognized: (1) foodborne botulism whereby preformed toxin (and sometimes cells and spores) are ingested in foods, (2) infant botulism in which *C. botulinum* colonizes

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the infant gut and produces BoNTs, (3) adult intestinal botulism analogous to infant botulism, (4) wound botulism due to colonization and BoNT formation in surface wounds, (5) inhalational botulism which is extremely rare naturally but a concern of bioterrorism, and (6) iatrogenic botulism caused by poisoning from inappropriate injection of BoNT for pharmaceutical and aesthetic purposes. In all six categories, the symptoms and pathology are very similar. BoNT is absorbed into the lymphatic system and then into the blood stream, and is then transported to peripheral nerve terminals, primarily cranial and somatic nerves. BoNT is internalized into the nerve terminals at the neuromuscular junction and prevents the release of acetylcholine to muscles innervating the eyes, face, and mouth, and then the toxicity descends bilaterally to motor nerves of the torso causing a generalized paralysis. There is no antidote for botulism once it enters nerves, and currently the only means for treatment of the disease is by thorough nursing care, and in severe cases mechanical ventilation and parenteral feeding. Serum therapy by administration of human (BabyBIG®) in infants or heptavalent equine antibodies (currently HBAT) in adults into the circulation of poisoned toddlers and adults, can decrease the severity of the paralysis and duration of the illness while BoNT remains in the blood stream. This chapter focuses on aspects of neurotoxigenic clostridia and botulinum neurotoxins that have not been extensively reviewed recently, including the importance of BoNTs as a public health risk, properties of BoNT-producing strains and BoNTs, laboratory criteria needed for a definitive diagnosis of botulism, the pathology, recovery and treatment of botulism, and lastly a brief section on strategies to prevent foodborne and infant botulism.

Keywords *Clostridium botulinum* • Botulinum neurotoxin • Endospores • Flaccid paralysis • Select agent

Introduction

Lamanna proposed in 1959 that botulinum neurotoxin type A (BoNT/A) is the most poisonous substance known to humankind (Lamanna 1959); and his prediction has stood the test of time (Gill 1982; Morton 1961; Johnson and Montecucco 2008; Scott and Suzuki 1988). BoNTs have an estimated lethal dose in humans of 0.1–0.5 ng per kg by intravenous exposure, 0.10–1.5 μg per kg by the oral route, and 7–12 ng per kg by inhalation (BoNT/A). Botulism is a true intoxication caused solely by BoNT actions, while it is dependent on neurotoxigenic clostridia that produce the toxin. Botulism is a rare disease in humans, and is much more common in certain animals, particularly in fishes and waterfowl for which BoNT has caused massive outbreaks, involving thousands of animals every year (Anniballi et al. 2013; Eklund 1987; Smith and Sugiyama 1988).

From a global perspective, the worldwide impact of foodborne and waterborne diseases on human health and economics is vast (WHO 2015). Systematic evalua-

tions of the global impact of foodborne infections and intoxications on human morbidity, mortality, and economics have been evaluated for several industrialized countries by means of established infrastructure for estimates of epidemiology, disease diagnosis, treatment, and reporting (WHO 2015). In contrast, comprehensive information on the incidence and impact of foodborne disease is not yet available for many developing countries but the burden is undoubtedly also very large (Akhtar et al. 2014). Most foodborne infections and intoxications are caused by Gram-negative enteric bacteria with associated diarrheal syndromes, by certain vegetative Gram-positive bacteria including *Staphylococcus aureus* and *Listeria monocytogenes*, and enteric viruses, particularly norovirus (Doyle and Buchanan 2013). Natural toxins, including mycotoxins and algal toxins, are also an important cause of foodborne disease (Tu 1983–95).

Serious foodborne diseases of high health impact are also caused by spore-forming bacteria, most notably *Clostridium perfringens*, *Clostridium botulinum* and other neurotoxigenic clostridia (Doyle and Buchanan 2013; Hatheway and Johnson 1998; Johnson 2013). In particular, botulism is a paralytic illness that occurs worldwide and is caused by BoNT produced by neurotoxigenic clostridia. The primary categories are infant botulism, in which spores colonize the intestinal tract of infants and produce BoNT, foodborne botulism in which BoNT is ingested in foods, and wound botulism resulting from the colonization of *C. botulinum* and BoNT production in surface wounds (Hatheway 1995; Johnson and Montecucco 2008; Sam and Beynon 2010). Rare forms include adult intestinal botulism, inhalational botulism, and iatrogenic botulism due to injection of inappropriate formulations of pharmaceutical BoNT (Chertow et al. 2006; Ghasemi et al. 2012; Werner et al. 2000). BoNT-producing species include *C. botulinum*, and sporadic strains of *C. argentinense*, *C. butryicum*, *C. baratii*, and *C. sporogenes*. Owing to the high potency of BoNT and its stability in the gastric and respiratory tracts, BoNT is also considered to be a potential agent of oral and inhalational bioterrorism and a potential cause of mass casualties (Arnon et al. 2001). Currently there are no antidotes for botulism, and survival and recovery from botulism relies on intensive nursing care, and in severe cases mechanical ventilation, parenteral feeding, and rehabilitation (Brook 2006; Cherington 2004; Johnson and Monteuccco 2008). Serum therapy by administration of antitoxins has been used to alleviate the symptoms and shorten the duration of the disease. Remarkably, BoNTs have also been developed as highly effective pharmaceuticals (Schantz and Johnson 1992; Johnson 1999; Scott 1989) for the treatment of more than 100 neuronal disorders by direct injection of BoNT into the neuromuscular region of the disorders (Fig. 1) (Truong et al. 2013). Figure 1 also illustrates the diversity of neuromuscular symptoms that can occur in botulism. It is evident from phylogenomic studies that the genes encoding BoNTs have been horizontally transferred to other clostridia; furthermore many neurotoxigenic clostridia possess more than one BoNT gene cluster (Franciosa et al. 2003; Williamson et al. 2016). The interspecies transfer of genes encoding BoNT raises concerns of the generation of novel BoNT-producing organisms posing new health threats.

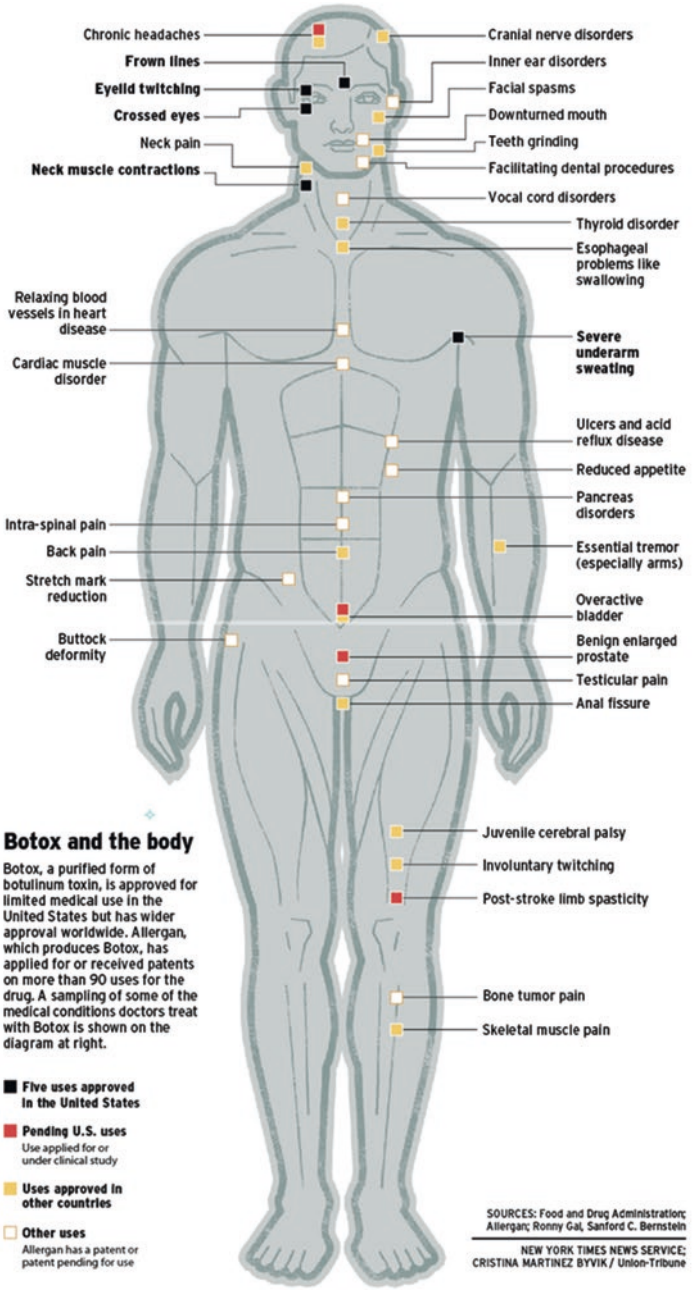

Fig. 1 Neuromuscular regions having shown successful treatment my pharmaceutical botulinum neurotoxin

Botulism as a Public Health Concern

Botulism is a serious public health concern to regulatory agencies, attending physicians and healthcare providers, and the food industry. Important considerations include:

1. Botulism is a reportable life-threatening disease (https://wwwn.cdc.gov/nndss/case-definitions.html; http://www.cdc.gov/nczved/divisions/dfbmd/diseases/botulism/#how_common). Botulism surveillance and emergency response measures have been described (Shapiro et al. 1997). Medical providers should be trained to recognize the signs and symptoms of botulism. Potential botulism cases with characteristic symptoms should be rapidly communicated to the appropriate state health department for possible occurrence and reporting of an incident, and management of the disease.
2. Botulism constitutes a public health emergency. The occurrence of a single case implies that other people may be at risk of contracting the disease.
3. In a probable botulism outbreak, the health care provider should contact their state epidemiology department for consultation. The CDC can also be contacted (www.cdc.gov; 770-488-7100) by the state epidemiologist for consultation and release of equine heptavalent antitoxin (HBAT). In the potential occurrence of infant botulism, the health care provider may contact the California Infant Botulism Treatment Program (www.infantbotulism.gov; 510-231-7600) who can provide valuable information and authorize the release of human-derived antitoxin (BabyBIG®). Both the CDC and Infant Botulism Treatment and Prevention Program provide 24-h, 7-day consultation (Maslanka et al. 2013).
4. The detection of early clinical symptoms should prompt appropriate medical observation and access to medical measures such as nursing care, neurological evaluation, mechanical ventilation, and administration of antitoxin as needed (Chalk et al. 2014; Maslanka et al. 2013; Shapiro et al. 1997).
5. A physical examination by a qualified physician for evaluation of typical symptoms of botulism should initially be performed. A confirmed case of botulism requires detection of BoNT in clinical samples and/or foods, generally by the mouse bioassay, and/or by culturing of neurotoxigenic clostridia from stool, wounds or foods (Hatheway 1988; Johnson 2013; Maslanka et al. 2013; http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070879.htm, accessed April 2016).

Current laboratory methods for the detection of botulinum toxin in clinical specimens and food samples generally require 2–4 days to perform (Hatheway 1998; Maslanka et al. 2013), and after consultation with the appropriate regulatory agency, samples should be refrigerated and sent as soon as possible to state health departments. Appropriate medical procedures and minimizing morbidity and mortality in a botulism outbreak depends on recognition of clinical signs in humans, an epidemiologic investigation, and rapid detection of botulinum neurotoxin and/or *Clostridium botulinum* in clinical specimens and foods (see Emergency Preparedness and Response procedures (http://emergency.cdc.gov/agent/botulism/, accessed April 2016; Arnon 2001).

Publicity Affecting the Public and Companies Involved in Putative Botulism Outbreaks

Botulism is a rare foodborne disease with unique symptoms compared to the more common diarrheal diseases. Botulism can be quite severe and may cause fatalities in humans and animals. Botulism often attracts much interest from the media and the public. The most common class of botulism in the USA and certain other countries is infant botulism, and illnesses in infants (as well as in adults) can be a particularly sensitive issue, and can lead to considerable publicity, often much of it presented in a sensational manner (Dickson 1920; Satin 2008). Important points should be kept in mind for industry and other responses to media releases and public perception:

1. Botulism is a foodborne illness that has been recognized for more than a century; it is not a new disease posing new threats to the human population.
2. Botulism is an extremely rare disease, and in the USA about 120 cases occur per year. Other countries for which epidemiologic data are available also have a very low incidence of botulism.
3. Botulism can be treated by appropriate nursing care and in severe cases by mechanical ventilation, parenteral feeding, and serum therapy.
4. Botulism is not a chronic disease and full recovery generally occurs unless there are underlying diseases. Botulinum toxin in foods is destroyed by heating the food for 10 min at 100 °C or for 30 min at 80 °C (Siegel 1993). However, spores are not destroyed by these heat treatments and the heated food should be discarded and not used in reformulated product.

Brief Historical Description of the Pathology and Microbiology of Botulism: The Foundation for Understanding the Disease and for Control of *C. botulinum* Intoxications

Although anecdotal evidence indicates that botulism was first recognized in ancient civilizations (Smith and Sugiyama 1988), botulism was first convincingly recognized as a distinct disease syndrome in Germany by Müller and Kerner in Germany in the 1700s and 1800s (Ergbuth 2007; Pellett 2012). The disease was noted to initially cause cranial neuronal disturbances of the eyes, mouth, and pharynx, followed by generalized muscle paralysis of the torso, and suffocation in severe cases. In 1820, Kerner published a seminal paper on the association of the disease with consumption of raw blood sausages by 230 people in Germany and termed the disease botulism, which derives from the Latin word “botulus” for sausage (reviewed in Pellett 2012). This important observation of the association of a toxic substance in food with a paralytic and often fatal human illness by Kerner was achieved several

decades before Pasteur, Koch and other founding microbiologists framed the germ theory of disease. Botulism was referred to as Kerner's disease for decades, and this discovery provided a background for studies of investigations of early outbreaks, primarily in the USA, Europe, Japan, and Russia (Dolman 1964; Meyer 1956). In these early outbreaks, the fatality rate was 40–60% (CDC 1998; Dembek et al. 2007; Meyer 1956). Presently, the fatality rate in developed countries has decreased to 5–10%, due to rapid diagnosis, supportive nursing care, parenteral feeding and assisted mechanical ventilation when needed, and serum therapy by administration of antitoxin. Serum therapy is derived by the methods of Von Behring and Kitasato for their work on antitoxins to diphtheria and tetanus (Grundbacher 1992). Von Behring was awarded the first Nobel prize in Physiology or Medicine in 1897 for serum therapy (Winau and Winau 2002). Early in the 20th century, antitoxin was tested against botulinum toxin extracts during the period 1897–1921 for neutralization of toxicity in animals using extracts from the van Ermengem strain (nonproteolytic type B) and *C. botulinum* type A strains (Dickson and Howitt 1920; Burke et al. 1921). They found that antitoxins against botulinum extracts were highly effective in neutralizing toxicity in animals. Serum therapy using antitoxins, although currently considered an archaic method for therapy of infectious diseases and intoxications (Manohar et al. 2015), is still the only method of preventive treatment for botulism today and new treatment modalities are urgently needed that can enter nerves and eliminate symptoms of botulism.

The fundamental information regarding the toxin-forming bacterium, toxicology of culture extracts, pathology, and inactivation and prevention of BoNT formation in foods was described in the late 1800s by van Ermengem (1897), and the principles he established are still valid today. In a remarkable investigation in 1894–96, the microbiologist Emile Pierre van Ermengem, who studied under Claude Bernard and Robert Koch and was eminently capable of recognizing new diseases, investigated a foodborne outbreak associated with 23 illnesses and 3 deaths that occurred from eating ham at a funeral ceremony in Ellezelles, Belgium (van Ermengem 1897). He discovered an anaerobic bacterium he termed "*Bacillus botulinus*" which produced a highly toxic substance. His primary findings of the properties of the bacterium and BoNT are integral for the current control of foodborne botulism: (i) foodborne botulism is an intoxication due to a heat-labile toxin and is not an infection; (ii) the toxin is produced by an anaerobic sporeforming bacillus; (iii) the toxin is active in the absence of the bacterium as revealed by filtration of culture extracts and microscopic examination (iv) the toxin is not inactivated by digestive enzymes in the gut of humans and animals; (v) the toxin is inactivated by moderate heat treatment such as boiling for a few minutes; (vi) the toxin is stable at low pH and in moderate NaCl concentrations; and (vii) BoNT is highly toxic to humans and to certain animals species via oral, intraperitoneal, and intravenous routes.

Following van Ermengem's pioneering study in 1894 and his discovery of BoNT type B (by today's *C. botulinum* taxonomy and BoNT criteria), five different serotypes (A-E) of BoNT were identified during the next 50 years (Johnson 2005). A serotype is defined by toxin neutralization and lethal protection in mice by antibodies raised against BoNTs of a homologous serotype, but not by heterologous

antibodies raised against different serotypes (Giménez and Giménez 1993; Hatheway 1988). Serotypes F and G were subsequently discovered, and currently there are seven known serotypes. Recently, a new serotype "H" was proposed (Barash and Arnon 2014; Dover et al. 2014), but this BoNT has been demonstrated to be a chimeric toxin comprised of BoNT/A and BoNT/F components, is neutralized by antibodies to type A, and thus by definition has been termed BoNT/FA and not BoNT/H (Maslanka et al. 2016; Pellett et al. 2016). Nonetheless, the discovery of a naturally occurring chimeric BoNT comprised of domains of BoNT/A and /F highlights the proclivity of BoNT gene transfer and recombination BoNT genes in the environment or animal hosts.

Researchers in the USA and in Europe mainly provided the foundations for the pathology of botulism in the early 20th century. The first detailed understanding of the pathology of botulism derived from studies of symptoms in animals and humans suffering from botulism (Bishop and Bronfenbrenner 1936; Dickson 1918; Dickson and Shevky 1923a, b), as well as by injection of BoNT into the intact gastrocnemius muscle of animals and careful measure of nerve and muscle activity, which is a technique that is widely used today in the study of BoNT (Guyton 1947; Payling Wright 1955). These studies revealed that BoNT acts on peripheral nerves that innervate striated muscle (Ambache 1949), and the toxin primarily acts at the neuromuscular junction (NMJ) of cholinergic neurons. In particular, BoNT acts on cranial and somatic motor nerves, and may affect the central nervous system (Bishop and Bronfenbrenner 1936; Dickson 1918; Dickson and Shevky 1923a, b). These early studies revealed that BoNT predominantly affects muscles controlling the eyes, mouth, pharynx, and gradually the muscles of the limbs and torso of the animal.

The molecular mechanisms of BoNT remained largely unknown until Burgen and colleagues (1949) used the isolated rat phrenic nerve-diaphragm preparation (Bülbring 1946) and later neuromuscular preparations to examine the effects of BoNTs A and B at the neuromuscular junction (NMJ) (Drachman 1964, 1971; Sellin 1981; Thesleff 1976). Administration of BoNT illicited paralysis of the phrenic diaphragm preparation in a dose-dependent manner with a substantial delay of paralysis compared to other NMJ toxins such as curare and saxitoxin. Kinetic studies and comparison with other toxins revealed that entry of the toxin into the nerves did not occur by penetration of the membrane, but involved a much slower process. This observation of a delay in action is a hallmark of BoNT intoxication, and was explained by the hypothesis that, unlike certain other neurotoxins such as saxitoxin which reach the nerve cytosol by rapid membrane penetration, BoNT requires several steps in the intoxication process, including entry into the lymphatic system and circulation, binding to receptors, endocytosis of BoNT, internalization into the nerve cytosol, and cleavage of SNARE substrates (Montecucco et al. 1994; Schiavo et al. 2000). These findings are highly relevant today since many substances can cause mouse lethality, but most of the nonspecific deaths by non-BoNT toxicants generally occur rapidly (within a few minutes to 1–2 h), whereas botulinum toxin action usually occurs after 4–24 h by oral gavage or intraperitoneal injection depending on the dose. An exception to the slow onset is intravenous injection of

high doses (e.g., 10,000 LD_{50s}), usually into the mouse tail vein (Boroff and Fleck 1966), and in this assay, paralytic symptoms and mouse death generally occur within 20–80 min depending on the dose. Burgen (1948) also provided evidence that acetylcholine was released from nonpoisoned neurons, but was blocked by BoNT in intoxicated neurons. Rats immunized with botulinum toxin were resistant to intoxication and of isolated neuromuscular preparations. Duchen (1971) and Duchen and Strich (1971, 1972) determined there were striking changes in the ultrastructure of motor endplates of NMJs following injection with BoNT. Taken together, these findings in intact animals and in diaphragm and NMJ preparations reveal the relatively slow onset of symptoms, including death, and provided the basis for our current understanding of the molecular mechanism of BoNT.

The fundamental aspects of the epidemiology and microbiology of *C. botulinum* and BoNTs were recognized during the period of 1920–1970 by prodigious microbiologists and physicians in the USA, Japan, Russia, and Europe (Hauschild 1989, 1993; Johnson 2005; Meyer 1956). In the USA, from 1913 – until his death in 1974, Karl Friedrich Meyer studied botulism and other diseases in California at the Hooper Foundation at the University of California San Francisco, which was the first (1914) medical research foundation incorporated into a university setting in the USA (history.library.ucsf.edu/hooper/html). It has been stated that *"Meyer influenced more microbiological and epidemiological domains than other scientists of his time; and he was driven by his deep-seated concern for the welfare of the people"* (Elberg et al. 1976). Meyer and colleagues (1956) discovered many cardinal properties of *C. botulinum*, its endospores, and BoNTs. They determined that the distribution of *C. botulinum* spores occurred worldwide and were present in approximately 35% of soil samples examined in the USA. Further, many regions of Europe had similar prevalences of *C. botulinum* spores. They also determined the high heat resistance of the spores and defined the thermal requirements for inactivation of *C. botulinum* spores (Esty and Meyer 1922), which led to the advent of the 12D "botulinum cook" treatment, which is still in use today and has been amazingly successful in preventing commercial outbreaks of botulism and spoilage in hermetically sealed (canned and pouched containers) (Lynt et al. 1975; Pflug 2010; Pflug et al. 1981). Meyer and Sommer also initiated studies on the isolation of botulinum neurotoxins, and determined that more than 90% of toxicity is precipitated from cultures by the addition of inorganic acids such as sulfuric acid to pH 3.5 (reviewed in Schantz and Johnson 1992). Acid precipitation is the first step in nearly all BoNT purifications of the seven serotypes currently performed. Meyer and colleagues also determined that growth of group I *C. botulinum* was inhibited at pH values $\leq$4.6 or $\geq$10% salt, and that growth of most strains did not occur at temperatures below 4–10 °C or above 50 °C, depending on the physiological group (I-IV) (Holdeman 1977) of *C. botulinum*. These parameters have served as the basis for formulation and extrinsic control of botulinogenic safe foods through the present.

During evaluation of early botulism cases, it was recognized that symptoms of botulism generally began 14–30 h after ingestion of the contamined food (Meyer 1956; Johnson and Montecucco 2008; Sobel 2005). Initial sporadic symptoms frequently are nausea, vomiting and diarrhea, but these gastrointestinal symptoms are

Table 1 Primary symptoms of botulism (Johnson and Montecucco 2008; Cherington 2004)

Symmetrical cranial neuropathies
Blurred near vision, blurred distant vision, dilated or nonreactive pupils, diplopia, drooping eyelids
Difficulty swallowing, dry mouth, difficulty speaking, facial ptosis
Descending bilateral flaccid paralysis, generalized muscle weakness progressing to neck, limbs and torso, paralysis of the respiratory diaphragm and death in the absence of mechanical ventilation

probably caused by the local action of the spoiled food in the gastrointestinal tract and not by BoNT itself. Nausea and vomiting are not usually seen in infant botulism, rather the first sign is constipation due to paralysis of intestinal musculature (Arnon 2006). In infants and adults, BoNT causes motor paralysis and the initial disturbances affecting cranial nerves, especially of the eyes, mouth and pharynx, including diplopia, mydriasis, nystagmus, strabismus, lack of response of the eye pupils, blepharoptosis, dysphagia, and aphonia (Table 1). The facial and mouth features include ptosis in the face, sluggish movements of the tongue, dry mouth, and accumulation of thick mucus in the pharynx (Cherington 1998, 2004; Johnson and Montecucco 2008; Shapiro et al. 1998; Sobel 2005). The intoxication then bilaterally descends to the torso and can paralyze all skeletal muscles. Other neurological signs are often seen in botulism depending on the BoNT serotype, including autonomic and parasympathetic symptoms. Retention of urine is commonly observed which is not due to kidney pathology but rather to weakened bladder contraction. Depending on the serotype, BoNT also causes disturbances in cholinergic-mediated secretions, including decreased sweating in the face, palms, and feet, lack of saliva formation, dysfunctional erection, constipation, and these symptoms are attributed to the effects of BoNT on the parasympathetic and autonomic nervous systems (Dressler and Benecke 2003; Merz et al. 2003; Tintner et al. 2005). BoNT does not cause fever (body temperature may be subnormal) or headaches in subjects, and thinking is clear except for the anxiety and depression due to the awareness of the disease (Cohen and Anderson 1986). Blood pressure and cardiac rate are generally unaffected. Analyses of recovered central and peripheral nerves have not shown permanent damage supporting that botulism is an acute and not a chronic disease syndrome.

In the 1940s and 1950s it was determined that the BoNT component existed in large protein complexes that formed paracrystals of high molecular weight (reviewed in Schantz and Johnson 1992), and, under certain conditions, the BoNT neurotoxic component could be separated from the complex (Sakaguchi 1983; Schantz 1964; Schantz and Johnson 1992). In the 1960s and 1970s, the neurotoxin component (BoNT) was isolated as a purified protein from the toxin complexes (DasGupta and Boroff 1967; Sugiyama 1980). BoNT was determined to consist of a heavy chain (ca. 100 kDa; HC) and a light chain (ca. 50 kDa; LC) linked by a disulfide bond (DasGupta and Sugiyama 1972). The availability of the purified BoNT component of the complex enabled definitive studies of biochemistry, structure and mechanisms of action. In the 1980s, the molecular mechanism of BoNT acting on the NMJ

was elucidated using purified BoNTs (reviewed in Schiavo et al. 2000; Rossetto et al. 2014). The three-dimensional structure of BoNT/A (Lacy et al. 1998, 1999) and later /B and /E (Swaminathan 2011) was determined along with variants (subtypes) within serotypes (Arndt et al. 2006), thereby providing critical insights into understanding the molecular mechanisms of BoNT, their immunological aspects, and the pathology of botulism.

The pathology of BoNTs has been extensively studied in mice (Simpson 2013), yet many aspects of the intoxication process are still unclear. BoNT intoxication involves a complex pathway, in which BoNT is absorbed into the lymphatic circulation and then into systemic circulation, from which it exits the main vasculature into capillaries and traffics to and binds at nerve terminals. The mechanisms of transport to the lymphatic system, into the circulation, and disposition from the circulation are not clear. After exiting the capillaries, the presynaptic mechanism of intoxication has been well studied (Montal 2010; Rossetto et al. 2014; Simpson 2000, 2013). The HC contains receptor-binding domains at its C-terminus, which have strong affinities to polygangliosides and, in some serotypes, for protein receptors including isoforms of SV2 and synaptotagmin within the neuronal membranes (Montal 2010; Rossetto et al. 2014). Following endocytosis and uptake in recycling synaptic vesicles, the HC N-terminal domain mediates translocation of the LC from the vesicle into the neuronal cytosol by formation of a channel within the H chain in the synaptic vesicle membrane (Montal 2010; Pirazzini et al. 2016; Rossetto et al. 2014). Once internalized in the cytosol, the LC acts as a protease with high specificity for SNARE (soluble N-ethylmalemide-sensitive fusion-associated) proteins (SNAP-25, VAMP, and syntaxin) which are responsible for trafficking and fusion of synaptic vesicles with the presynaptic membrane and release of acetylcholine (and other transmitters depending on the organ and innervation) at the neuromuscular junction (NMJ) (Schiavo et al. 2000; Rossetto et al. 2014). The deficiency in release of acetylcholine prevents muscle activation and contraction and results in the characteristic long-lasting flaccid paralysis, the hallmark property of botulism.

The inability of synaptic vesicles to fuse with neuronal membranes and to release neurotransmitter also prevents vesicle recycling and thus the nerve terminal presumably becomes resistant to further uptake of BoNTs by the major pathway of vesicular trafficking, and the toxin then affects other susceptible nerves (Dong et al. 2006), descending to large muscles with a comparatively low nerve-muscle ratio such as those in the limbs. The mechanism of vesicular trafficking within mammalian cells was elegantly revealed by in vitro reconstitution of the vesicle trafficking mechanisms by independent researchers. The elucidation of the mechanisms of SNARE proteins in trafficking of vesicles in cells resulted in the 2013 Nobel Prize in (Physiology or Medicine awarded to James Rothman, Randy Schekman, and Thomas Südhof http://www.nobelprize.org/nobel_prizes/medicine/laureates). The discovery of the function of SNARE proteins in vesicular trafficking and exocytosis, clarified the presynaptic mechanisms of BoNT since SNAP-25, syntaxin, VAMP I, II are the specific targets of BoNTs (Schiavo et al. 2000).

Clostridium botulinum and Other Neurotoxigenic Clostridial Species

Botulinum neurotoxins (BoNTs) are mainly formed by the species *Clostridium botulinum*, which is a diverse assemblage of clostridia comprising at least four independent clostridial lineages (Collins and East 1998; Collins et al. 1994; Hutson et al. 1993a, b; Smith et al. 2015). Neurotoxigenic clostridia have in common the requirements for anaerobic growth, complex nutrient needs, formation of resistant endospores, and elicitation of a highly potent neurotoxin. The group *C. botulinum* is artificially designated as a single species due to the common clinical property of producing BoNTs, but this "species" is highly variable from phylogenetic and evolutionary perspective, and in the ability to form the seven known serotypes (A-G) of BoNTs (Lawson and Rainey 2015). Sporadic isolates of *Clostridium butyricum, Clostridium baratii*, and *Clostridium argentinense* have also been shown to produce BoNTs /E /F, and /G, respectively (Hatheway 1993; Suen et al. 1988). Rare strains of *C. sporogenes* possess the genes for BoNT formation (Weigand et al. 2015), but no known human or animal cases of botulism have resulted from this organism. Nonetheless, the acquisition of genes encoding BoNTs by *C. sporogenes*, which often shows higher resistance properties of its spores to heat and vegetative cells to salt and acidity than *C. botulinum*, creates concern about horizontal gene transfer of genes for BoNT to more resistant organisms than *C. botulinum*. The occurrence in *C. botulinum* on plasmids harboring the genes for BoNTs, with some having conjugative properties, was initially identified in our laboratory for type A (Marshall 2007, 2010), and later confirmed (Smith et al. 2007, 2015) and expanded to other *C. botulinum* serotypes and to C. *butyricum* using experimental genetic and genomic approaches (Carter et al. 2016; Franciosa et al. 2003, 2009; Sebaihia et al. 2007).

The initial taxonomic classification of the neurotoxigenic clostridia was based on production of BoNTs and structural and physiological properties, especially a Gram-positive cell wall structure, formation of endospores, saccharolytic and proteolytic abilities, as well as cardinal intrinsic and extrinsic parameters governing growth, including temperature, pH and a_w (Franciosa et al. 2003; Holdeman et al. 1977; Hatheway 1993; Johnson 2005). *C. botulinum* has been classified for several decades into four physiological groups (I-IV) (Hatheway 1993; Holdeman 1977; Johnson 2005; Smith et al. 2015), and *C. butyricum* and *C. baratii* have been considered Groups V and VI by some researchers (Franciosa et al. 2003). Cardinal physiological properties control growth and BoNT formation by the different Groups of *C. botulinum* neurotoxigenic clostridia, and are paramount in formulating and processing various classes of foods to prevent botulism outbreaks (Franciosa et al. 2003; Glass and Johnson 2002; Hauschild 1989; Johnson 2013). Due to the severity of botulism, certain food laws have been implemented mainly to control growth and BoNT formation by *C. botulinum* in foods, including required heat treatments of foods in hermetically sealed containers (12D "bot cook"), low-acid food regulations (pH > 4.6), and critical values of water activity (≤0.93) in food

formulations. From an economic perspective, cases of botulism are among the highest costs per case than other foodborne diseases (Roberts 2000).

Since the 1970s, molecular properties have become the primary criteria for taxonomic delineations within genus *Clostridium.* These have been based primarily on the homologies and sequences of genes encoding 16S rRNA (Collins et al. 1994; Hutson et al. 1993a, b; Johnson and Francis 1975; Lawson and Rainey 2015). However, the genus *Clostridium* over time became a "general depository" for Gram-positive sporeforming bacteria, and eventually expanded to 228 species (Lawson and Rainey 2015). Recently, it was proposed to restrict the genus *Clostridium* Prazmowski to *Clostridium butyricum* (the type species) and related bacteria, designated *Clostridium sensu stricto* (clostridial rDNA group I) (Lawson and Rainey 2015). Group I includes *C. butyricum* (the type species of *Clostridium*), *C. botulinum* serotypes A-G *C. argentinense*, *C. baratii*, and *C. tetani*. Furthermore, *C. botulinum* and other pathogenic clostridia have been analyzed by complete and partial chromosomal gene sequences, particularly for genes encoding BoNTs and associated proteins that complex with the neurotoxin (Williamson et al. 2016). Detailed phylogenomic analyses of *C. botulinum* and related clostridia have been described (e.g. Williamson et al. 2016), and while these genomic studies provide phylogenomic insights, they will require considerable supplemental research to elucidate the biochemistry, physiology, and practical genetics for a better understanding of the biology of the neurotoxigenic clostridia and the functions of their neurotoxins, as well as food safety.

A breakthrough in the understanding of toxigenesis in *C. botulinum* took place during the study of unusual strains in which it was very difficult to neutralize toxicity using single serotype-specific antibodies, while multi-serotype mixtures provided protection against lethality in mice (Ciccarelli and Giménez 1972; Giménez and Ciccarelli 1970; Hatheway et al. 1981). By tedious serological neutralization experiments, these groups showed that the two *C. botulinum* strains (strain 84 and 657Ba) produced more than one serotype of BoNT. Surprisingly, *C. botulinum* strain 84 has recently been shown to produce three BoNTs (Dover et al. 2013). In multi-BoNT producing strains, one of the BoNTs is usually produced in greater quantities compared to the lesser BoNT and the major BoNT is designated in upper case, e.g. Ab. The regulation of the differential expression of the multi-BoNT-gene clusters is not currently understood (Connan and Popoff 2015; Johnson and Bradshaw 2001). Franciosa et al. (1994) expanded upon this work and showed that many strains of *C. botulinum* possess two toxin gene clusters, in which one of the clusters is often "silent" or unexpressed due to mutations in the unexpressed gene encoding an inactive BoNT.

The mobility of BoNT gene clusters was further supported by the isolation from infant botulism cases of strains of *C. butyricum* and *C. baratii* that produced BoNT/E and /F, respectively (Aureli et al. 1986; Hall et al. 1985; McCroskey et al. 1986). Since these initial isolations, several strains of *C. butyricum* and *C. baratii* have been isolated from infant botulism cases, foodborne botulism incidents, and the environment (Franciosa et al. 2003; Hatheway and Johnson 1998; Johnson 2013). Phylogenomic studies have supported that horizontal transfer of genes encoding BoNTs has occurred independently in the evolution of neurotoxigenic clostridia

(Peck 2009; Skarin et al. 2011, 2015; Skarin and Segerman 2011, 2015; Williamson et al. 2016). The food and medical importance of BoNT gene transfer may be substantial, since new neurotoxigenic clostridia could arise, but the mechanisms of molecular transfer and selective advantages of BoNTs for neurotoxigenic clostridia is unclear.

Botulinum Neurotoxins

C. botulinum produces seven serotypes of BoNTs (A-G), and rare neurotoxigenic strains of *C. butyricum*, *C. baratii,* and *C. argentinense* produce serotypes E, F, and G, respectively (Franciosa et al. 2003; Johnson 2013). Human disease is caused predominantly by BoNT serotypes A, B, and E. Serotypes C and F have been associated sporadically with human illness (Fenicia and Anniballi 2009; Koepke et al. 2008; Johnson and Montecucco 2008), whereas types D and G have not been reported to cause human botulism (Hatheway 1995). The specific toxicity of BoNTs measured in mouse LD_{50s} ranges from 3×10^7 to 3×10^8 per mg for the seven serotypes. As described above, seven distinct BoNT serotypes A through G are defined through neutralization by homologous polyclonal antitoxins (Giménez and Giménez 1993; Hatheway 1988). Antibodies for study of BoNTs are usually raised in rabbits, horses, goats, or mice, using intramuscular injections of formalized toxoid, recombinant toxoids, or BoNT fragments, particularly the receptor-binding domain of the HC, which has high immunogenicity compared to other regions of BoNTs (Karalewitz and Barbieri 2012; Webb and Smith 2013). Cross-reactivity with antibodies has been observed between serotypes C and D or E and F (Sugiyama 1980; Giménez and Giménez 1993). Certain BoNTs, particularly C, D, (Nakamura et al. 2013) and the newly described FA (Pellett et al. 2016), are comprised of mosaic BoNTs comprised of domains of different serotypes. These chimeras are usually less efficiently neutralized by standard methods and optimization of the antitoxin assays is required (Maslanka et al. 2016; Pellett et al. 2016). With new molecular biology techniques, the BoNT/B gene was genetically inactivated, which enabled the purification and definitive characterization of BoNT/FA (Pellett et al. 2016).

DNA sequencing of genes, encoding BoNTs have shown that there is a large diversity in the BoNT family (Fig. 2). Within the seven serotypes, more than 40 variants (designated subtypes) of BoNTs have been detected by *bont* gene sequencing (e.g. Williamson et al. 2006) and later, arbitrarily defined as having ≥2.6% differences in amino acid sequence (Hill et al. 2007; Hill and Smith 2013). This arbitrary classification, based on a fixed degree of change in amino acid sequence, does not necessarily represent substantive differences in BoNT biological and toxicological properties, since even a change of one or a few amino acids can affect BoNT characteristics and function (Montecucco and Rasetto 2015; Rossetto et al. 2014). For example, one amino acid change in the LC can destroy catalytic activity (Pier et al. 2008). Structural modeling of subtypes A1-A4 have revealed interesting differences in the location of the variant amino acids (Arndt et al. 2006) and these

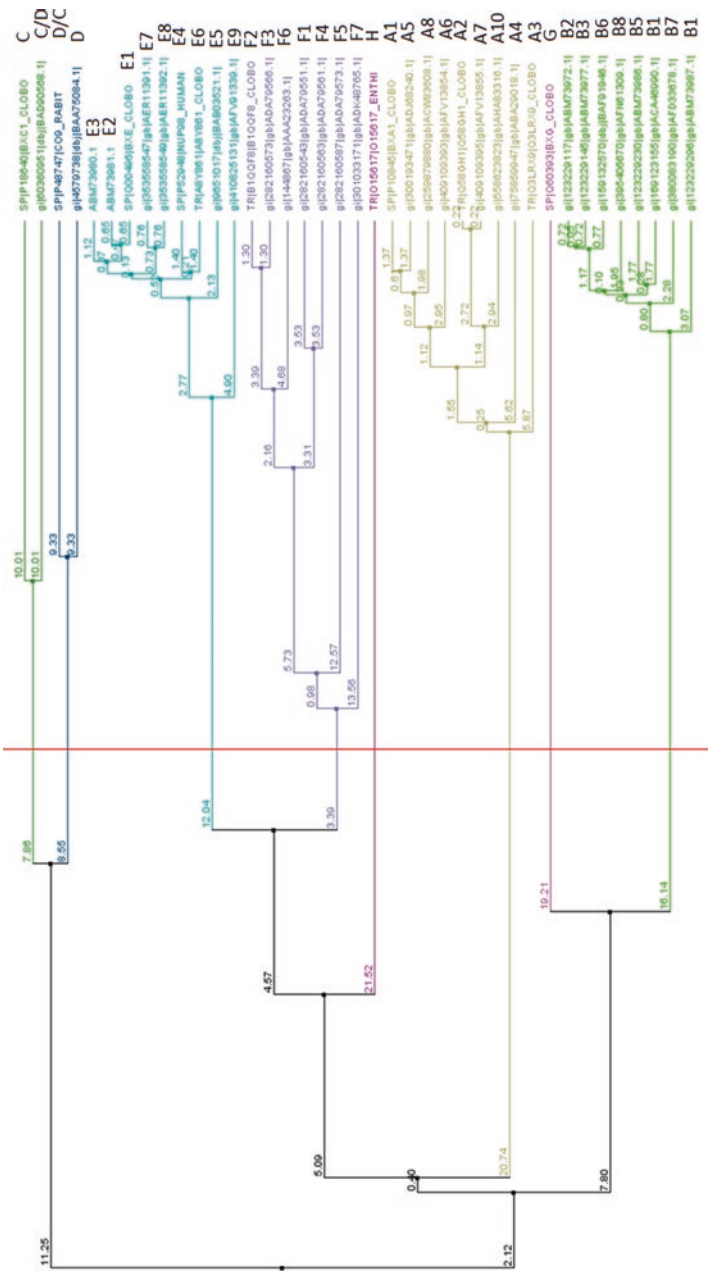

Fig. 2 Phylgenomic diagram of the different serotypes and certain subtypes of *Clostridium botulinum*

differences are likely related to differences in properties of the subtypes (Rossetto et al. 2014). Some of the subtypes within a serotype have distinct and intriguing neurological and pathologic properties in mouse models including rate of onset of symptoms, duration of action, effectiveness of cross-neutralization by antibodies raised against a heterologous subtype, and differences in pathological symptoms (Pellett et al. 2015).

The neurotoxic BoNT component is known to reside in protein complexes (Benefield et al. 2013; Inoue et al. 1996; Lin et al. 2015; Oguma et al. 1995; Sugiyama 1980; Sakaguchi 1983; Sugii and Sakaguchi 1975). The 150-kDa BoNTs exist naturally in two primary types of protein complexes. One class (HA; Type I complex) consists of BoNT associated with hemagglutinins and a protein termed non-toxic non-hemaglutinin (NTNH), and these complexes range in molecular weight from ~500–900 kDa. The second class of BoNT complex (NTNH ("orfX"), Type II) consists of BoNT associated only with NTNH, without the presence of hemagglutinins or other proteins, and has a molecular weight of ~300 kDa. The complexes are also known to contain ribonucleic acid (Schantz and Johnson 1992), but the RNA in the complexes is probably random sequences of rRNA binding through ionic interactions and would not be expected to have a functional role, except for changing the electronic charge of the complex. The complexes are well-known to protect the labile neurotoxins during passage through the gastrointestinal tract, during handling, and likely in the environment (Bonventre 1979; Schantz and Johnson 1992; Ohishi et al. 1977; Simpson 2013; Sugii et al. 1977).

BoNTs are produced as single chain ~150-kDa proteins and undergo post-translational cleavage to the toxic dichain form with HC and LC linked by a disulfide bond (DasGupta and Sugiyama 1972; Sugiyama 1980; Sakaguchi 1983). BoNTs from proteolytic strains of BoNTs A, B, and F are proteolytically cleaved (activated) in native cultures of *C. botulinum*. The inactive 150-kDa single chain BoNT/A is modified to the dichain form by removal of 11 amino acids by native proteases expressed by *C. botulinum* (Krigelstein et al. 1994). BoNTs from proteolytic strains of B and F also undergo native cleavage, but the mechanisms and amino acids removed during conversion of the dichain form remain to be elucidated. In nonproteolytic (physiological Group II) *C. botulinum* types B, E, and F the toxins are not cleaved in culture, and nicking to the dichain form can be artificially achieved by growth in the presence of trypsin or preferably by *in vitro* cleavage to the dichain form (generally with trypsin) (Sugiyama 1980). Following incubation with trypsin, soybean trypsin inhibitor is used in our laboratory to prevent undesired subsequent degradation of BoNT (Pellett et al. 2016). Trypsin treatment cleaves BoNTs at single residues and differs from the native cleavage by clostridial proteases in Group I *C. botulinum* which remove a series of amino acids. This non-native cleavage step by trypsin or other proteases is also necessary in BoNTs produced recombinantly in *E. coli* and other heterologous hosts, and it is not clear whether the differences of in vitro post-translational modification affect biological properties compared to naturally cleaved BoNTs. Cleavage to the dichain form in certain serotypes can result in a 10–100-fold increase in toxicity in mice (Oguma et al. 1995; Sugiyama 1980).

Molecular changes other than cleavage can lead to significant activation and increased potency, but these noncovalent modifications have not been revealed.

Other biochemical and physiological features of BoNTs contribute to their importance as natural intoxicants. BoNT is highly unique compared to most other proteins in that it is stable in the gastrointestinal tract and is absorbed in its intact form into the lymph and blood stream on ingestion (Simpson 2013). In contrast, most other ingested proteins in the diet are degraded in the intestinal tract and are not translocated through the intestinal mucosa to the circulatory system except in the case of sepsis or disruption of intestinal barriers. The stability in the gastric tract and uptake through the intestinal lumen and to the lymph and blood stream may be a common feature of certain microbial enteric protein toxins. The mechanism of intestinal transfer of BoNTs is an active area of study, and current proposed mechanisms have been reviewed (Simpson 2013).

Pathology of Botulism in Humans

The historical basis for the pathology of BoNT was described in section "Brief Historical Description of the Pathology and Microbiology of Botulism: The Foundation for Understanding the Disease and for Control of *C. botulinum* Intoxications", and newer aspects of pathology are described in this section. BoNT affects the neuromuscular system, in which the functional structure is the motor unit (Buchtal et al. 1980). The motor unit emanates from the anterior horn of the spinal cord and the cranial motor nerve nucleus in the brain stem (Ropper et al. 2014). Motor and sensory nerves of the motor unit innervate muscle at the neuromuscular junction (NMJ) (Pytel and Anthony 2015; Sanes and Lichtman 2001). Both striated cranial and skeletal muscle and unstriated smooth muscle are affected by BoNT-intoxicated nerves. Botulinum toxin inhibits vesicular neurotransmission from nerve terminals, resulting in blockage of exocytosis of neurotransmitters (Rizo and Xu 2015; Rossetto et al. 2014). BoNT acts specifically on the nervous system due to its affinity for ganglioside and protein receptors, and by its ability to be internalized into the nerve cytosol by endocytosis and channel formation (Pirazzini et al. 2016), and cleavage of the SNARE proteins SNAP-25, VAMP I and II, and syntaxin (Schiavo and Montecucco 2000; Rossetto et al. 2014). Vesicular neurotransmission also occurs in non-neuronal organs such as the endocrine system for release of hormones, for instance, insulin. However, non-neuronal organs do not possess polysialogangliosides and the protein receptors for BoNT, and some organs may not contain the BoNT-specific SNARE protein substrates (SNAP-25, VAMP I and II, and syntaxin). One example is endocrine cells that contain SNAP-23, which is not cleaved by native BoNTs. When genetic modifications of BoNT are made for cleavage of SNAP-23 (Sikorra et al. 2015), inhibition of release of hormones can occur (Masuyer et al. 2014). The severity of symptoms and duration of botulism in cell and animal models depends on the serotype and subtype of BoNT, generally increasing in the severity

and duration in the order BoNT/A ≥ BoNT/C1 > BoNT/B > BoNT/F > BoNT/E (Foran et al. 2003a, b; Johnson and Montecucco 2008).

Botulism also affects the autonomic nervous system, including, the intestinal musculature, bladder, intestines, sweat glands, and eye pupils, (Dressler and Benecke 2003; Jenzer et al. 1975; Merz et al. 2003; Tintner et al. 2005). BoNT/B intoxication is often associated with autonomic symptoms (Goode and Shearn 1982; Merz et al. 2003). Case reports have described disturbances in the autonomic system, such as dry mouth and throat, postural hypotension, anhydrosis (lack of sweating), urine retention due to absence of bladder contraction, sialorrhea, erectile dysfunction, pupil dilation, and effects on smooth muscle in the esophagus and intestinal tract (Dressler and Benecke 2003; Merz 2003).

Although the effects of BoNT on smooth muscle are less studied than striated muscle, BoNT causes a long lasting paralysis of smooth muscle. Smooth muscle is a major component of the walls of hollow organs, including the gastrointestinal tract, the trachea, bronchi of the respiratory system, blatter, uterus, blood vessels in the cardiovascular system, and the urogenital system. Smooth muscle contracts more slowly than skeletal muscle, thus often exhibits sustained contraction, and also relaxes more slowly than skeletal muscle (Brozovich et al. 2016). Pharmacological treatments of smooth muscle with BoNT can cause long-lasting denervation, often of a year or more (Truong et al. 2013).

The pathology of botulism intoxication and recovery follows these general steps (Montecucco et al. 1994; Schiavo et al. 2000; Foran et al. 2003a, b; Simpson 2013):

1. Absorption of BoNT into the lymphatic system and then into the vasculature through the gastrointestinal tract, wounds, or by inhalation
2. Exit from the vasculature and binding to gangliosides and receptors on neurons
3. Vesicular endocytosis and entry of LC into the neuronal cytosol, leading to cleavage of SNARE protein substrates
4. Catalytic cleavage of the SNARE substrates SNAP-25, VAMP I and II, and syntaxin according to serotype
5. Inhibition of trafficking of vesicles containing acetylcholine (or certain other neurotransmitters) to the membrane and stopping of exocytosis
6. Postsynaptic effects on muscle, including denervation of muscle fibers is followed by atrophy and disruption of ion movement in muscle membranes
7. Eventual recovery of the NMJ block occurs through neurite sprouting and reestablishment of the original NMJ
8. Gradual neuronal and muscle recovery from the intoxication. Since nerve and muscle cells are not killed, the original NMJs are replenished.

Irrespective of the category of botulism, the primary clinical signs are similar (Table 1). The characteristic symptoms of botulism can be principally ascribed to the blockade of acetylcholine transmission at neuromuscular junctions of cranial and skeletal muscle (Dickson 1918; Koenig 1971; Cherington 1998, 2004). The disease initially affects the 12 cranial nerves in the facial region and presents in patients with bilateral vision impairment, ptosis of facial features, dry mouth (due

to lack of production of saliva), slurred speech, and difficulty chewing and swallowing. This pathology is followed by weakness in the neck, shoulders, chest, muscles of respiration, particularly the diaphragm with accompanying labored breathing, and eventually weakness in the legs, arms and hands (Cherington 1998, 2004; Johnson and Montecucco 2008). Although the different forms of botulism show similar clinical signs and symptoms, there are certain distinctions. Before the onset of foodborne paralytic botulism symptoms, patients may have gastrointestinal symptoms such as nausea, vomiting, abdominal cramps and diarrhea (Hughes et al. 1981; Johnson and Montecucco 2008). These symptoms are probably not due directly to BoNT but from components of the spoiled food. Infant botulism differs from other forms of botulism in the ages of the affected individuals and intestinal colonization by neurotoxigenic clostridia Infant botulism often begins with severe constipation, lasting 3 days or longer, that precedes the appearance of other neurologic signs affecting cranial and somatic neuromuscular systems (Arnon 2013; Johnson and Montecucco 2008). BoNT does not usually cause a fever, affect consciousness or alter blood pressure. Physicians often refer to botulism symptoms as a classical triad: bulbar palsy and descending paralysis, lack of fever, and clear mental status (Arnon 2013).

An intriguing question is the progression of symptoms in botulism. Botulism symptoms nearly always first begin with cranial neuropathies and then descend bilaterally to the respiratory diaphragm and skeletal muscle. The initial effects on cranial nerves is likely due to the high level of innervation of cranial muscles and the size of the musculature, i.e. precise and small muscles affecting the eyes (Ruff 2002). The number of muscle fibers within each motor unit throughout the body differ markedly (Ropper and Adams 2014). The degree of innervation is higher for the cranial musculature than for skeletal muscle. Muscles with high precision movement, such as eye muscles, have a high neuron to muscle fiber ratio (ca. 1:10), whereas muscles with relatively extensive and broad movements such as calf muscles, have a much lower neuron to muscle ratio (ca. 1:2000) (Ropper et al. 2014). The high level of nerve to muscle ratio in the cranial motor units probably is the reason that the initial symptoms of botulism always involves cranial nerves, particularly eye movement and vision as well as cranial nerves of the face and mouth, and then is followed by paralysis of the mouth and skeletal muscle of the torso where the ratio of nerve to muscle in the motor units is less. Other factors could be involved in the progression of symptoms in botulism such as differential exit from the blood stream and differences in NMJ structure and composition, but more studies are needed to further understand the progression of symptoms in botulism.

In humans, BoNT does not appear to accumulate in cells or tissues and when exocytosis of neurons is disrupted by intoxication they are generally not sensitive to further uptake of BoNT by primary recycling vesicular transport (Dong et al. 2006; Harper et al. 2016). A minor proportion of BoNT is internalized into nerves by non-recycling vesicles (Harper et al. 2016). Recent evidence supports that similar to tetanus neurotoxin, BoNT can undergo transynpatic transport to the CNS and this is an exciting and active area of study (Mazzochio and Caleo 2015; Wang et al. 2015).

Epidemiology of Botulism

The USA has one of the highest incidences of botulism worldwide, with about 120–150 confirmed cases per year (CDC 2012). The annual occurrence of botulism in the USA usually comprises 10–30 cases of foodborne botulism, 70–130 cases of infant botulism, and 10–30 cases of wound botulism. (www.cdc.gov; www.cdc.gov/nationalsurveillance/pdfs/botulism_cste_2014.pdf). Various countries have different incidences of foodborne and infant botulism (Hauschild 1989, 1993; Johnson and Goodnough 1998), which is related to the level of spores in the environment and in the raw commodity or formulated foodstuff, food processing and handling practices (Dodds 1993; Hauschild 1993). The differences in the incidence of infant botulism worldwide is related to the number of spores in infant foods and the environment (Dodds 1993; Koepke et al. 2008). The food supply for various countries is increasingly global and spores in foods such as honey, which is often formulated from different geographical regions, and caution is needed in interpreting the causative geographic region leading to different incidences of infant and foodborne botulism. The epidemiology of botulism has been described in several reviews (Hauschild 1993; Johnson and Goodnough 1998; Sobel et al. 2004; Koepke et al. 2008), although most of these reviews do not cover the past two decades so updates will be valuable.

Categories of Botulism

Foodborne Botulism

Foodborne botulism is mainly caused by the ingestion of foods containing preformed BoNT. On ingestion, BoNT is absorbed from the small intestine into the lymphatic system and is trafficked to lymph nodes and then into the circulation. BoNT then exits the circulation through capillaries and is bound by nerves in the peripheral nervous system. The period from exposure to BoNTs to onset of symptoms ranges from 4 h to as long as 10 days, but typically presents within 8–36 h (Johnson and Montecucco 2008). The onset period, duration, and severity of foodborne botulism is correlated with the quantity of BoNT ingested (Nishiura 2007). Although infrequently described, it is conceivable in many cases of foodborne botulism that the consumed food contains not only preformed BoNT but a mixture of BoNT, vegetative cells, and spores of *C. botulinu*m. The presence of cells and spores in the ingested foods may permit some growth and BoNT formation in the gastrointestinal tract, potentially prolonging the duration and the severity of botulism.

Most foodborne botulism outbreaks worldwide are due to home-prepared and preserved foods, mainly involving vegetables, fruits, and fish (CDC 1998; Hauschild 1989, 1993; Johnson and Montecucco 2008). Commercial- and restau-

rant-prepared foods have also been responsible for foodborne botulism outbreaks in accordance with a changing epidemiology involving restaurant-associated botulism (MacDonald et al. 1986). Prominent recent commercial examples include canned and frozen chili and carrot juice (Juliao et al. 2013; Sheth et al. 2008). Botulism occurring in prisons from fermented "Pruno" (Walters et al. 2015) illustrates how BoNT can easily be formed in beverages and foods using common food substrates with high levels of spores such as potatoes (Angulo et al. 1998). Fermented potatoes have been involved in all the outbreaks of Pruno botulism (Vugia et al. 2009; Walters et al. 2015).

In nearly all cases in the USA, foodborne botulism is caused by proteolytic *C. botulinum* (Group I) strains due to the heat and chemical resistance of the spores, which can survive home-canning and many commercial food processes and formulations, and can outgrow and produce BoNT in the product (Johnson 2013). Most of the outbreaks in commercial foods have been due to insufficient processing to kill spores of proteolytic strains of serotypes A and B retaining viable spores, and the major contributing factor then is temperature abuse of the food (Glass and Johnson 2002; Setlow and Johnson 2013). In current food production, there is a trend for "natural" foods and "clean labels" with elimination of preservatives and traditional secondary barriers. Many of these foods rely primarily on refrigeration for microbiological safety and do not have secondary barriers to prevent growth during temperature abuse (Glass and Johnson 2002; Johnson 2013). Ensuring safety of minimally processed foods by refrigeration is a particularly susceptible risk factor in food safety (Glass and Johnson 2002).

The level of spores in food commodities is directly related to the incidence of botulism (Hauschild 1989, 1993; ICMSF 2005). Many soil-grown vegetables have higher levels of spores than tree fruits, milk, and meats, including poultry, beef, and pork (ICMSF 2005). Fish and shellfish as well as marine mammals and products such as fish eggs may contain high levels of type E spores (ICMSF 2005). Certain foods grown in soil, including mushrooms, garlic, onions and potatoes or foods that may be cultivated in or fertilized with manure such as *Agaricus* mushrooms can contain high levels of spores and have been responsible for outbreaks of foodborne botulism (Johnson 2013; CDC 1998; Smith and Sugiyama 1988). The intestinal contents of many animals (but not humans) contain spores of *C. botulinum* and it is important during processing that the intestinal contents be separated from the meat to the extent feasible. Although healthy humans are not a known carrier of botulinum spores, it is interesting that gorillas (*Gorilla gorilla*) appear to harbor botulinum spores (Bittar et al. 2014). *C. botulinum* spores have also been reported from an 11th century A.D. pre-Columbian Andean mummy (Santiago-Rodriguez et al. 2015). Microbiome studies have begun to explore the microbiota of colonized infants (Shirey et al. 2015), and this approach will be useful in defining the effect of competitor organisms on the ability of neurotoxigenic clostridia to colonize the intestinal tract of humans of various ages and health.

Infant Botulism

Infant botulism, also known as floppy baby syndrome, is the most common form of botulism in the USA, with 70–130 confirmed cases per year (CDC 2012). Infant botulism is also the predominant form in certain other Western countries (Arnon 2013; Koepke et al. 2008), whereas it is very rare in certain European countries such as the United Kingdom (Johnson et al. 2005). Infant botulism is caused by oral ingestion of spores, their colonization of the large bowel and formation of BoNT that is absorbed into the lymphatic system from the cecum. Infants are susceptible from a few days following birth up to 12 months, although >90% of cases occur within 6 months of age (Arnon 2013). There have been very few known deaths from infant botulism, although it has been suggested that fulminant cases may be the cause of a substantial number of cases of sudden infant death syndrome (Bartram and Singer 2004; Byard et al. 1992; Marx 1978; Nevas et al. 2005). Symptoms of infant botulism usually begin with constipation, and then cranial and somatic nerves are affected, including eye movements, ptosis of the face, weak suck, difficulty feeding, lethargy, and generalized weakness (Arnon 2013). In severe cases, a near complete flaccid paralysis is observed together with respiratory impairment. A higher proportion of infants compared to adults need to receive mechanical ventilation, possibly due to the smaller size of muscles involved in respiration at their age (Johnson and Montecucco 2008).

Nearly all infant botulism cases have been caused by proteolytic *C. botulinum* (Group I) *C. botulinum* strains producing BoNTs A and B (Arnon 2013). Several cases of infant botulism in the USA and Italy have also been caused by *C. butyricum*-producing BoNT/E and *C. baratii*-producing type F (Arnon 2013; Franciosa et al. 2003; Hannett et al. 2014; Hatheway 1995; McCroskey et al. 1991). Rare cases of infant botulism have been caused by *C. botulinum* serotypes C, E, and FA (Barash and Arnon 2014; Lúquez et al. 2010; Oguma et al. 1990). *C. baratii*-producing BoNTs E and F have become more prevalent in causing infant botulism (Arnon 2013; Franciosa et al. 2003). The properties of the BoNTs that cause type E and F infant botulism are similar to their counterparts in foodborne botulism from *C. botulinum* types E and F. The ability for spores to colonize the cecum is likely related to undetermined virulence traits in neurotoxigenic clostridia, including attachment within the intestinal mucus, selective nutrient acquisition, and by the competitive microbiota in the infant intestine (Smith and Sugiyama 1988; Sugiyama personal communication). The main competitive flora affecting *C. botulinum* colonization have not been defined, although studies in animals have revealed that lactic acid bacteria may be important competitors (Moberg and Sugiyama 1979; Wells et al. 1982). As few as 10–100 spores have been suspected to cause infant botulism based on the spore levels in retained foods, including honey (Arnon 2013), and levels of spores colonizing animal models (Wang and Sugiyama 1984). Honey is the only food proven to be a vehicle of infant botulism, while many foods that contain *C. botulinum* spores such as soil-grown vegetables could be potential sources if used as infant foods. However, current evidence suggests that most spores are derived not

from food but from dust and possibly other natural vectors in the environment. Many outbreaks have occurred in sites that are undergoing construction or soil movements, or possibly from persons such as parents that work in jobs where they frequently contact dust (Arnon 2013; Nevas et al. 2005).

Adult Intestinal Botulism

Botulism in patients 1 year of age or older typically results from the ingestion of BoNT in foods or during infection of wounds. The CDC initially had a category "classification undetermined" that included cases in patients over 1 year of age in which it was not possible to implicate a food source (Chia et al. 1986; Morris and Hatheway 1979; Sam and Beynon 2010; Shen et al. 1994; Sheppard et al. 2012). It was postulated that these patients had intestinal infections due to *C. botulinum* with accompanying production of BoNT (Morris and Hatheway 1979). Intestinal infections by *C. botulinum* in adults had been proposed in 1925 (Starin and Dack 1925) and this hypothesis was supported by feeding adult animals large doses of spores of *C. botulinum* (cited in Chia et al. 1986). Adult intestinal botulism resembles infant botulism in that *C. botulinum* colonizes the adolescent or adult intestinal tract and produces BoNTs. Adult intestinal botulism was initially confirmed in a woman following a truncal vagotomy antrectomy, (Chia et al. 1986), with BoNT/A detected in serum and stool by the mouse bioassay and *C. botulinum* but not pre-formed BoNT/A was detected in a jar of cream of coconut in her refrigerator that she had consumed and in her stools. In another intriguing incident, two seemingly unlinked type E botulism cases in Italy occurred in 1995 and 1997 in a 9-year-old child and a 19-year-old woman (Fenicia et al. 1999). These patients had undergone a laparotomy, and the surgery and associated antibiotic use may have permitted colonization by *C. butyricum* type E. The isolates were genetically and phenotypically identical to the *C. butyricum* type E strains isolated from infant botulism in 1984–85 in Italy (Franciosa et al. 2003). *C. botulinum* has not been detected in feces of healthy human adults (Dowell et al. 1977), although spores are present in many animals and possibly in nonhuman primates (Bittar et al. 2014; Shirey et al. 2015; Smith and Sugiyama 1988). C. *botulinum,* but not preformed botulinum toxin, has also been found in certain foods causing botulism, and in some cases these have been reported to be more severe. In an intriguing case, a patient with obstruction of the terminal ileum from Crohn's disease developed complete paralysis, and was suspected of having Guillain-Barré syndrome (Griffin et al. 1996) over several weeks. BoNT/A was detected in serum and stool specimens and a *C. botulinum* type A strain was isolated from stools confirming a diagnosis of botulism. Antibodies to BoNT/A were detected in the patient's serum after 19 weeks and remained at a protective level for more than a year. These cases highlight the presence of intestinal botulism in adolescents and adults, usually following surgery, possibly antibiotic use, or GI tract alteration of the microbiota.

Wound Botulism

Wound botulism, formerly a rare type of botulism, was a suspected form of botulism for many years, and was confirmed in the 1940s and 1950s (Davis 1951; Hatheway 1995; Weber et al. 1993). In the past two decades, wound botulism has become more frequent due to "skin popping" of street drugs and from "snorting" of cocaine (Maselli et al. 1997; Roblot et al. 2006; Werner et al. 2000; Sandrock and Murin 2001; Tucker and Frazee 2014; Yuan et al. 2011). These actions cause skin disruptions that can provide an anaerobic environment for spore exposure, outgrowth and vegetative cell colonization and production of BoNTs.

Latrogenic Botulism

Botulism related to injection of commercial pharmaceutical BoNT preparations and non-approved preparations, has caused serious cases of botulism in recent years (Ghasemi et al. 2012; Johnson and Montecucco 2008). Some of the cases in which approved commercial preparations were used involved treatment of patients with underlying diseases involving the NMJ, especially Myasthenia Gravis and Guillain Barré Syndrome (Dressler 2010; Watts et al. 2015). Counterfeit pharmaceutical botulinum toxin preparations not produced under appropriate GMP facilities and without required quality control have been widely recognized, particularly on the internet, and use of these products could lead to iatrogenic botulism (Pickett and Mewies 2009). The severity of symptoms caused by illicit injection of high quantities of botulinum toxin was vividly illustrated in 4 patients treated with very high doses of nonapproved BoNT for aesthetic purposes (Chertow et al. 2006). The patients were injected with as much as ca. 2857 times the estimated human lethal dose. Serum samples contained 21–43 times the estimated human lethal dose. The clinical findings were typical of other categories of botulism, and the patients required ventilator support for 36–171 days, although administration of antitoxin was helpful in sequestering BoNT in the serum and enhancing recovery. In a concise and well-controlled medical study, high doses of BoNt/A were injected into large muscles in the neck for cervical dystonia without detrimental effects (Dressler et al. 2015), thereby indicating that properly injected BoNT, even at relatively high therapeutic doses, does not usually spread systemically.

Inhalational Botulism

Due to their high toxicity and potential for causing mass casualties, BoNTs have been considered as bioterrroism weapons (Arnon 2001). A potential route of botulism in bioterrorism incidents, analogous to other nerve agents, is by exposure to

aerosols. Animal studies have revealed that intoxication can occur by inhalation and passage through mucosal membranes and entry into the circulatory system (Park and Simpson 2003; Pitt and LeClaire 2005). Since inhalation exposure is not a known natural route of BoNT intoxication, the estimated doses to cause botulism have been based on rodent and primate exposure and little data is available in the public domain. The LD_{50} in rhesus macaques has been estimated at 200–500 and 21,600 LD_{50s} for types A and B, respectively (Franz et al. 1993; Sanford et al. 2010). Inhalational botulism has been extremely rare in the laboratory environment, and only one incident has been attributed to inhalation of BoNT from animal fur in a laboratory accident (Holzer 1962).

Diagnosis of Botulism

The initial diagnosis of botulism is based on detection of characteristic clinical signs (Cherington 1998, 2004; Sobel 2005) that include oculobulbar disturbances initially affecting eyes, face and mouth, followed by generalized weakness, and fatigue (Johnson and Montecucco 2008; Cherington 1998, 2004; Sobel 2005). The diagnosis of botulism is often supported by an epidemiologic survey surrounding the incident, with information including illnesses occurring in persons who consumed food in the same household or restaurant, the different foods consumed, preparation of the foods, temperature profile of the suspect foods, and other relevant aspects that would enable *C. botulinum* to grow and produce BoNT. Clinical diagnosis of botulism can be difficult since the symptoms can mimic other neurological diseases and intoxications affecting the NMJ (Table 2) (Francisco and Arnon 2007; Merriggioli et al. 2004; Shapiro et al. 1998). Being an extremely rare disease, many physicians do not have experience in diagnosing botulism. Electrodiagnostic testing can be valuable in the diagnosis of botulism (Cherington 2004; Merriggioli et al. 2004). Guidelines for electrodiagnostic testing for botulism have been outlined

Table 2 Case definitions of potential botulism and confirmed botulism and their diagnosis

I. Potential botulism. This category includes a person who has myasthenic symptoms typical of botulism and several other diseases. Recognition of clinical symptoms and signs. Neurological examination, including electromyograpy
II. Confirmed botulism. A confirmed case of botulism involves two or more of the symptoms listed in Table 1 and who meet one of the following conditions:
1. The identification of botulinum neurotoxin in an implicated food; or in serum, stool, gastric aspirate, or vomitus collected from the person. The confirmatory test usually utilizes the traditional mouse bioassay but detection of BoNT and the gene encoding BoNT in certain cases can include indirect methods including ELISA, Mass Spectroscopy, and/or PCR
2. The isolation of *C. botulinum* organism from the persons' stool, serum, or gastric aspirate vomitus
3. A history of eating the same implicated (confirmed) food as a person meeting one of the first two conditions

(Cherington 1998, 2004; Maselli and Bakshi 2000; Merriggioli et al. 2004). However, review of botulism case reports has revealed that electrodiagnostic testing may be difficult, variable, and inconsistent in the diagnosis of botulism (Cherington 1998, 2004; Merriggioli et al. 2004). Furthermore, EMG testing capabilities may not be available for botulism outbreaks in many hospitals and in countries having limited neurological diagnostic capabilities.

Laboratory Confirmation of Botulism

Clinical symptoms must be substantiated by laboratory tests for BoNT and/or *C. botulinum* to be confirmed as a case of botulism (Cherington 2004; Hodowanec and Bleck 2015; Maslanka et al. 2013; Sharma and Whiting 2005; Solomon and Lilly 2003; Woodruff et al. 1992). The case definition of botulism has been described by the Centers for Disease Control and Prevention (CDC 2015, (http://www.cdc.gov/mmwr/pdf/rr/rr4610.pdf)). A laboratory-confirmed case of botulism must meet at least 1 of 3 criteria: (1) detection of BoNT (usually by mouse bioassay) in a clinical specimen obtained from the patient, including serum, vomitus and/or stool; (2) the detection of BoNT in the suspect food and/or, (3) isolation of *C. botulinum* from the patient's feces and/or the ingested food (Table 3). Diagnostic tests must be

Table 3 Differential diagnosis of botulism from other disorders (CDC 2006; Caya et al. 2004; Meriggoli et al. 2004)

Differential diagnoses for botulism in adults, adolescents, and infants	
Adults and children	Infants
Meningitis	Sepsis, meningitis
Guillain-Barré Syndrome (GB)	Guillain-Barré Syndrome (GB)
Myasthenia Gravis	Myasthenia Gravis
Lambert-Eaton syndrome	Acute infantile neuropathy
Cerebrovascular accidents	Meningitis/Encephalitis
Acute intermediate porphyria	Metabolic disorders, e.g. electrolyte imbalance
Carcinomatosis of cranial nerves	Reye's syndrome
Neoplasm of CNS	Neoplasm
Tick paralysis	Congenital myopathy
Diphtheritic neuropathy	Enteric virus
Polymyelitis	Poliomyelitis
Miller-Fischer variant of GB	Werdnig-Hoffman disease
Food poisoning (e.g. Saxitoxin)	Leigh disease
Chemical neurotoxin exposure	Chemical neurotoxin exposure
Mushroom poisoning	Food poisoning
Neuronal viral infection	Neuronal viral infection

performed in an approved laboratory according to biological safety and Select Agent requirements (http://www.selectagents.gov/Regulations.html) and laboratory safety guidelines BMBL (http://www.cdc.gov/biosafety/publications/bmbl5/). When animals are used, the facilities must be AALAC approved), and in accordance with appropriate protocols. The laboratory personnel must have expertise for safe handling of BoNT, as well as necessary materials for a confirmatory assay, including reference toxins, validated antitoxins, and standard media and protocols for detection of *C. botulinum*. In certain putative cases of botulism, it can be difficult to detect BoNT in the clinical and food samples, which is often due to delays or inability to obtain clinical specimens and food items, handling of the samples, and the presence of toxicants other than BoNT (Hatheway 1979, 1988; Maslanka et al. 2013; Merson and Dowell 1973). In complex matrices such as foods and stools, the assay method needs to be robust and can tolerate nontoxic and non-botulinum lethal materials that are often present (Hatheway 1988).

Assays used for definitive determination of BoNTs should embody all four physiological steps involved in the intoxication process to reach an unambiguous conclusion of BoNT toxicity related to pathology. Currently the mouse bioassay and neuronal cell-based assays (for BoNT therapeutic preparations) are the only assays approved that require the activity of all four steps. These four steps entail (Johnson and Montecucco 2008):

1. Binding of circulating BoNT to polygangliosides and protein receptors on the surface of nerves or neuronal cells
2. Endocytosis in vesicles into the nerve cytosol
3. Translocation of the light chain from the vesicles
4. Specific enzymatic cleavage of SNARE substrates

The "gold-standard" assay for BoNT detection, and the only method currently accepted by the FDA and CDC, is the intraperitoneal mouse bioassay (Cunniff 1995; Hatheway 1988; Kautter and Solomon 1977; Maslanka et al. 2013; Schantz and Kautter 1978). The mouse assay is extremely sensitive, and the quantity of BoNT for 50% death in a population of ~20-g-mice (MLD_{50}) is generally 5–10 pg, depending on the serotype. For the mouse bioassay, samples are usually diluted in sodium phosphate buffer within a pH range of 6–6.8, and with 0.2% gelatin to stabilize BoNT in the presence of potential interfering compounds and at high dilutions of the toxin. In the standard assay, 0.5 ml is intraperitoneally (ip) injected into mice. Treatment with trypsin or other proteases for BoNT activation is required for certain BoNT serotypes, particularly BoNT/B, /E and /F produced by nonproteolytic strains. Certain controls need to be employed, including heating of samples (e.g., 80 °C for 10 min), to destroy BoNT. A second essential control is antibody neutralization of BoNT activity. Samples are mixed with serotype-specific antibodies to confirm the lethality is due to BoNT and not nonspecific toxic substances often present in food and clinical samples. A toxin standard should also be included in the assays. Mice are observed for 1–4 days for characteristic botulism symptoms and death (Hatheway 1988; Maslanka et al. 2013; and by the FDA method, (http://www.fda.gov/food/foodscienceresearch/laboratorymethods/ucm070879.htm)). Mouse

symptoms depend on the serotype but typically include ruffled fur, labored abdominal breathing, a pinched or wasped waste, weakness of the limbs, dragging of the hind legs, gasping for breath, and spasticity immediately prior to death. Depending on the serotype and quantity of toxin in the samples, mice generally become sick within 4–12 h and fatalities often occur within 12–48 h, while some serotypes and subtypes of BoNT require observation for up to 7 days. The sensitivity of the mouse LD_{50} assay is 5–10 pg of BoNT, with the limit of detection being 0.01 ng per ml (Smith and Sugiyama 1988). A more rapid assay and using fewer mice involves the injection of 0.1 ml by the intravenous route and BoNT titer is determined from a standard curve of LD_{50s} of a specific serotype of BoNT vs. time to death (Boroff and Fleck 1966; Malizio et al. 2000). However, this assay causes massive systemic botulism and confirmation should be performed with an ip assay with appropriate dilutions of the extracted samples.

Certain difficulties are often encountered in the mouse ip bioassay for BoNT, especially nonspecific deaths in mice due to toxic lethal non-BoNT substances present in clinical samples and in foods, and sensitivity of detection in serum and food matrices (Hatheway 1998; Johnson and Montecucco 2008; Wheeler et al. 2009). Non-BoNT substances can show similar signs to those of botulism, and thus death must be reached as an endpoint to confirm the presence of BoNT. To definitively demonstrate the presence of BoNT in complex matrices it is necessary to conduct neutralization assays with serotype-specific antibodies to ensure toxicity is due to BoNT and not to other components in foods and clinical samples. It may be necessary to prepare high dilutions of the samples to exceed the threshold of substances causing nonspecific deaths relative to BoNT. Other potential confounding issues with the mouse bioassay need to be considered, including assay of BoNT toxicity from *C. botulinum* strains that produce more than one serotype of BoNT, often with one of the toxins in excess (Giménez and Giménez 1993; Hatheway 1988). In extracts that contain more than one serotype of BoNT, mixtures of antibodies may be required for neutralization. Reviews of the problems that may be encountered in the mouse bioassay have been expertly described (Hatheway 1988). Other important issues include the time-consuming labor involved, the requirement for animal facilities and highly trained scientists to perform the test, and the ethical use of animals.

Neuronal cell-based assays also require all four steps of intoxication in the measurement of BoNT toxicity (Pellett 2013). Two neuronal cell assays have been approved by the FDA and by regulatory agencies in certain European countries for determination of the concentration of BoNT in pharmaceutical preparations (http:/agn.client.shareholder.com/release detail.cfm?ReleaseID = 587,234; https://www.merz.com/blog/news/botulinum-neurotoxin/). Neuronal cell assays can have greater sensitivity and a smaller variation in results compared to the mouse bioassay (Pellett 2013). Neuronal cell assays have been useful for detection of BoNT in serum (Pellett 2013), but they have not been qualified for detection of BoNT in foods or feces. Neuronal cells may not be as robust as mice injected intraperitoneally with samples in complex matrices, and further research is required to develop and validate neuronal assays for food products and clinical samples. Other limitations

compared to the in vivo mouse bioassay is that cell-based assays do not provide a model for BoNT distribution, clearance, transport and other properties of in vivo assays in mice and other animal models.

The rodent hemidiaphragm assay has been used for determination of BoNT activity, particularly in Europe. Although the assay is sensitive and can determine approx. 1 mouse LD_{50} depending on the serotype, it requires considerable expertise and still uses animals yet at reduced numbers. Generally the sample preparation must possess relatively pure BoNT, and usually the serotype must be known prior to testing.

Another major class of BoNT assays uses antibodies for detection of BoNTs (Capek and Dickerson 2010; Ferreira et al. 2004; Linstrom et al. 2006; Maslanka et al. 2011, 2013; Scotcher et al. 2010). Antibody-based tests have been developed in a variety of platforms (Capek and Dickerson 2010). Some of the most commonly used formats are sandwich ELISA, Western blot, and ELISA coupled to cleavage of BoNT SNARE substrates (Capek and Dickerson 2010; Hallis et al. 1996). Current ELISA platforms generally have sensitivity to detect 0.5–10 mouse LD_{50s}. Other antibody platforms include flow through cells, immuno-PCR, immunoprecipitation coupled with enzymatic assays, Western blot, and electochemiluminescence (Capek and Dickerson 2010; Grenda et al. 2014). ELISA formats can be multiplexed to detect more than one serotype of BoNT (Singh et al. 2015). A highly sensitive antibody-based assay (ALISSA) for BoNTs has been developed by capturing BoNT on beads followed by assay Kalkum and colleagues (Bagramyan et al. 2008). For antibody-based assays to give definitive results, a high signal to background ratio and high reproducibility among different laboratories is required. One of the primary factors affecting sensitivity and specificity is the quality and uniformity of the antibodies used in the assays. ELISA has been useful for screening of the toxicity of BoNT in foods and clinical samples (Lindström and Korkeala 2006). A number of variations of antibody-based assays for enhanced sensitivity and specificity have been described, including increased sensitivities and specificity for BoNT in food systems (Capek and Dickerson 2010). Monoclonal antibodies have been developed for capture which can increase the specificity and discrimination of epitopes of BoNTs (Scotcher et al. 2009; Stanker et al. 2008). There are certain practical drawbacks to ELISA and other antibody-based tests for BoNTs. All antibody-based tests can yield false-positive reactions since inactivated BoNT as well as BoNT fragments can be detected leading to inaccurate results. Components of the food matrix and clinical samples can also interfere with the detection of BoNTs.

A third commonly used group of assays for BoNTs is based on determining single steps in the four-step intoxication process, but these must be interpreted with caution since the full intoxication process is not evaluated. Several platforms have been developed to detect the catalytic step of BoNT acting on its specific SNARE substrates (Capek and Dickerson 2010). A similar class entails binding of BoNTs to protein receptors and/or gangliosides, and this step may be coupled with its catalytic activity, or a combination of both (Capek and Dickerson 2010). A rapid and sensitive test is mass spectroscopy for detection of BoNT substrate cleavage products (Kalb et al. 2015). Mass spectrometry also enables analyses of kinetics and

determination of the cleavage site on the substrate. BoNTs in food and clinical matrices can be captured with specific antibodies and then analyzed by mass spectroscopy. Mass spectroscopy is currently being considered as a replacement for the mouse bioassay in certain academic and governmental laboratories, but as with all SNARE cleavage assays, it has the drawback of measuring the catalytic activity which is only one of the four steps of intoxication, and this could lead to false-positive or negative results. This assay needs to be carefully considered and evaluated before it is widely implemented by governmental and commercial testing laboratories.

Another approach for indirect detection of BoNTs and *C. botulinum* cells is PCR for the detection of genes encoding BoNTs or associated complexing proteins (Lindtröm and Korkeala 2006). PCR has been useful in epidemiologic determinations of the presence of *C. botulinum* in food products and clinical samples (Lindström and Korkeala 2006; Fach et al. 2009). PCR has known drawbacks including inhibition of amplification by substances in complex matrices, including foods and clinical samples. Importantly, the detection of a BoNT gene or gene fragment is not necessarily indicative of the presence of BoNT, and results need to be interpreted with caution.

A future milestone for the rapid detection of BoNTs in clinical samples would occur at the point of care (POC), whereby an assay could be performed in the clinic or hospital, ideally within a few hours of collection of clinical samples (Drancourt et al. 2016). POC detection offers the potential to accurately and efficiently identify pathogens and facilitate rapid diagnosis and initiation of therapy. A POC method would also be useful for distinguishing botulism from other more common myasthenic diseases such as Myasthenia Gravis and Lambert-Eaton syndrome. POC methods have been developed for *Clostridium difficile*, *Staphylococcus aureus* MRSA, *Mycobacterium tuberculosis*, and other pathogens in the clinic (Bomers et al. 2015; Catanzaro and Cirone 2012; Drancourt et al. 2016; Goldenberg et al. 2014). POC methods have the potential to decrease patient morbidity and mortality, enhance health care outcomes, and reduce the high costs associated with botulism cases. Currently, most POC methods utilize nucleic acid amplification (Drancourt et al. 2016; Spencer et al. 2015). Mass spectrometry targeting gas emission (volatile organic compounds) in stool samples is also under evaluation for on-site detection of *C. difficile* and potentially other clostridial pathogens (Bomers et al. 2015) and POC methods for determination of proteins such as BoNT are in development. A POC method for detection of BoNT using the protease activity of BoNT LC as a prototype has been proposed (Park et al. 2016).

Culturing of *Clostridium botulinum*

Several approaches have been used for culturing of *C. botulinum* cells from clinical samples and foods (Hatheway 1988; Holdeman and Moore 1977; Maslanka et al. 2013; Lindström and Korkeala 2006; FDA Bacteriological Analytical Methods,

BAM, http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm2006949.htm). For isolation of strains, an anaerobic environment and nutrient-rich media are required. Commonly used media are Trypticase peptone glucose yeast extract medium (TPGY), cooked meat medium (CMM), reinforced clostridial medium (RCM) (Lindström and Korkeala 2006; Maslanka et al. 2013; BAM) and egg yolk agar which is used for detection of lipolytic and lecithinase reactions (Glasby and Hatheway 1985). Enrichment cultures are often required to recover a suitable number of *C. botulinum* cells from samples for enumeration and toxicity testing. Selective enrichment is also useful to dilute out competitor microbiota and enable the growth of *C. botulinum* since the organism is usually a poor competitor in bacterial populations (Glass and Johnson 2002). Quantitation of viable *C. botulinum* cells can be attained by using the most probable number method. Strains may be distinguished by colony morphology and physiological reactions on agar plates or in customized media (Holdeman and Moore 1977).

Molecular Typing of *C. botulinum*

Molecular typing of neurotoxigenic clostridia is valuable for studies of the epidemiology of botulism outbreaks, including gathering information of suspect food products in foodborne botulism (Gerner-Smidt et al. 2006; Lindström and Korkeala 2006). For example, typing of *C. botulinum* strains isolated from an infant with botulism and infant formula present in the household showed that the two strains were different, hence the infant formula was a less likely source of the illness (Johnson et al. 2005). Currently, the most commonly used method of molecular typing for epidemiologic purposes is pulsed-field gel electrophoresis. PFGE was useful in establishing the source of *C. botulinum* in a case of infant botulism in the United Kingdom (Johnson et al. 2005). Methods for PFGE of genomic and plasmid DNA have been established (Lin and Johnson 1995; Lúquez et al. 2015). Standard methods for PFGE used by the CDC have been developed for use in PulseNet (www.cdc.gov/pulsenet/PDF/c-botulinum-protocol-508c.pdf). Isolation of high quality DNA from neurotoxigenic clostridia and particularly from endospores can be problematic because *C. botulinum* is Gram-positive with thick cell walls and spores are encased in a highly impermeable and lysis-resistant form. Additionally, certain serotypes and strains produce nucleases that can degrade the DNA (Linström and Korkeala 2006). Certain *C. botulinum* strains lack S layer proteins which can facilitate lysis and DNA purification.

In addition to PFGE, other methods have been successfully used for molecular typing of *C. botulinum,* including multi-locus-sequence-typing (MLST), DNA microarrays, ribotyping, and several PCR-based methods, such as amplified fragment length polymorphism (AFLP), repetitive element sequence-based PCR (rep-PCR) (Lindström and Korkeala 2006). These methods have been useful in epidemiologic and phylogenomic studies, and identification of strains involved in foodborne and infant botulism outbreaks (Deng et al. 2016; Grenda et al. 2014).).

However, it is becoming apparent that whole genome sequencing (WGS) by next generation sequencing (NGS) methods will supersede methods that only determine a nominal number of genes (Hasman et al. 2014) and extrachromosomal elements of foodborne pathogens, including *C. botulinum*, and it is anticipated that WGS will likely replace PFGE and be adapted for PulseNet and other epidemiologic systems (Carter and Peck 2015; Deng et al. 2016).

Treatment of Botulism

At present, there are no FDA-approved antidotes or treatments for botulism once the BoNT has entered the nerves (Simpson 2013; Johnson and Montecucco 2008). Untreated botulism has a mortality rate of 40–60%, whereas with intensive supportive care, mechanical ventilation, and serum therapy, the fatality rate has decreased to 5–10% (Dembek et al. 2007; Johnson and Montecucco 2008).

Serum therapy, using antitoxins to BoNT for sequestration of the toxin in serum, has helped to improve the outcome of botulism by preventing entry of circulating toxin into nerves (Manohar et al. 2015; Mayers et al. 2001; Thanongsaksrikul and Chaicumpa 2011). Prompt administration of antitoxin can shorten the duration of hospitalization and the time for recovery. Currently, serum therapy is considered an archaic method for control of pathogens or their toxins and is rarely used for infections or intoxications, except for certain natural toxins in the bloodstream (Manohar et al. 2015). For most diseases that were formerly treated by serum therapy, such as diphtheria and tetanus, vaccination provides early and more efficacious protection. However, it is not practical to vaccinate civilian humans against BoNT since botulism is extremely rare and vaccination would also prevent the use of the toxin as a pharmaceutical. Several domesticated animals, including poultry, cattle, horses, goats, fur-farm animals such as minks, foxes and sheep, and wet wild birds may receive vaccinations to prevent botulism (Anniballi et al. 2013).

Antitoxin treatment has advantages and limitations in the treatment of botulism. Some patients have hypersensitivity reactions to antitoxin administration (Black and Gunn 1980; Tacket et al. 1984), but this is likely to be less common in more current preparations such as HBAT. Antitoxin can only be successfully utilized for a limited time after food ingestion and observation of the first clinical signs, typically within 12–36 h. During this initial period, BoNT is circulating in the bloodstream and can physically react with antibodies. Once BoNT enters nerve terminals it is unavailable physically to react with the antibodies. Passive antibody therapy has shown utility in large foodborne outbreaks, where the onset time is variable among the victims, and the window of onset and circulation duration is widened. In certain foodborne botulism cases where a large quantity of toxin is ingested and remains in the sera, serum therapy can also be very effective (Chertow et al. 2006). In some foodborne cases of botulism, spores and cells are ingested in addition to or independent of toxin, and BoNT may be produced for an extended time in the gastrointestinal tract. Serum therapy would be of utility in bioterrorism events, in which toxin

may be ingested or inhaled by humans or animals at various time intervals and doses, and antioxins can be administered prophylactically.

Several antitoxin preparations have been used over the years (Thanongsaksrikul and Chaicumpa 2011). In the 1920s serum therapy was shown to be effective in monkeys (Dack and Wood 1928). Trivalent antitoxin to serotypes A, B, and E prepared against crude toxin precipitates in the 1960s and 1970s was the mainstay therapy until 2010 and was used successfully in many foodborne botulism outbreaks (Hatheway 1988). In 2010, an immunoglobulin ($F(ab')_2$)) hepatavalent antitoxin (HBAT) prepared against purified BoNTs (A, B, C, D, E, F, G) became available for treatment (CDC 2010). Despeciation of HBAT is expected to significantly reduce the risk of hypersensitivity reactions, anaphylaxis, and serum sickness that was occasionally encountered with trivalent antitoxin (Black and Gunn 1980; Hill et al. 2013). In 2003, the FDA approved human botulinum immune globulin for the treatment of infant botulism (Arnon et al. 2006). This product was derived from pooled plasma of adult humans that had been immunized against pentavalent botulinum toxoid. The human-derived product poses fewer risks of anaphylaxis or sensitivity reactions compared to equine antitoxins (Robinson and Nahata 2003).

Several factors affect the efficacy of serum therapy using antitoxins, particularly the affinity for BoNT and duration of the antitoxins in serum (Fagan et al. 2009; Hatheway et al. 1984). Limited studies are available on the half-life of BoNTs and antitoxins in serum. Hatheway and collaborators (1984) provided preliminary data from one patient that received A, B, and E (trivalent) antitoxin which had a half-life of about 6–7 days in serum. A steady state was reached and then the antitoxin was gradually eliminated. There are limited data on the half-life of HBAT. In serum the half-life of HBAT in serum was estimated to be 7.5–34.2 h for type F botulism (Fagan et al. 2011), but these results need substantiation, and with its current more frequent use, a definitive duration should become established. Monoclonal antibodies (MAbs) are also in development to treat human botulism. A three-MAb mixture prepared with human or humanized domains that target different regions of BoNT/A1 was very effective in neutralizing and clearing BoNT from the system in mouse studies (Nowakowski et al. 2002). The three-MAb mixture was well-tolerated and was detected for a minimum of 4 weeks in humans (Nayak et al. 2014).

Recovery from Botulism

Recovery from botulism can be slow, gradual and tedious (Colebatch et al. 1989; Eleopra et al. 1998; Mann et al. 1981; Wilcox et al. 1990). Presynaptic and postsynaptic functions at the NMJ must be restored and muscle strength needs to be replenished. Patients vary in the duration of paralysis from days to several months and the rate of repair and time to full recovery is correlated with the severity of botulism. Botulism is unique compared to certain other more common myasthenic diseases such as Myasthenia Gravis and Guillain Barré Syndrome, in being an acute and not chronic disease, and neuronal and muscle tissue is fully repaired, and complete

recovery is generally achieved unless there are other underlying disease syndromes. During recovery, nerves sprout neurites that may provide minimal innervation of the NMJ. However, the original presynaptic nerve terminals are gradually repaired and the neurites are eliminated (Foran et al. 2003a, b; Johnson and Montecucco 2008). Similarly, BoNT causes paralysis and atrophy of muscle fibers comprised of myocytes, but these cells are not killed by the actions of BoNTs and they fully recover. During intoxication, muscle tissue can undergo atrophy and temporary loss of ion channel activities (Foran et al. 2003a), but these are restored during recovery. Compared to the presynaptic molecular actions of BoNT, relatively little is known of the molecular mechanisms leading to muscle recovery.

Symptoms generally repair gradually during the extended recovery period and different neuromuscular groups vary in progressive recovery (Johnson and Montecucco 2008). Recovery of speech and the ability to swallow returns relatively early. Torso muscular paralysis and weakness also usually returns relatively early in the recovery from botulism. The oculobulbar disturbances are usually the last symptoms to clear. Some patients continue to experience weakness, fatigue, and symptoms of impaired autonomic nervous system dysfunction such as dry mouth, constipation, and impotence even after 1–2 years following onset of botulism (Johnson and Montecucco 2008). Certain botulism survivors have claimed to have chronic botulism, but these conclusions have been based on questionnaires of symptoms such as weakness, while definitive neurological examinations were not performed to substantiate the chronicity of botulism (Sobel 2014). Evidence strongly indicates that patient nerve and muscle function recover completely following the intoxication and full activity of affected persons is complete.

Prevention of Botulism

The primary technologies for preventing the hazard of *C. botulinum* and many other pathogens in foods are: (a) preventing contamination of the raw food commodity; (b) inactivating pathogens including spores by physical treatments such as an extensive thermal treatment (e.g. 12D botulinum cook); (c) formulating botulinal-safe foods by using inhibitory values of pH, a_w, ORP, temperature control, and (d) use of efficacious antimicrobials and other secondary barriers (Glass and Johnson 2002; Johnson 2013). The different parameters for controlling *C. botulinum* growth and BoNT formation can act in combination or ideally in synergy, and this forms the basis of "hurdle" technology for production of safe foods (Gould and Jones 1989; Leistner 1995). Control of growth of *C. botulinum* and BoNT formation should not depend on refrigeration alone, as temperature is difficult to control in the food supply chain and in the home and epidemiologic data reveals that temperature abuse is the most common contributing factor for commercial outbreaks of botulism (Glass and Johnson 2002; Johnson 2013). The prevention of infant botulism is more difficult. The prevalence of *C. botulinum* spores in the baby's household environment ideally should be minimized, including the feeding of foods that may contain spores

such as honey. Precautionary avoidance of infant foods up to 1 year of age, including home-prepared baby foods from ingredients that are known to contain *C. botulinum* spores such as soil-cultivated vegetables could reduce the incidence of infant botulism. The control of spores in the environment of a child is difficult to prevent, but minimal exposure to dust such as occurs in construction sites or on clothing would be beneficial.

Perspectives and Conclusions

The global impact of foodborne disease is of high significance in affecting the morbidity and mortality of humans and animals, safety and sustainability of the food supply, as well as having an enormous economic impact on society. *C. botulinum* produces a characteristic neurotoxin (BoNT) that is the most poisonous substance known to humankind. Botulism is an acute disease that can cause a long-lasting paralysis of several weeks to months. Severe cases require intensive nursing support, parenteral feeding, and mechanical ventilation. The confirmed diagnosis of botulism involves the recognition of characteristic symptoms of botulism, and detection of BoNT in clinical or food samples. Despite the rare occurrence of the disease, botulism is a continual concern in medicine, the food industry, and by regulatory agencies. Our understanding of the actions of BoNT and its pathophysiology is gradually growing, largely due to its phenomenal success in the treatment of humans for neuronal diseases that has led to many studies of pathology and recovery. Certain aspects *C. botulinum* and BoNT related to foods offers many opportunities for future research and development, including improved assays that depend on all molecular steps of intoxication, a more rapid diagnosis and differentiation from other myasthenic diseases, the development of improved countermeasures to diminish the impact of the disease and enable a more rapid recovery from botulism, and novel processing technologies and formulation strategies to assure food safety related to botulism.

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Mitigation of Foodborne Illnesses by Probiotics

Valerie Ryan and Arun K. Bhunia

Abstract Foodborne illness is a serious global health concern. There are over 200 known microbial, chemical, and physical agents that have the potential to cause foodborne illness. There have been several advances in foodborne pathogen control and prevention, which include antibiotics, antimicrobial molecules, ionizing particles, ultraviolet radiation, and heat; however, foodborne illness remains a critical problem to the world's food supply. Probiotics have been used for over 200 years to promote, not only general, but also gastrointestinal health. These probiotics offer a unique strategy for control and prevention of foodborne illness, while conferring the same additional health benefits that probiotics have been known to confer for years. This chapter will highlight both wild-type and bioengineered probiotic strains in order to control foodborne illness. Further, proposed modes of action will also be expanded upon. While probiotics hold promise as strategies for foodborne pathogen control and treatment, challenges remain in the realm of characterization, administration and dosing, as well as disparities in host-strain specificities.

Keywords Probiotics • Foodborne pathogens • Bacterial disease • Viral disease • Immunomodulation • Prebiotics • Control • Prevention

Introduction

Enteric foodborne pathogens continue to be an increasingly severe global health concern. While the total global burden of foodborne illness is very difficult to investigate. In 2010, the World Health Organization (WHO) estimated that 22 foodborne pathogens were responsible for approximately 2 billion illnesses, resulting in over

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1 million deaths (Kirk et al. 2015). The Centers for Disease Control and Prevention (CDC) estimated that, in the United States alone, approximately 48 million cases of foodborne disease occur annually, resulting in 128,000 hospitalizations and 3000 deaths (Scallan et al. 2011). Foodborne illness outbreak data from 2006 to 2010 revealed that bacterial pathogens were responsible for 40% of the outbreaks, norovirus for 49%, chemicals 6%, parasites 1%, and other multiple miscellaneous agents were responsible for 4% when etiologic agents were known.

Foodborne Pathogens and Disease

There are many bacterial, viral, fungal, and parasitic adulterants in the global food supply; however, in order to cause illness, agents typically must have the potential to (A) cause significant morbidity and mortality with a relatively low infectious dose, (B) be ubiquitous in nature, and (C) be able to persist or multiply in food. According to WHO, the major foodborne enteric pathogens include nontyphoidal *Salmonella* (viz., *enterica enterica*)*, Campylobacter jejuni,* Shiga-toxin-producing *E. coli* (STEC)*, Listeria monocytogenes, Vibrio parahemolyticus,* and norovirus (Table 1). Among foodborne diseases, gastrointestinal disorders are most predominant, resulting in abdominal cramps, nausea, vomiting, diarrhea and dysentery. This illness may be induced by the foodborne illness-related microorganisms *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella enterica*, *Shigella* spp., *Vibrio* spp., norovirus, Ebola virus, rotavirus, *Entamoeba histolytica*, *Cryptosporidium*, *Cyclospora*, *Giardia*, *Isospora*, *Taenia* spp. and many more. In addition, infections caused by some foodborne pathogens may lead to reactive arthritis, Reiter's syndrome, and rheumatoid arthritis, as sequelae to *Campylobacter*, *Salmonella*, *Shigella*, and *Yersinia* infections. Debilitating Guillain-Barre Syndrome (GBS) can also follow a *Campylobacter jejuni* infection. Hemorrhagic colitis, hemolytic uremic syndrome (HUS) and renal failure can result from exposure to Shiga toxins (Stx) produced by a STEC subtype, enterohemorrhagic *E. coli* (EHEC) and *Shigella* spp. Hepatitis and jaundice may also result from food and waterborne hepatitis A virus (HAV) and hepatitis E virus (HEV) infections. Pregnancy-related complications, miscarriage, and stillbirth, as well as neonatal and perinatal infections can result from *Listeria monocytogenes* and *Toxoplasma gondii* infections. Central nervous system disorder, meningitis and meningoencephalitis can be caused by *Listeria monocytogenes*, bovine spongiform encephalopathy (BSE), and Nipha virus, in addition to *Cronobacter* spp. that causes infections in neonates. Neurological disorders and paralysis may result from consumption of toxins produced by *Clostridium botulinum* and seafood toxins, as well as from *Campylobacter* infection. Acute or chronic disease, including malignancies and auto-immune illnesses, may result from mycotoxin exposures, in addition to allergic responses by seafood toxins, such as histamine, saurine, cadaverine and other small amines (Amalaradjou and Bhunia 2012).

Table 1 List of some common foodborne pathogens, possible food sources, incubation period, symptoms, and global burden

Foodborne pathogens	Examples of food sources	Incubation period	Symptoms	Estimated global infection burden (2010)
Bacillus cereus	Meat, milk, rice, potatoes, pasta, vegetables, and cheese	30 min to 15 h	Diarrhea, abdominal cramps, vomiting	256,775
Campylobacter jejuni	Raw milk, eggs, poultry, raw beef, water, cake icing	1–7 days	Diarrhea, abdominal cramps, nausea, headache	166,175,078
Clostridium botulinum	Low-acid canned food, meat, sausage, fish	12–36 h	Diarrhea, nausea, headache, vomiting, dry mouth, fatigue, double vision, slurred speech, repertory distress, flaccid paralysis	475
Clostridium perfringens	Undercooked meats, roast beef, and gravies	8–24 h	Diarrhea, abdominal cramps, dehydration	3,998,164
Cryptosporidium parvum	Contaminated water or milk, person-to-person transmission, or undercooked food	2–10 days	Watery diarrhea accompanied by mild stomach cramps, nausea, loss of appetite	64,003,709
Escherichia coli O157:H7 *and other* Shiga toxin-producing *E. coli* (STEC)	Ground or undercooked beef, raw milk, apple, green leafy vegetables	2–4 days	Hemorrhagic colitis, hemolytic uremic syndrome	2,481,511
Giardia lamblia	Contaminated soil, water food, or surfaces	1–2 weeks	Diarrhea, loose or watery stool, stomach cramps, lactose intolerance	183,842,615
Hepatitis A	Water fruits, vegetables, ice drinks, shellfish, and salads	4–6 weeks	Fever, malaise, nausea, abdominal discomfort, hepatitis, jaundice	46,864,406

(continued)

Table 1 (continued)

Foodborne pathogens	Examples of food sources	Incubation period	Symptoms	Estimated global infection burden (2010)
Listeria monocytogenes	Contaminated vegetables, milk, cheese, meats, sea food, smoked fish, ready to eat foods	2 days to 3 weeks	Meningitis, septicemia, miscarriage, stillbirth, neonatal listeriosis	14,169
Norwalk virus, Norwalk-like virus or Norovirus	Raw oysters, shellfish, water and ice, salads, frosting, person-to-person contact	12–60 h	Diarrhea, abdominal cramps, vomiting, nausea	684,850,131
Nontyphoidal *Salmonella* serovars	Meat, poultry, eggs, milk products	12–24 h	Diarrhea, abdominal cramps, headache, fever, chills, prostration	153,097,991
Staphylococcus aureus	Custard or cream-filled baked goods, ham, poultry dressing, gravy, eggs, potato salad, cream sauces, sandwich filling	1–6 h	Severe vomiting, abdominal cramps, diarrhea	1,073,339
Shigella spp.	Salad, raw vegetables, dairy products, poultry	12–50 h	Fever, cramps, abdominal pain, vomiting	190,849,501
Toxoplasma gondii	Domestic cat, bird, or rodent feces, raw or undercooked foods	5–23 days	Swollen lymph glands, fever, headache, muscle ache, spontaneous abortion. Severe infection in the immunocompromised	190,100

Adapted from Amalaradjou and Bhunia (2012)

Probiotics and Foodborne Pathogen Prevention

Over the last few decades, our understanding of the relationship between the diet and general health has evolved. Originally, there was a simplistic perception of, primarily, only an acquisition of nutrients was needed for metabolism, and now there is a greater understanding of how specific foods promote the overall

Table 2 Commonly used probiotic bacteria and yeasts

Resident strains	Transient strains	Mixtures
Lactobacillus acidophilus	*Lactobacillus casei*	VSL#3: *Streptococcus thermophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis, Lactobacillus acidophilus, Lactobacillus plantarum, Lactobacillus casei, Lactobacillus bulgaricus*
Lactobacillus salivarius	*Lactobacillus rhamnosus* GG	Lacteol Fort: *L. acidophilus*, lactose monohydrate, anhydrous lactose
Bifidobacterium bifidum	*Lactobacillus bulgaricus*	BLO: *B. breve* Yakult, *L. casei* Shirota, oligossacharides
Bifidocacterium infantis	*Lactobacillus yoghurti*	Ecologic 641: *L. acidophilus, L. casei, L. lactis, B. bifidum, B. bacterium lactis*
Bifidobacterium longum	*Lactobacillus brevis*	Bio-three: *Enterococcus faecalis* T-110, *Clostridium butyricum* TO-A, *Bacillus mesemtericus* TO-A
Bifidobacterium animalis	*Lactobacillus kefir*	
Streptococcus faecalis	*Lactobacillus delbrueckii*	
Streptococcus faecium	*Lactobacillus plantarum*	
Saccharomyces boulardii	*Lactobacillus gasseri*	
	Lactobacillus reuteri	
	Enterococcus faecium	
	Streptococcus lactis	
	Streptococcus thermophilus	

well-being of an individual (e.g., "superfoods," antioxidants, anti-inflammatories, etc.). This understanding fostered the use of the term "functional foods." Foods that, beyond simply providing nutritional value, can benefit additional specific functions within the body; hence, why these foods are considered functional foods (Figueroa-Gonzalez et al. 2011). Because of their ability to specifically enhance gut health and function, probiotics may be considered to be a functional food (Nagpal et al. 2012). Probiotics are typically nonpathogenic microorganisms that promote health benefits when administered in clinically-appropriate dosing. The most common probiotics are *Lactobacillus* and *Bifidobacterium* (Table 2). To be considered a probiotic, a microorganism must exert benefits in three ways: (i) Provide the host with organic acids (short chain fatty acids) through anaerobic fermentation of carbohydrates that can be positively utilized by the host. There has been a wealth of recent studies investigating the wide reach of these fermentation end products, suggesting that they play a major role in brain function and cognition. End products (ii) stimulate or prime the host immune system, without causing inflammation within the

gastrointestinal tract (GIT). Furthermore, they (iii) exclude pathogens in the GIT from causing disease by outcompeting them for the limited resources and space (Stecher and Hardt 2011). In general, probiotics are known to prevent and/or alleviate chronic inflammatory bowel disease, colorectal cancer, metabolic disorders, allergic response, obesity, and osteoporosis (Amalaradjou and Bhunia 2012; Azcarate-Peril et al. 2011; Cate et al. 2015; Kim et al. 2016; Ly et al. 2011). They are also administered in preterm neonates to allow early colonization of beneficial microorganisms (Deshpande et al. 2011).

Further, probiotics must be administered in adequate amounts to be beneficial. Widespread colonization of the gut by probiotics can obstruct pathogen access to the physical niche required for attachment and infection (Gaggia et al. 2010). If physical displacement is insufficient and a pathogen is able to colonize, probiotics have several functional attributes which allow them to effectively subvert pathogen infections within the gut. Like many microorganisms trying to survive within a complex ecosystem, several probiotics can inactivate pathogens through the secretion of antimicrobial peptides, called bacteriocins (Amalaradjou and Bhunia 2012; Klaenhammer 1993). As previously stated, probiotics stimulate the host immune system, allowing for a prompt host immune response to host-pathogen interaction (Gourbeyre et al. 2011; Oelschlaeger 2010).

The Safety of Probiotic Therapy

Bacteria have been added to food for many years to enhance flavor, produce texture changes, and provide specific health benefits. They are added to food pre-production (starter cultures) as well as post-production (functional food additives). When probiotics are added to prevent or treat enteric pathogenic disease, the probiotic itself must be nonpathogenic to the host. Commonly-used probiotic lactic acid bacteria (e.g., *Lactobacillus,* and *Bifidobacterium*) are Generally Regarded as Safe (GRAS); however, some strains have been known to be opportunistic human pathogens in individuals with underlying, immunocompromising health conditions (Boyle et al. 2006). Despite these findings, there have been few systematic safety studies performed.

The Agency for Healthcare Research and Quality (AHRQ) released a study in 2011 (Hempel et al. 2011) seeking to evaluate the potential health risks of probiotic administration. Within this review, they evaluated 622 studies of probiotic microorganisms; the review concluded that, while there was no apparent elevated risk of human illness due to supplementation with the probiotic bacteria administered in these studies, there is a significant lack of investigation of potential risk factors associated with probiotic supplementation within probiotic studies in current literature. In summation, the study found that current literature was unable to address safety concerns regarding probiotic supplementation. The WHO/FAO released a report in 2002, which suggested that there are four potential side effects of probiotics: (i) translocation to the bloodstream, leading to systemic effects, (ii) "*deleterious*

metabolic activities," (iii) overstimulation of the host immune system, and (iv) gene transfer to commensal microbiota. The report illustrated gene transfer to commensal microbiota leads to an adverse effect within the gut microbiota or directly to the host, such as transfer of antibiotic resistance genes to opportunistic pathogens commonly found in the gut microbiome or overproduction of lactic acid leading to lactic acidosis in the patient (Doron and Snydman 2015). Given this, and the strain specificity of probiotic action, it is important to screen potential probiotics for the presence of virulence factors, antimicrobial resistance, antibiotic sensitivity and antibiotic gene transfer, as well as the potential for the uptake of pathogenic virulence genes (Amalaradjou and Bhunia 2012; Didari et al. 2014). It is important to note that while these complications might occur in certain rare and isolated settings, the transient nature of probiotics in the GIT decreases the likelihood that they will occur. Both the Scientific Committee on Animal Nutrition (SCAN) and the FAO have developed guidelines for probiotic additives to animal feed and food consumed by humans, respectively. Both strategies employ methods that test for the aforementioned factors of safety risk (i.e., genotypic and phenotypic analyses for virulence factors, antimicrobial resistance, gene transfer, etc.), with a notable exception, that the FAO also requires probiotics to pass double blind, randomized, placebo-controlled human clinical trials. Within these studies and reviews two areas of probiotic used have been identified, probiotic use for the general public through food additives and dietary supplements and probiotic use on an individual basis for health intervention as a drug or vaccine. The risk assessment for general use of probiotics as a food additive or dietary supplement reveals that the potential risks (lactic acidosis, persistent gut inflammation, and antibiotic resistant gene transfer to potential pathogens) are greater than the potential general health benefits (gut microbiome balance or flavor enhancement). When probiotics are used on an individual basis to treat specific health problems, such as enteric pathogens, acute infectious and antibiotic associated diarrhea, and irritable bowel syndrome, the potential risks are balanced by the specific health benefits conferred to combat the specific diagnosis (Sanders et al. 2010).

Probiotics

The term probiotic is derived from the words "pro" and "bios," meaning "for life." In the early twentieth century, Eli Metchnikoff, Nobel Prize winner and grandfather of modern probiotics, observed that lactic acid bacteria (LAB) played a positive role in both digestion and the immune system. Metchnikoff believed that with adequate consumption of appropriate foods, these "good bacteria" could displace "bad bacteria" within the gut (Mackowiak 2013). Since the discovery that certain "good bacteria" can confer health benefits, there has been a wealth of probiotic research into a wide variety of areas (ranging from protection against bacterial pathogens and viral infection to cancer prevention), attempting to exploit these microbes for human benefit (Sanders et al. 2013; Sanders et al. 2014).

Probiotic Definition

There have been many iterations of the definition of probiotics over the years; however, in 2001, FAO/WHO (2002) (FAO/WHO 2002) defined probiotics as *"live microorganisms that, when administered in adequate amounts confer health benefits on the host"* (Hill et al. 2014). Notably, this definition fails to address the probiotic mechanism of action (i.e., balancing intestinal microbiota, immunomodulation, etc.) that previous definitions included. The change in definition was to accommodate the claim that certain organisms are probiotic, while not fully elucidating the mechanism by which the health benefit is conferred (Sanders et al. 2014).

Probiotics are divergently categorized based on their ability to colonize the intestinal tract. Resident strains (e.g., *Lactobacillus* spp.) are commonly found in the human gut flora and, when supplemented, can readily colonize the human GIT. Transient strains (e.g., *Bifidobacterium* spp.) cannot establish themselves within the GIT and are excreted by the host. The duration of residence within the host is both strain- and host-specific. The ability of a probiotic to confer its health benefits is dependent on many factors, including strain specificity, host microbiome profile, mode of application (e.g., capsule, kefir, or yogurt), and ecology of the supplement (monoculture or mixture). Prebiotics, which will be addressed later in this chapter, are sometimes included with the probiotic to enhance the probiotic activity of the strain or strains.

Like all microorganisms, probiotics are identified taxonomically by their genus, species, and strain. Over the past few decades, however, many probiotic species have been identified, most of which are Gram-positive, bile-resistant lactic acid bacteria (LAB). The most important genera include *Lactobacillus, Lactococcus, Enterococcus, Streptococcus, Pediococcus, Leuconostoc* and *Bifidobacterium,* while the most common types used are *Lactobacillus* and *Bifidobacterium* (Didari et al. 2014; Volzing et al. 2013). A list of common resident and transient probiotic strains is in Table 2.

To be considered a probiotic, microbes must meet certain criteria (Hill et al. 2014). Table 3 denotes the notable characteristics of probiotics, but generally a probiotic must be Generally Recognized as Safe (GRAS), able to survive in the low pH environment of the stomach and be resistant to bile and pancreatic juices, persist in the gut, confer general health benefits to the host, and be maintained stably during processing, storage, and application.

Application of Probiotics and General Health Benefits

Probiotics can be administered in a variety of ways, in either mixed or monoculture. They can be consumed as a supplement in pill or capsule form, or in foods including fermented milk (kefir or yogurt), smoothie drinks, fruit juices, wine, chocolate, fermented sausage, fresh sauerkraut or fresh pickles, and cheeses. There has recently

Table 3 Criteria for an ideal probiotic

1.	Accurate taxonomic identification
2.	Accurate and acceptable antibiotics resistance/sensitivity profile
3.	Ability to colonize host (transient or resident)
4.	Generally Recognized as Safe (GRAS)
5.	Maintain viability in host physiological conditions (e.g., bile, hydrochloric acid, pancreatic secretions, high and low pH of the intestines and stomach)
6.	Persistence in host GIT
7.	Adherence to receptors in host intestinal epithelium
8.	Immunostimulation and immunomodulation activities
9.	Nonpathogenic
10.	Maintain antimicrobial activity at target site
11.	Maintain cell viability at target site
12.	Inability to exchange genetic information (genetic stability)
13.	Remain viable during processing, storage, and delivery

Adapted from Amalaradjou and Bhunia (2012), Sanders et al. (2013), Hill et al. (2014)

been an increase in probiotic-containing yogurt used to coat an assortment of nutritional supplement bars, dried fruits, pretzels, and a host of "healthy" desserts. An effective dose of probiotics must include a minimum of 5 billion colony-forming units of probiotic bacteria (CFU)/day that administered daily for a period of 5 days provides health benefits (Gronlund et al. 1999; Williams 2010). It is generally understood that the health benefits of the gut microbiome are due to a synergistic effect among the strains within the biome, and this synergistic effect can be extended to the probiotic culture, thus, mixed cultures tend to confer greater benefits than monocultures.

While many probiotics confer health benefits, the benefits and mechanism of action are strain-specific and may not be applicable for all probiotics (Hill et al. 2014). *Lactobacillus,* for example, elicits variable cytokine profiles that are dependent upon the species and strain administered (Christensen et al. 2002; Latvala et al. 2008; Vissers et al. 2010). The health benefits of probiotics have been well documented by a wealth of published research results, e.g., Nagpal et al. 2012; Shen-Shih and Tzu-Ming 2012; Williams 2010), which include food digestion and nutrient metabolism, short-chain fatty acid (SCFA) production through carbohydrate fermentation, cholesterol reduction, mucoidal immune modulation, enhanced mucus production within the GIT, antimicrobial peptide production, enhancement of epithelial barrier integrity, and equilibration of host gut microbiota.

Probiotics Mechanisms of Action

Several mechanisms of probiotic action have been proposed (Table 4); several, of which, are discussed in this section.

Table 4 Proposed probiotic mechanisms of action against human health conditions and benefits

Health condition or benefit	Proposed mechanism
Enteric pathogen resistance	Antagonism Increased antibody production Colonization resistance Limiting access of enteric pathogens (pH control by acid production, bacteriocins, antimicrobial peptides) Modulation of tight junction integrity, production of SCFA
Aid in lactose metabolism	Bacterial lactase acts on lactose in the small intestines
Small bowel bacteria overgrowth	Decrease toxic metabolite production Normalize small bowel flora Antimicrobial activity
Immune system modulation	Production of SCFA Regulate Th1/Th2 cell activation Modulate IL-10/IL-12/IL-6 activity Stimulation from phagocytic and dendrocytic cytokines
Anticolon cancer effect	Anti-mutagenic and anti-carcinogenic activity Detoxification of carcinogenic metabolites Immune stimulation and modulation
Toxic microbial metabolite production	Modulation of host microbiota increases carbohydrate metabolism of the community
Antiallergenic activity	Prevention of antigen translocation into blood stream Modulation of immune response to increase in antigen
Heart disease, blood lipids	Uptake of cholesterol by gut microflora Modulation of bile salt hydrolase (BSH) activity
Urogenital infections	Competitive exclusion pH modulation SCFA production Modulation of urogenital microflora
Necrotizing enterocolitis	Reduce IL-8 response
Rotovirus gastroenteritis	Increased viral specific IgA response
Inflammatory bowel disease	Enhance mucosal barrier function Decreased inflammatory response Immune modulation
Crohn's disease	Reduced pro-inflammatory cytokines Reduced inflammation
Human immunodeficiency virus (HIV)	Competitive exclusion Modulation of urogenital flora Regulation of mucosal T-cell populations, Mucosal immune stimulation and modulation

Adapted from Amalaradjou and Bhunia (2012), Nagpal et al. (2012)

Antimicrobial Activity of Probiotic Bacteria

Microorganisms have evolved to express several antimicrobial properties to improve their ability to compete for limited ecological recourses, and probiotic bacteria are no exception (Amalaradjou and Bhunia 2012). Within the ecology of the human GIT, probiotics express these antimicrobial factors to enhance their survival and,

inadvertently, enhance host protection against enteric pathogens. Probiotics suppress enteric pathogens both directly and indirectly through the production of antimicrobial factors and modulation of host cell expression of antimicrobial peptides, respectively.

The secretion of SCFAs by both probiotic and commensal gut microflora provide several positive effects on host endocytes and the gut microbiota themselves, including antimicrobial effects against enteric pathogens. SCFA have the ability to disrupt the outer membrane of Gram-negative pathogens (e.g., *Salmonella enterica* serovar Typhimurium and various *E. coli* strains) by inhibiting growth, disrupting osmotic pressure and increasing permeability to other antimicrobial factors, produced by both probiotic bacteria and host cells that can disrupt pathogen cell membranes (Alakomi et al. 2000). Aside from direct bacteriostatic or bactericidal properties, the SCFA's propionic and hexanoic acid promote host antimicrobial production (Alva-Murillo et al. 2012). Oral administration of butyrate can possibly stimulate host defense peptides leading to the clearance of *Salmonella* (Sunkara et al. 2012). Interestingly, SCFA-producing bacteria are apparently immune to the antimicrobial effect of SCFAs, providing them an additional selective advantage within the ecology of the gut microbiota (Alva-Murillo et al. 2012). SCFAs produced by probiotic bacteria reduce the pH of the intestinal lumen thereby inactivating several enteric pathogens (Carey et al. 2008), and SCFAs also regulate colonic T reg cell homeostasis for improved gut health (Smith et al. 2013).

Bacteriocins and microcins are antimicrobial agents commonly secreted by Gram-positive and Gram-negative bacteria, respectively. Bacteriocins are a heterogeneous group of secretory molecules that bind to species-specific extracellular receptors on enteric bacteria (sensu lato), including pathogens, that cause morphological (typically pore formation) or metabolic changes leading to cell death (Bhunia et al. 1991; Daw and Falkiner 1996; Jack et al. 1995). Depending on the molecular weight and the inhibitory spectrum of bacteriocins, there are four general classes of bacteriocins produced by lactic acid bacteria (Cotter et al. 2005; Klaenhammer 1993). In contrast, microcins are antimicrobial peptides that target the enzymes involved in DNA, RNA, and protein structure and synthesis (Duquesne et al. 2007). There have been many studies in the past two decades elucidating the bactericidal or bacteriostatic activities of bacteriocins produced by probiotic bacteria. *Lactobacillus acidophilus*, a commonly used and commercially-available probiotic, can inhibit *Salmonella, Shigella, Pseudomonas,* and *Vibrio* species *in vitro,* likely by the inhibitory activity of the bacteriocin, acidophilin (Brown 2011; Vila et al. 2010). A bacteriocin secreted by *L. plantarum* has broad spectrum inhibitory activities against both Gram-positive and Gram-negative bacteria, including many pathogens such as *S. aureus, L. monocytogenes,* and many *E. coli* strains (Kumar Tiwari and Sheela 2008). A broad spectrum bacteriocin (ABP-118) secreted by *L. salivarius* UCC 118, although ineffective against other *Lactobacillus* species, is highly inhibitory to *Listeria, Bacillus,* and *Staphylococcus* (Dunne et al. 2001; Flynn et al. 2002). In further *in vivo* studies, a mutant strain of *L. salivarius* was unable to produce ABP-118 and had no protection against *L. monocytogenes* infection, whereas the WT control probiotic conferred protection (Corr et al. 2007; Sherman et al. 2009).

Some probiotic bacteria also directly exert their antimicrobial properties by adhering to the host intestinal epithelial cells and effectively reducing pathogen adherence (Collado et al. 2009). Several *in vitro* studies involving pretreatment with individual strains of *Lactobacillus* species (viz., *L. acidophilus, L. rhamnosus, L. helveticus,* and *L. paracasei*) revealed the successful inhibition of adhesion and translocation of several pathogens, including enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), *L. monocytogenes*, and *Salmonella* spp. (Johnson-Henry et al. 2008; Medellin-Pena and Griffiths 2009; Zareie et al. 2006).

Alternatively, probiotic bacteria can indirectly suppress enteric pathogen function by eliciting an enhanced antimicrobial response by the host intestinal cells. The two major classes of antimicrobial peptides produced by human intestinal cells are defensins and cathelicidins. Although cathelicidins are constitutively produced in the human GIT, butyrate produced by both commensal and probiotic bacteria can also induce cathelicidin production (Schauber et al. 2003; Sunkara et al. 2011). Prophylactic oral administration of butyrate has been used to reduce and control both *Salmonella* (Fernandez-Rubio et al. 2009) and *Shigella* infections (Raqib et al. 2006).

Human intestinal Paneth cells and neutrophils constitutively secrete α-defensin, while various other epithelial cells constitutively secrete β-defensin. Defensins have antimicrobial properties and are produced in the intestine to suppress enteric pathogens (Kudryashova et al. 2015). Several studies have revealed that probiotic bacteria (e.g., *E. coli* Nissle 1917, *E. coli* DSM 17252, and VSL#3 consisting of four strains of lactobacilli (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, and *L. delbrueckii subsp*. *bulgaricus*), three strains of bifidobacteria (*Bifidobacterium longum*, *B. breve*, and *B. infantis*), and *Streptococcus salivarius subsp. thermophilus*) can induce the secretion of β-defensins (Schlee et al. 2008; Schlee et al. 2007; Seo et al. 2012; Wehkamp et al. 2004). In studies with the probiotic *E. coli* Nissle, one of the best characterized probiotics, human β-defensin-2 was determined to be flagellin-mediated (Seo et al. 2012).

Immunomodulation by Probiotic Bacteria

The intestine is an important mucosal organ responsible for the absorption of nutrients from digested food; however, the GIT is also a prominent immune organ consisting of approximately half of the immune cells in the human body. It was suggested in the 1970s that the commensal bacteria of the gut play an important role in immune modulation as the IgG response to antigens, which was found to be much lower in germ-free (GF) mice than in conventional (CV) mice (Ohwaki et al. 1977). Additionally, it has been revealed that the presence of gut microbiota markedly influences $CD4^+$ and $CD8^+$ cell pools within gut-associated lymphoid tissue (GALT) (Bandeira et al. 1990). Probiotic bacteria are of particular importance in modulating the mucosal immune system of the gut. These tissues are collectively known as the gut-associated lymphoid tissues and primarily consist of the mesenteric lymph node, Peyer's patches, and hematopoietic-derived cells such as lymphocytes,

dendritic cells, and isolated lymphoid follicles, which are the primary location of intestinal B cells (Caballero-Franco et al. 2007). Studies have revealed that colonization of the gut leads to increased numbers of intraepithelial lymphocytes, antibody-producing cells, and antibody concentration in sera (Hakansson and Molin 2011). The lamina propria secretes IgA (sIgA) that is luminal bacteria-specific in CV mice; however, sIgA is not present in the GIT of GF mice, suggesting that the gut microbiota stimulate the production and secretion of intestinal IgA. The sIgA protects the host against the luminal microbiota that penetrates the epithelial barrier (Macpherson and Harris 2004). Furthermore, *Bacteroides thetaiotaomicron*-colonized gnotobiotic mice implanted with hybridoma cells producing *B. thetaiotaomicron*-specific IgA had a decrease in cecal antigen (Peterson et al. 2007). These results suggests that the commensal gut microbiota and probiotic bacteria influence the intestinal immune system and specific host immune tolerance of the commensal microbiota.

There are three postulated mechanisms for probiotic modulation of the innate immune system: (i) functional regulation of intestinal epithelial cells through direct contact, (ii) probiotic bacteria transported through micro-fold (M) cells activate phagocytic cells within the Peyer's patches, and (iii) dendritic cells (DC) in the lamina propria recognize probiotic bacteria through pattern recognition receptors (PRR), such as Toll-like receptors (TLR) (Hardy et al. 2013).

Many probiotic bacteria adhere and interact directly with intestinal epithelial cells, and it is believed that this is the mechanism they exert to modulate host immunity. A mixture of probiotic bacteria, VSL#3, increased the production and secretion of mucin *in vitro* in both human colon epithelial cells (LS 174 T) and rat colonic loops (Caballero-Franco et al. 2007). Probiotic and commensal bacteria have the ability to influence the inflammatory signaling pathway through direct contact with intestinal epithelial cells. The nuclear factor kappa B (NF-κB is the prominent inflammatory signaling pathway. Under anti-inflammatory (non-stimulatory) conditions, the cytoplasmic NF-κB is bound to an inhibitor molecule, which renders it inactive; however, when inflammation is stimulated, the inhibitor molecule is phosphorylated, making NF-κB active and subsequently activates transcription of proinflammatory genes in the nucleus. Several studies have revealed that probiotic strains are able to inhibit phosphorylation of the NF-κB inhibitor and thereby negate inflammation. Interestingly, this also inhibits the secretion of IL-8, a proinflammatory cytokine, by intestinal epithelial cells, which further negates intestinal inflammation (Thomas and Versalovic 2010).

Probiotic bacteria taken up by M cells have the potential to activate phagocytic cells (macrophages, monocytes, and neutrophils) and natural killer cells (NK), as well as dendritic cells. In this way, probiotic as well as commensal bacteria have the ability to "tune" the innate immune system, which enables its rapid response to enteric pathogens. This ability to "tune" or prime the immune system is demonstrated by the ability of probiotic bacteria to elicit pro-IL-1β production by intestinal macrophages; thus allowing for rapid conversion to active IL-1β (Franchi et al. 2012). This can lead to a variety of cytokine expression and subsequent activation of immune cells. Two key cytokines dictate the direction of an immune response,

IL-10 and IL-12. IL-12 is involved in activation of cellular immunity and cytotoxic cell function, whereas IL-10 suppresses IL-12 function and reduces inflammation leading to a reduction in immune response (Shida et al. 2006). Konieczna et al. (2012) determined that *Bifidobacterium* spp. can induce high levels of IL-10 production in human DCs, whereas *Lactobacillus* cannot. The activation of IL-10 is a key factor in priming the host immune system, while negating a negatively inflammatory state. In a human study in which elderly participants were supplemented with *L. rhamnosus,* results revealed that the probiotic-supplemented group experienced an increase in peripheral blood NK cell counts compared to the control (Gill and Rutherfurd 2001a, b). *In vitro* studies have revealed that probiotic bacteria induce IL-12 activity in macrophages, subsequently stimulating NK activity (Dong et al. 2010), which could be an insight into the mechanism by which certain strains of *Lactobacillus* spp. potentially activate NK cells. Not only do probiotic bacteria increase the number of peripheral blood lymphocytes, but they also enhance their phagocytic capacity (Gill 2003). *In vitro* studies in RAW264.7 macrophages exposed to cell wall extracts of *B. adolescentis* BBMN23, *B. longum* BBMN68, and *L. salivarius* Ren revealed enhanced phagocytic activity via increased production of IL-6 and TNF-α (Yuanmin et al. 2011). Similarly, Kaushal and Kansal (Deepti and Vinod 2014) determined that mice orally administered with *L. acidophilus* and *B. bifidum* had an increased production of reactive oxygen intermediates and enhanced phagocytic activity in macrophages. Many of the immune functions that are carried out by immune cells are mediated by cytokines. Administration of probiotic bacteria has a major effect on modulating cytokines. For example, specific strains of probiotic bacteria have increased the expression of IFN-γ, IFN-α, and IL-12 (Arunachalam et al. 2000). Similarly, long-term consumption of probiotic bacteria in yogurt increased production of IL-1, IL-1β, IL-6, IL-10, IL-12, IL-18, IFN-γ, and TNF-α by monocytes and DC (Cross 2002; Gill and Guarner 2004; Niers et al. 2005).

Probiotic and pathogenic bacteria have conserved extracellular features that interact with host immune cell PRR like their interaction with epithelial cells, discussed previously. For example, the flagellin expressed by some probiotic bacteria for chemotaxis and cellular adhesion also interacts with dendritic cells sequestered in the lamina propria and stimulates production of cytokines which are responsible for initial immune responses, chemokine production and antimicrobial peptide secretions (Kinnebrew et al. 2012). Probiotic bacteria have a potent ability to stimulate innate immunity, which plays a role in the stimulation of adaptive immune modulation. Probiotic bacteria have an "adjuvant" effect, meaning they possess the ability to stimulate a humoral immune response (Belkaid and Hand 2014; Hall et al. 2008). For example, antigen-presenting dendritic cells stimulate a T-cell response. Several studies have revealed that *L. rhamnosus* can regulate Th_1/Th_{17} cells by an increased pro-Th_1/Th_{17} cytokine release by DC (Lin et al. 2009; Yu et al. 2010). B-cell-secreting IgA is an important part of humoral mucosal immunity. Orally administered *L. gasseri* increased intestinal IgA secretion and induced TGF-β production in DC (Sakai et al. 2014). There have been several animal and clinical studies conducted in an attempt to elucidate the immunomodulatory effects of probiotic

bacteria. Together, they present a multifaceted and very complex network of cooperation among probiotic bacteria, epithelial cells, GALT, and immune cells (Table 4).

Enhancement of Intestinal Barrier Function by Probiotic Bacteria

The physical barriers that separate self from non-self are the first line of defense in the innate immune system. With respect to enteric pathogens, this is the epithelial barrier of the GIT. This intestinal epithelial barrier has mechanisms that enhance barrier function such as mucus secretion, chloride and water secretion, and cellular junction maintenance (Thomas and Ockhuizen 2012). Loss of barrier function is commonly referred to as "leaky gut." It is well-established that disruption of intestinal barrier function can lead to inflammatory bowel disease (IBD), several diarrheal diseases, enteric infection, and other autoimmune diseases (Xavier and Podolsky 2007). Probiotic bacteria can prevent or improve leaky gut resulting from food antigens, enteric pathogens, proinflammatory cytokines, and immune dysregulation (O'ara and Shanahan 2007).

The epithelial barrier is covered by a protective layer of mucus largely produced by goblet cells throughout the GIT. The proportion of goblet cells to epithelial cells is 4–16% depending on the location within the GIT. Goblet cell occurrence and mucus thickness is least in the duodenum, increasing in occurrence and thickness through the small intestine until the descending colon (Goto and Kiyono 2012). Several probiotic and commensal bacteria are able to influence goblet cell number and mucus production (Ng et al. 2013; Pelaseyed et al. 2014; Tomas et al. 2013). To translocate across the epithelial barrier, pathogenic bacteria must first penetrate the protective gel layer of proteolytic-resistant mucin. Many bacteria have developed mechanisms to degrade the mucin to uptake nutrients or gain access to the epithelial cells underneath (Ohland and MacNaughton 2010). Intestinal inflammation plays an important role in pathogen invasion of mucus layer because under inflammatory conditions the mucus layer becomes thin, granting access to potential pathogens that would otherwise not have been able to penetrate the mucoid layer (Ng et al. 2013). While it is well-established that several probiotic strains of *Lactobacillus* and VSL#3 can stimulate mucus production (Caballero-Franco et al. 2007; Mack et al. 2003), the mechanism of action has been elusive. However, in a recent study using *Ruminococcus gnavus* E1 revealed there was an increased expression of the mucin-encoding genes MUC1, MUC 2 and MUC3 which resulted in increased glycosylation by goblet cells that correlated with an increase in mucin production (Graziani et al. 2016).

The integrity of the epithelial barrier is largely due to cell to cell junction complexes. These complexes consist of the tight junctions, adherent junctions, gap junctions and desmosomes. The tight junction is comprised of some 50 proteins that consist of transmembrane proteins that are anchored to the actin cytoskeleton. Regulation of these proteins are paramount to the integrity of the junction, and subsequently the epithelial barrier. Several enteric pathogens have evolved mechanisms to circumvent and cross the epithelial barrier via both intracellular and paracellular

means (Goto and Kiyono 2012; Guttman and Finlay 2009). Similar to the thinning of the mucus layer, chronic inflammation plays a role in barrier function and promotes pathogen permeation of the barrier. In *in vitro* and *in vivo* studies, *L. plantarum* restored tight junction protein integrity after disruption with unconjugated bilirubin administration (Zhou et al. 2010). Similarly, pretreatment with *L. acidophilus* or *S. thermophilus* both decreased permeability *in vitro* and *in vivo* (Ahrne and Hagslatt 2011).

Probiotic Bacteria Control of Enteric Pathogens

There has been substantial research into the use of probiotic bacteria to control enteric pathogens over the past five decades. The scope of the research has narrowed from understanding which probiotic strains or mixtures could positively enhance the resistance of humans or feed animals to pathogenic bacterial infections to bioengineering designer probiotic bacteria that elicit specific protective qualities against specific pathogens. There is great potential for these bioengineered probiotics to provide twofold protection, including (i) a bioengineered defensive function, and (ii) conferring the native health benefits previously discussed in this chapter such as enhanced intestinal barrier function and production of antimicrobial compounds. Several studies have revealed the efficacy of wild-type and recombinant, bioengineered probiotics in suppressing enteric pathogen infection (Bhunia 2012; Dobson et al. 2012; Fijan 2014; Salminen et al. 2010). This next section addresses probiotic-based mediation of enteric pathogens.

Bacterial Pathogens

Salmonella

Salmonella is a predominant cause of human foodborne disorders worldwide and is associated with a wide variety of food, including raw poultry and eggs, fish, and fruits and vegetables, nuts, dry milk, infant formula, spices, etc. (Carrasco et al. 2012). It is a zoonotic pathogen with a natural reservoir in livestock, especially chickens, which, when consumed, transfers the live pathogen to the GI tract of the host (Das et al. 2013; Foley and Lynne 2008). Multiple strategies have been employed to address exclusion of this pathogen from food production and subsequently the food supply (Vandeplas et al. 2010). Several studies have revealed that administration of probiotic microbes directly to broiler chickens may reduce *Salmonella* contamination, thus reducing human exposure through contaminated food (Higgins et al. 2008; Van Coillie et al. 2007). Higgins et al. (2008) determined that challenging neonatal broiler chicks with *Salmonella* Enteritidis and subsequently feeding them increasing doses of *Lactobacillus*, resulted in up to an 85%

reduction in the prevalence of *Salmonella*. Another study revealed that *L. rhamnosus* reduced epithelial cell stress due to *S*. Typhimurium adhesion and invasion *in vivo* (Burkholder and Bhunia 2009). Not only did administration of *L. plantarum* improve the growth performance of pigs, but it also reduced fecal shedding of *S.* Typhimurium (Gebru et al. 2010). Oral administration of lactic acid bacteria to broiler chickens not only reduced the transmission of *S.* Typhimurium from the GI tract to the spleen and liver, but also down-regulated SPI-1 virulence gene expression, which is necessary for intracellular multiplication (Xiaojian et al. 2014). Interestingly, in an *in vitro* study using an intestinal fermentation system with immobilized fecal microbiota, *Bifidobacterium thermophilum* RBL67 inhibited *S.* Typhimurium when administered either pre- or post-challenge (Tanner et al. 2016). In contrast, hens and broiler chickens receiving a combination of probiotics that included *Bacillus subtilis, Lactobacillus acidophilus, L. casei, Enterococcus faecium*, and *Bifidobacterium longum* in their feed had no significant reduction in the carriage of *S.* Enteritidis (Murate et al. 2015). This difference in results highlights the strain-specific attributes of probiotic bacteria and their interactions with pathogenic bacteria, host cells, and host microbiota, which is further supported by a meta-analysis study of competitive exclusion of *Salmonella* in broiler chickens conducted by the Public Health Agency of Canada and the University of Guelph in 2013 (Kerr et al. 2013). These investigators concluded that of the 214 probiotic trials considered, the reduction in prevalence or amount of *Salmonella* varied considerably (Kerr et al. 2013). *Lactococcus lactis* IL1403 has been engineered to secrete the antimicrobial peptides (AMP) Alyteserin-1a and A3APO, both of which are effective against Gram-negative bacteria, but not Gram-positive bacteria. When AMP-expressing *L. lactis* is co-cultured with *S.* Typhimurium or *S.* Infantis, the growth of both serovars was significantly inhibited compared to the control (Volzing et al. 2013).

Because the high health and economic burden of *Salmonella,* considerable effort has been directed toward determining the underlying mechanism of antimicrobial and protective effects of probiotic bacteria against *Salmonella*. Asahara et al. (2011) identified a correlation between an increase in intestinal organic acids which reduced the luminal pH and the inhibition of *S.* Typhimurium growth in mice administered *L. casei* Shirota. Inhibition of *Salmonella* growth due to lowered pH has been shown in many studies (Asahara et al. 2011; Dobson et al. 2012; Fayol-Messaoudi et al. 2005).

Immunomodulation of the innate and adaptive immune system by probiotic bacteria correlates with the reduction of *Salmonella* intestinal and tissue counts in *in vivo* mouse studies. *L. casei* CRL administration reduced inflammation, increased bacterial clearance due to activated phagocyte activity, increased IgA^+-producing cells, and increased production of pathogen-specific sIgA (de LeBlanc et al. 2010). Hence, it appears that the mechanism of reducing pathogen levels in hosts by probiotic bacteria is multifaceted and involves reducing the luminal pH by increased organic acid concentrations, in addition to the production of bacteriocins and modulating the innate and adaptive immune systems (Dobson et al. 2012). Although probiotic bacteria have antimicrobial activity against *Salmonella* in an animal's intestinal tract, these bacteria can also enhance the growth performance of these food animals.

Campylobacter

Campylobacter jejuni is predominantly a poultry-origin human pathogen which colonizes the ileum and colon of the GI tract and causes gastroenteritis in humans, characterized by fever, abdominal cramps, and diarrhea (Dasti et al. 2010; Young et al. 2007). Pathogenesis is well-defined and can be attributed to disruption of the mucosal epithelial barrier that occurs during campylobacter adhesion and invasion in the GIT (Boehm et al. 2011). Several studies both *in vitro* and *in vivo* have revealed that *Lactobacillus* spp. and *Bifidobacterium* spp. can reduce *C. jejuni* adhesion to and invasion of cells (Neal-McKinney et al. 2012; Schachtsiek et al. 2004; Tareb et al. 2013; Wine et al. 2009). A recent study revealed that reducing *C. jejuni* adhesion by the probiotic *L. gasseri* SBT 2055 (LG) was due to co-aggregation of the probiotic bacteria and *C. jejuni* by an interaction between the proteinaceous cell surface of LG and *C. jejuni*. When protease K-treated LG cells were used in *in vitro* studies, co-aggregation was reduced and adhesion and invasion of Int-407 cells were increased compared to Int-407 cells not treated with probiotic bacteria, suggesting that surface proteins of *L. gasseri* play a significant role in reducing the adhesion and invasion of *C. jejuni* (Nishiyama et al. 2014). Similarly, *L. helveticus* R0052, which efficiently adheres to epithelial cells, was able to competitively exclude *C. jejuni*, resulting in a 55% reduction in invasion into Int-407 cells (Wine et al. 2009). Wagner et al. (Wagner et al. 2009) through *in vivo* studies using defined human microbiota-associated Balb/C mice dosed with lactobacilli and bifidobacteria after being orally infected with either *Salmonella* or *C. jejuni*, determined that the probiotic bacteria could successfully colonize the gut and competitively exclude both pathogens. Enhanced colonization resistance against both pathogens was observed. A *Bacillus subtilis* probiotic isolated having increased motility and an increased ability to occupy sites of *Campylobacter* adhesion within the GIT, provided enhanced protection against *Campylobacter* infection when administered orally to chickens (Aguiar et al. 2013).

Shiga Toxin-Producing *Escherichia coli* (STEC)

STEC are becoming an increased concern as traditionally non-Shiga toxin (STX)-producing *E. coli* are discovered to produce Shiga toxin (one or both of the STX proteins, STX1 and STX2). In 2011, in Germany, an outbreak caused by an STX-producing enteroaggerative *E. coli* (EaggEC), typically STX negative, hospitalized 3842 people, with hundreds developing hemolytic uremic syndrome (HUS). Interestingly, 88% of those cases were healthy adults (Beutin and Martin 2012; Muniesa et al. 2012). This would suggest that STEC are capable of causing systemic infection in not only immunocompromised individuals but also in healthy people. STEC especially the EHEC subtype that possesses the STX and the LEE (locus of enterocyte effacement) pathogenicity island is responsible for sporadic cases and outbreaks characterized by hemorrhagic colitis, largely resulting from ingestion of meat and vegetables contaminated by STEC containing fecal matter.

HUS is the more severe form of STEC infections, occurring in 5–15% of cases (Davis et al. 2013). When the pathogen enters the intestine it begins the synthesis of STX, which crosses the epithelial barrier, gains entry into the blood stream, and eventually invades and damages kidney cells. The key receptor for STX is globotriaosylceramide (Gb3), whereas globotetraosylceramide (Gb4) is the receptor associated with the STX variant responsible for the disease in swine (Degrandis et al. 1989). Knowledge of the host cell receptor responsible for pathogen attachment can be exploited to develop strategies for conferring resistance to the pathogen. In a recent study, recombinant probiotic *E. coli* R1 expressing globotetraose, which mimics an STX receptor on its surface, were twice daily administered to piglets 24 h after receiving a dose of STEC. The recombinant *E. coli* significantly reduced fecal toxin excretion by day 3; however, despite the reduction of intestinal toxin, the probiotic was not successful in reducing the frequency of vascular lesions and clinical disease (Hostetter et al. 2014). Many other studies with probiotic bacteria in mice have shown efficacy in reducing or preventing disease when wild-type probiotics, especially *Lactobacillus,* are administered prior to infection with STEC (Eaton et al. 2011; Tsai et al. 2010). In contrast, it appears the globotetraose expressing probiotic bacteria were effective in neutralizing STX, but not attenuating the disease. As described above in the section on *Salmonella, L. lactis* has been engineered to secrete anti-Gram-negative bacterial AMP. The recombinant *L. lactis*-secreting alyteserin-1a inhibited the growth of *E. coli* O157:H7 when co-cultured (Volzing et al. 2013). With additional studies, this recombinant probiotic bacterium may provide useful for suppressing STEC in both human and animal systems.

Listeria monocytogenes

Listeria monocytogenes is a rare enteric pathogen because it is generally not associated with gastric distress, rather it causes a severe invasive systemic infection in high-risk individuals such as pregnant women, newborns, the elderly, and the immunocompromised (Swaminathan and Gerner-Smidt 2007). While *L. monocytogenes* is associated with a relatively low morbidity when compared to other enteric pathogens such as *Salmonella*, the mortality rate is very high (20–30%) (Voetsch et al. 2007). Kirk et al. (2015) estimated that there were 14,169 cases of *L. monocytogenes* infections leading to 3175 deaths globally in 2010. This equates to a staggering 22.4% mortality rate. *L. monocytogenes,* after surviving the gastric juices of the stomach and arriving at the intestinal tract, attaches to and invades epithelial cells both intracellularly and paracellularly, and disseminates to the mesenteric lymph nodes (MLN), liver, spleen, and can eventually penetrate both the blood-brain and blood-placental barriers (Burkholder et al. 2009; Ribet and Cossart 2015). After penetrating the blood-placental barrier, *L. monocytogenes* can induce microabscesses and necrosis, resulting in preterm spontaneous abortion, stillbirth, and miscarriage (Bakardjiev et al. 2006; Jiao et al. 2011; Vazquez-Boland et al. 2001). Several studies investigating the feasibility of probiotic intervention of listerial infection have been performed. *L. casei* Shirota-fed rats

not only had a reduced *L. monocytogenes* load in the GIT, spleen, liver, and feces, but also had a delayed hypersensitivity to the pathogen suggesting an increased cell-mediated immune response (de Waard et al. 2002). Similarly, *L. delbrueckii* provided a protective effect against colonization, advanced clearance of *L. monocytogenes,* and an increase in IFN-γ and IL-10 (dos Santos et al. 2011). This study highlights the role of host immune modulation in probiotic-induced protection against and clearance of *Listeria*. Additionally, *L. plantarum* can reduce the production of proinflammatory cytokines necessary for *Listeria*-mediated cytotoxic effect (Puertollano et al. 2008). Bacteriocin production by probiotic bacteria can directly inhibit pathogens. *L. salivarius* produces a bacteriocin that provides protection against listerial infection (Corr et al. 2007). In contrast, *Lactococcus lactis*, which produces lacticin, provides strong protection *in vitro*, but failed to protect against *Listeria* infection in *in vivo* mouse and pig studies (Dobson et al. 2011; Rea et al. 2007; Rea et al. 2011). Similarly, in a recent study, Fernandez et al. (2016) determined that a pediocin producer, *Pediococcus acidilactici* UL5 was able to affect the metabolic activity (increased production of acetic and propionic acids) of immobilized intestinal microbiota in a bioreactor, but it was unable to inhibit *L. monocytogenes* growth within the reactor. These studies highlight the species and strain specificity of the antimicrobial action of probiotic bacteria and the potential lack of relationship between *in vitro* and *in vivo* study results. A recombinant *L. paracasei* strain expressing *Listeria* adhesion protein (Burkholder and Bhunia 2010; Jagadeesan et al. 2010) developed by Koo et al. (2012) had reduced *L. monocytogenes* adhesion, invasion and translocation in Caco-2 cell culture model. The recombinant probiotic bacterium expresses LAP, an essential virulence factor that aids in adhesion to and paracellular translocation of *L. monocytogenes* across intestinal epithelial cells that are anchored to the cell wall. LAP interacts with the host Hsp60 (receptor) and facilitates *L. monocytogenes* extraintestinal dissemination across the epithelial barrier (Burkholder and Bhunia 2013; Kim and Bhunia 2013). LAP not only enhances probiotic bacteria binding to the host GIT and attached to a *L. monocytogenes* receptor, but also may allow the probiotic bacteria to be administered frequently to the host for enhanced binding of the probiotic bacteria.

Viral Pathogens

Norovirus (NoV) is the leading cause of foodborne and waterborne acute gastroenteritis worldwide (Jianrong et al. 2012; Rodriguez-Lazaro et al. 2012; Rubio-del-Campo et al. 2014). In the United States, it is responsible for an estimated 23 million cases of gastroenteritis and 50,000 hospitalizations each year (Mattison 2011; Patel et al. 2009). The resulting symptoms of a NoV infection continue for 12–60 h and include gastroenteritis, self-limiting diarrhea, vomiting, and, in some cases, dehydration and death (Rubio-del-Campo et al. 2014). Recently, a recombinant probiotic

Lactobacillus paracasei (rLbp) secreting 3D8 single-chain variable fragment (3D8 scFv), an antinucleic acid antibody which can penetrate a cell and hydrolyze DNA, has antiviral activity in both *in vitro* RAW 264.7 cell culture models and *in vivo* mouse models (accepted model for NoV) (Phuong Mai et al. 2015). Mice pretreated with rLbp secreting 3D8 scFv (3 administrations orally over 6 days) had a 20-fold reduction in mRNA expression of the viral polymerase gene compared to mice pretreated with the empty vector rLbp; however, no change in viral-associated cytokine levels were observed. This could be the result of the small number of oral administration of probiotic bacteria (Phuong Mai et al. 2015). In a human study, fermented milk containing *L. casei* Shirota was given to patients suffering from NoV infection. The treatment group had a decreased duration of fever and gastrointestinal distress compared to the control group (Nagata et al. 2011).

Prebiotics and Synbiotics

The WHO/FAO defines prebiotics as a nonviable food component (typically a fiber) that confers health benefits on the host via modulation of host gut microbiota. Prebiotics are digestion-resistant carbohydrates that are not readily taken up by host cells and are fermentable by the gut microbiota, thus enhancing the beneficial health attributes of the commensal gut microbiota. The definition of prebiotic does not include probiotics, but rather is a fiber that is fermentable by any gut microbiome member (Hutkins et al. 2016). There are three characteristics that a fiber must possess to be considered a prebiotic: (i) maintain its characteristic integrity in the harsh environment of the stomach and GIT (pH, acids, bile, etc.), (ii) avoid absorption in the upper GIT, and (iii) be fermentable by gut microflora (Shiu-Ming 2013). Common prebiotic sources include soybeans, raw oats, unrefined wheat, and unrefined barley, which are nondigestible carbohydrates (Manigandan et al. 2012; Pandey et al. 2015; Pokusaeva et al. 2011). These sources are not well-characterized in composition or dosage requirements for optimal beneficial effects, so the definition of prebiotics was revisited by Roberfroid et al. 2010 (Biplab and Mandal 2014; Roberfroid 2007; Roberfroid et al. 2010). They stated that, "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health". Within this definition, two prebiotics stand out as functional: inulin and (trans) galacto-oligosaccharides (GOS, a hydrolytic product of inulin) (Pokusaeva et al. 2011). When the host benefit-enhancing effects of prebiotics are combined with the enhancement of probiotic bacteria, a new term, called synbiotics, was applied (de Vrese and Schrezenmeir 2008). Some of the probiotic bacteria mixtures listed in Table 2 contain the prebiotics lactose or oligosaccharides, thus these combinations can be termed synbiotics. Manipulating prebiotics and the microbiota composition in the gut may aid in the prevention and treatment of chronic diseases in humans (Shanahan and Quigley 2014).

Conclusions and Future Perspectives

The effects of probiotic bacteria on enteric pathogens have been widely studied both *in vitro* and *in vivo* and there is substantial evidence that probiotics are effective at mitigating foodborne diseases, especially when administered prophylactically. In addition to mitigation of foodborne illness, probiotic bacteria can be beneficial against diarrhea, atopic eczema, colorectal cancers, cardiovascular disease, lactose intolerance, allergic reactions, and treatment of obesity. While there are many specific health benefits to the administration of probiotic bacteria, there remains uninvestigated risks; hence, it is critical that the potential toxic and metabolic effects in human subjects are investigated, particularly in the young, old, pregnant, and immunocompromised. Several areas of research are needed before the application of probiotics can be fully realized, including probiotic strain specificity (not all probiotics are created equal), dosage, specific mechanisms of antimicrobial action, interaction between the applied probiotic bacteria and the host microbiome, and understanding probiotic-host specificity (not all probiotic bacteria are effective in all individuals). Because of this, there is a need for more human clinical trials to address the interactions of probiotic bacteria. Bioengineered probiotic bacteria, expressing specific antimicrobial molecules, possessing pathogen receptor-mimic sites, antigen-specific antibodies, and host-specific receptor sites present exciting areas for future development and study; however, the creation and application of genetically-modified organisms (GMOs) is controversial.

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