Salmonella spp. Interactions with the Gallbladder during Chronic Carriage

DISSEPTION

Presented in Partial Fulfllment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Geoffrey Javier Gonzalez-Escobedo, Microbiologist

Graduate Program in Microbiology

The Ohio State University

2013

Dissertation Committee:

John S. Gunn, PhD., Advisor

Daniel Wozniak, PhD.

Stephanie Seveau, PhD.

Jesse Kwiek, PhD.
Abstract

*Salmonella enterica* serovar Typhi (S. Typhi) is an important human pathogen and the etiologic agent of typhoid fever. Despite advances in modern medicine, typhoid fever is still a significant cause of mortality worldwide. Although most studies focus on the initial stages of the disease, this successful enteric pathogen is able to persist chronically in the gallbladder of some patients. Shedding of S. Typhi by these asymptomatic carriers can contaminate food and water supplies, especially in underdeveloped countries, and as such be a source of recurring *Salmonella* infections. This chronic carrier state is often associated with the presence of gallstones in the gallbladder. Biofilm formation on the surface of cholesterol gallstones has been demonstrated to be a mechanism of chronic colonization and carriage in the gallbladder of mice and human carriers.

In this study, we identified genes specifically up-regulated during biofilm development on cholesterol surfaces by using an in vitro model that mimics the gallbladder and gallstones environment. These genes encode the surface proteins type 1 fimbriae and YcfR. To validate the role of these genes in vivo, we used our mouse model of *Salmonella* chronic carriage. This study strongly suggests that type 1 fimbriae are important for persistent attachment on cholesterol gallstones whereas *ycfR* seems to negatively modulate biofilm formation on cholesterol gallstones. This is the first study to profile gene expression of *Salmonella* during biofilm development on gallstones with
further evaluation in a mouse model of chronic carriage. As part of our effort to fully understand the mechanisms of *Salmonella* persistence, we also examined the gallbladder epithelium as a potential additional niche for chronic carriage. Here we present evidence demonstrating the ability of *Salmonella* to also persist in gallbladder tissue both intracellularly and extracellularly (as micro-colonies or biofilms) in/on the epithelium and that heavily infected host cells can be extruded from the epithelium.

In addition, we also analyzed the histopathological features of chronic carriage up to 1 year post-infection demonstrating that chronic cholecystitis and hepatitis were present in infected mice regardless of the presence of gallstones. At most time points, the mere presence of gallstones caused more gallbladder inflammation than chronic salmonellosis. However, only *Salmonella* chronic inflammation induced pre-malignant lesions (atypical hyperplasia and metaplasia) of the gallbladder and pancreas epithelium. This study has implications regarding the role of *Salmonella* chronic infection and inflammation in oncogenesis. Finally, the genome sequencing of a hyper-biofilm forming strain isolated from the gallstone of a carrier mouse nine months post-infection revealed 14 single nucleotide polymorphisms that may represent a specific adaptation of *Salmonella* to the gallbladder and gallstones environment during long-term carriage.

Taken together, these results reveal the complicated nature of the mechanisms governing chronic carriage in the gallbladder (with or without gallstones) including biofilm formation on gallstones and gallbladder epithelium, intracellular persistence in the gallbladder epithelium, chronic inflammation with development of pre-malignant lesions and mutational adaptation during long-term carriage. Gaining a better understanding of the specific mechanisms of chronic carriage in the gallbladder may contribute to the
development of effective strategies to prevent/treat this chronic, biofilm-mediated infection.
Dedication

To my grandparents Wilfredo and Adriana,
you are my best example of life, strength and integrity.

To my parents Nieves and Godofredo and my sisters Vanessa and Susan,
thank you for your limitless support.

To my love and partner Ruby,
thank you for enduring with me every difficulty regardless of the distance.

To my friends at The Ohio State University and Columbus;
Ran, Tracy, Lynette, Ingrid, Kristi, Mohini, Gustavo, Shilpa, Nrusingh, David, Andrea,
Dekel, Shira, Karla, Antonio, Binjie, Diego, Meredith, Fiorella, Maria Elena and Michael;
I do not conceive this journey without your loyalty.
Acknowledgments

I specially thank my advisor John S. Gunn, PhD., for his continuous support and mentorship in every aspect of my graduate studies. I also thank Daniel Wozniak, PhD.; Jesse Kwiek, PhD.; Stephanie Seveau, PhD.; Joanne Turner, PhD.; Jordi Torrelles, PhD.; and Larry Schlesinger, M.D.; for also providing valuable suggestions and for contributing to my career development. Also, I thank all members of the Center for Microbial Interface Biology (CMIB). I am grateful I had the opportunity to work in this highly interactive environment.

In addition, I want to thank staff members from the OSU Campus Microscopy and Imaging Facility, especially to Brian Kemmenoe; and the OSU Comparative Pathology and Mouse Phenotyping Shared Resource (CPMPSR), especially to Krista La Perle; for their support and contributions of electron microscopy and histology/immunohistochemistry, respectively.

I also thank Dr. Sum P. Lee and Christopher Savard from the Division of Gastroenterology at the University of Washington for their donation of the primary dog gallbladder epithelial cells used in this study. In addition, I also thank Andreas Baumler, PhD. (U. of California, Davis); Steve Libby, PhD. (U. of Washington); Michael McClelland, PhD. (San Diego Institute for Biological Research) as well as Matthew
Chapman, PhD. (U. of Michigan); and Dieter M. Schifferli, PhD. (U. of Pennsylvania) for their donation of strains and antibodies. Finally I thank Sarah Chaney (OSU) for helping in the interpretation of histopathology results.

This work was supported by a grant from the US National Institutes of Health (AI066208) and by a Pelotonia Graduate Fellowship (2011-2012).
Vita

1999-2003 ................................................. B.S. Biological Sciences
          Universidad Nacional de Trujillo, Peru
2004-2005 ................................................. Professional Degree in Microbiology
          Universidad Nacional de Trujillo, Peru
2005-2007 ................................................. Research assistant
          International Potato Center, Lima, Peru
2007 ........................................................ Visiting scholar, Department of Horticulture
          and Crop Sciences, The Ohio State
          University
2008 to present ......................................... Graduate student, Department of
          Microbiology, The Ohio State University
Publications


Fields of Study

Major Field: Microbiology
Table of Contents

Abstract ................................................................................................................................. ii
Dedication .............................................................................................................................. v
Acknowledgments ............................................................................................................... vi
Vita......................................................................................................................................... viii
Table of Contents ................................................................................................................ x
List of Tables ....................................................................................................................... xv
List of Figures ...................................................................................................................... xv

Chapter 1: Introduction ....................................................................................................... 1
  1.1. *Salmonella* and Typhoid Fever ................................................................................. 1
    1.1.1. The genus *Salmonella*: Taxonomy, genetics and clinical importance ........... 1
    1.1.2. Typhoid fever ....................................................................................................... 5
      1.1.2.1. Historic background ..................................................................................... 5
      1.1.2.2. Epidemiology ............................................................................................... 6
      1.1.2.3. Pathogenesis and clinical manifestations ..................................................... 7
      1.1.2.4. Virulence factors of *Salmonella enterica* ..................................................... 10
      1.1.2.5. Diagnosis ..................................................................................................... 16
      1.1.2.6. Treatment and vaccines .............................................................................. 17
      1.1.2.7. Host-pathogen interactions and immunity ...................................................... 18
    1.1.3. Chronic typhoid carriage ...................................................................................... 22
      1.1.3.1. Historic background ..................................................................................... 22
      1.1.3.2. Epidemiology and risk factors ....................................................................... 23
      1.1.3.3. Treatment ..................................................................................................... 25
      1.1.3.4. Complications .............................................................................................. 26
      1.1.3.5. Animal studies of chronic carriage ................................................................. 27
      1.1.3.6. Immune response during chronic carriage ...................................................... 29
  1.2. The Gallbladder: Anatomy, physiology, infection and inflammation .................... 30
    1.2.1. The Gallbladder and bile .................................................................................... 30
1.2.2. Cholelithiasis ................................................................. 35
1.2.3. Cholecystitis ................................................................. 36
1.3. Salmonella interactions with the gallbladder ......................... 38
  1.3.1. Salmonella and bile signaling ........................................ 38
  1.3.2. Acute cholecystitis caused by Salmonella ....................... 41
1.4. Salmonella biofilms .......................................................... 44
  1.4.1. Biofilms........................................................................ 44
  1.4.2. Factors mediating Salmonella biofilm formation ............... 45
    1.4.2.1. Flagella ................................................................... 46
    1.4.2.2. Fimbriae .................................................................. 47
    1.4.2.3. Cellulose and colanic acid ........................................ 48
    1.4.2.4. O-antigen capsule and other exopolysaccharides .......... 49
    1.4.2.5. Biofilm associated proteins and extracellular DNA ...... 50
  1.4.3. Coordinated regulation during Salmonella biofilms ........... 51
    1.4.3.1. CsgD ...................................................................... 51
    1.4.3.2. Cyclic di-guanylic acid ............................................ 52
    1.4.3.3. Small RNAs .............................................................. 54
    1.4.3.4. Quorum sensing ...................................................... 54
    1.4.3.5. Global metabolic shift .............................................. 56
1.5. Salmonella biofilms on gallstones ......................................... 57
  1.5.1. In vitro biofilm formation on gallstones ......................... 57
  1.5.2. Biofilm initiation on gallstones ...................................... 58
  1.5.3. Biofilm maturation on gallstones .................................. 60
  1.5.4. Mouse model of typhoid carriage .................................. 61
  1.5.5. Human studies of typhoid carriage ............................... 62
1.6. Hypothesis and goals ........................................................... 65

Chapter 2: Identification of Salmonella Genes Regulated During Biofilm Formation on Cholesterol (Gallstone) Surfaces ......................................................... 68
  2.1. Abstract .......................................................................... 68
  2.2. Introduction ..................................................................... 69
  2.3. Materials and Methods .................................................... 72
    2.3.1. Ethics Statement ....................................................... 72
    2.3.2. Bacterial strains and growth conditions ....................... 73
    2.3.3. Biofilm growth on microtiter plates ............................. 73
    2.3.4. Flow-through system assays ..................................... 74
2.3.5. Crystal violet assays ................................................................. 75
2.3.6. Differential gene expression analysis ............................................. 75
2.3.7. Generation of mutants and cloning procedures ................................. 76
2.3.8. Biofilm treatment with DNase I, proteinase and cellulase .................. 77
2.3.9. FimA and CsgA detection by immunofluorescence ............................ 78
2.3.10. Mouse infections ........................................................................ 78
2.3.11. Confocal microscopy ................................................................... 79
2.3.12. Scanning electron microscopy ....................................................... 79
2.4. Results ............................................................................................. 80
2.4.1. Cholesterol coated-surfaces enhance biofilm formation ....................... 80
2.4.2. Differential gene expression between planktonic versus. biofilm cells (± cholesterol-coated surfaces) ...................................................... 82
2.4.3. Type 1 fimbriae structural genes (fimAICD) and ycfR were specifically up-regulated during biofilm development on cholesterol-coated surfaces ...................... 83
2.4.4. Mutations of type 1 fimbriae and ycfR increased biofilm formation on cholesterol-coated surfaces ........................................................................ 85
2.4.5. Expression of FimA and ycfR increased during biofilm maturation in vitro ... 88
2.4.6. Increased curli expression is observed in biofilms of fimAICDHF and ycfR mutants on cholesterol-coated surfaces ........................................... 90
2.4.7. Type 1 fimbriae and ycfR mutants colonized Nramp1+/+ mice during early and chronic time points regardless of the presence of gallstones ...................... 91
2.4.8. In vivo, type 1 fimbriae and ycfR mutants showed different biofilm capacities depending on the time of infection .............................................. 95
2.5. Discussion .......................................................................................... 98

Chapter 3: The Gallbladder Epithelium as a Niche for Chronic Salmonella Carriage 103
3.1. Abstract .............................................................................................. 103
3.2. Introduction ........................................................................................ 104
3.3. Materials and Methods ..................................................................... 106
3.3.1. Bacterial strains and growth conditions ........................................... 106
3.3.2. Growth of gallbladder epithelial cells .............................................. 107
3.3.3. Attachment, invasion and intracellular survival assays ..................... 108
3.3.4. Monitoring of attachment and invasion by microscopy .................... 109
3.3.5. Flow-through system assays ......................................................... 109
3.3.6. Mouse infections ......................................................................... 110
3.3.7. Immunohistochemistry ................................................................. 110
3.3.8. Confocal microscopy ........................................................................................................ 111
3.3.9. Electron microscopy .......................................................................................................... 112
3.4. Results .................................................................................................................................. 113

3.4.1. S. Typhimurium bacteria attach to the surface of polarized DGEC resembling microcolonies ............................................................... 113
3.4.2. S. Typhimurium invades polarized gallbladder epithelial cells in vitro .......... 113
3.4.3. S. Typhimurium can replicate intracellularly in DGEC regardless of previous exposure of the epithelium to bile .................................................. 114
3.4.4. S. Typhimurium forms extensive cellular aggregations/biofilms on the surface of DGEC ........................................................................ 119
3.4.5. Curli and yciE affect invasion into and biofilm formation on DGEC .......... 120
3.4.6. In vivo assays recapitulate the invasion, intracellular survival, extrusion, and microcolony formation shown in vitro .......................................................... 123
3.4.7. Inflammation of the gallbladder is primarily macrophage related during chronic salmonellosis ................................................................. 129
3.5. Discussion ............................................................................................................................... 132

Chapter 4: Histopathological Analysis of Salmonella Chronic Carriage in the Hepatopancreatobiliary System .......................................................... 141
4.1. Abstract .................................................................................................................................. 141
4.2. Introduction ............................................................................................................................. 142
4.3. Materials and Methods .......................................................................................................... 147

4.3.1. Mice infections and bacteria enumeration ........................................................................ 147
4.3.2. Histopathology of the hepatopancreatobiliary system of chronically infected mice ................................................................. 148
4.3.3. Immunohistochemistry .................................................................................................... 148
4.4. Results .................................................................................................................................. 150

4.4.1. Salmonella spp. was recovered at all time points from the feces and spleen of mice without gallstones, but inconsistently observed in other tissues/fluids .......... 150
4.4.2. Salmonella spp. was detected by immunohistochemistry in the liver, gallbladder and pancreas of some infected mice .............................................. 151
4.4.3. Chronic cholecystitis and hepatitis are the hallmark during Salmonella carriage ......................................................................................... 156
4.4.4. Biliary epithelial hyperplasia was observed as a result of gallstone disease and Salmonella carriage ......................................................... 161
4.4.5. Atypical hyperplasia/dysplasia was only observed in the gallbladder of infected mice regardless of the presence of gallstones .......................................... 162
4.4.6. Pancreatic mucinous metaplasia was only evident in infected mice regardless of the presence of gallstones ......................................................... 163
4.5. Discussion ............................................................................................................................... 164

xiii
Addendum: Characterization of a Hyper-Biofilm Strain Isolated from Mouse Gallstones

5.1. Identification of a hyper-biofilm forming strain .................................................................172

5.2. Methods and Results ........................................................................................................174
  5.2.1. Biofilm formation of JSG3538 on various surfaces .....................................................174
  5.2.2. Biofilm treatment with DNase I, proteinase and cellulase .................................176
  5.2.3. Sequencing ..................................................................................................................177

5.3. Discussion ..........................................................................................................................178

Concluding remarks ..............................................................................................................182

References ..................................................................................................................................187

Appendix A: List of genes differentially regulated in S. Typhimurium planktonic versus biofilms cells grown on glass or cholesterol (>5 fold change) ..............................................230

Appendix B: Biofilms from Type 1 fimbriae and ycfR mutants at 72 hours post-inoculation .................................................................................................................................238
List of Tables

Table 1. Bacterial strains and plasmids used in Chapter 2..........................74
Table 2. Oligonucleotide primers used in Chapter 2 ..................................77
Table 3. Bacterial strains and plasmids used in Chapter 3 ............................107
Table 4. Histological scoring of macrophage and neutrophil recruitment in mouse
gallbladder tissue at different days post-infection ......................................130
Table 5. Histopathological grading system for hepatopancreatobiliary lesions.........149
Table 6. List of genes containing single nucleotide polymorphisms in JSG3538.......178
List of Figures

Figure 1. Strategies that allow *Salmonella* spp. to cross the intestinal barrier, survive in intestinal tissues and spread systemically .........................................................15

Figure 2. The gallbladder and biliary tract .................................................................31

Figure 3. Gallbladder histology .........................................................................................34

Figure 4. The physical-chemical processes involved in formation of cholesterol gallstones .................................................................37

Figure 5. *Salmonella* spp. acute infection of the gallbladder ........................................43

Figure 6. Biofilm formation on human gallstones was observed only in patients seeking cholecystectomy that were positive for typhoid carriage ..........................................64

Figure 7. Cholesterol coated-surfaces enhance biofilm formation ..................................81

Figure 8. Type 1 fimbriae genes (*fimAICD*) and *ycfR* were induced during biofilm development on cholesterol-coated surfaces .........................................................84

Figure 9. Quantitative Real-Time PCR validated the increased expression of *fimC* and *ycfR* during biofilm formation on cholesterol coated-surfaces .........................................84

Figure 10. Type 1 fimbriae and *ycfR* mutants showed enhanced biofilm formation on cholesterol-coated surfaces in vitro .................................................................86

Figure 11. Biofilms from type 1 fimbriae and *ycfR* mutants are thicker on cholesterol-coated surfaces in vitro .............................................................................................................87

Figure 12. FimA expression during biofilm formation on cholesterol-coated surfaces ....89
Figure 13. Expression of \textit{ycfR} increases during biofilm maturation.................................90
Figure 14. Proteinase treatment of established biofilms drastically affected biofilm formation of the wild-type, type 1 fimbriae and \textit{ycfR} mutants ........................................92
Figure 15. CsgA expression during biofilm formation on cholesterol-coated surfaces....93
Figure 16. Type 1 fimbriae and \textit{ycfR} mutants colonized \textit{Nramp1}^{+/+} mice during early and chronic time points regardless of the presence of gallstones ........................................94
Figure 17. In vivo colonization and biofilm formation on mouse gallstones of \textit{S. Typhimurium} wild-type and mutants ......................................................................................97
Figure 18. \textit{S. Typhimurium} attaches and invades into/on polarized DGEC in vitro.......115
Figure 19. Exposure of DGEC with bile decreases invasion by \textit{S. Typhimurium}..............116
Figure 20. SPI-1, curli fimbriae and \textit{yciE} but not type 1 fimbriae mutants decrease invasion into DGEC in vitro ..................................................................................................................117
Figure 21. Representative SEM image of \textit{S. Typhimurium} invading DGEC in the absence of bile ..........................................................................................................................117
Figure 22. \textit{S. Typhimurium} can replicate intracellularly in DGEC .................................118
Figure 23. \textit{S. Typhimurium} forms extensive cellular aggregations/biofilms on the surface of DGEC .................................................................................................................................119
Figure 24. SPI-1 is also important for attachment to the surface of DGEC.................121
Figure 25. Curli and \textit{yciE} affect biofilm formation on DGEC .......................................122
Figure 26. The presence of gallstones enhances \textit{Salmonella} colonization at 60 days post-infection .................................................................................................................................125
Figure 27. In vivo assays recapitulate invasion, intracellular survival and extrusion.....126
Figure 28. In the absence of gallstones, inflammation of the gallbladder is mostly macrophage related during infection .........................................................................................127
Figure 29. S. Typhimurium can form microcolonies on the surface of the mouse gallbladder epithelium.................................................................128

Figure 30. In the presence of gallstones, inflammation of the gallbladder is both macrophage and neutrophil related.........................................................131

Figure 31. Current model of typhoid chronic carriage .............................................................138

Figure 32. Salmonella detection in organs and bodily fluids of chronically infected mice up to 1 year post-infection ...............................................................152

Figure 33. Salmonella LPS staining was frequently detected in the gallbladder of infected mice regardless of the presence of gallstones.............................................................153

Figure 34. Salmonella LPS staining was detected in the liver of infected mice regardless of the presence of gallstones...............................................................154

Figure 35. Salmonella LPS staining was detected in the pancreas of infected mice regardless of the presence of gallstones.............................................................155

Figure 36. Chronic cholecystitis occurs as a result of both gallstone disease and Salmonella carriage.................................................................158

Figure 37. Chronic hepatitis is exacerbated in infected mice at 3 and 6 months post-infection.........................................................................................159

Figure 38. Chronic pancreatitis is exacerbated in infected mice at 3 and 6 months post-infection.........................................................................................160

Figure 39. Dystrophic mineralization of venous fibrin thrombi was only present in the gallbladder and liver of infected mice with gallstones........................................161

Figure 40. Atypical hyperplasia was observed in the gallbladder of infected mice regardless of the presence of gallstones.............................................................162

Figure 41. Pancreatic mucinous metaplasia was evident in infected mice regardless of the presence of gallstones .................................................................163
Figure 42. Biofilm capacity of ten different isolates recovered from mouse gallstones 9 months post-infection .................................................................173

Figure 43. Hyper-biofilm formation by JSG3538 did not occur in other sites of the same mouse. ..................................................................................................................173

Figure 44. Hyper-biofilm formation by JSG3538 does not occur on every surface. .....175

Figure 45. JSG3538 does not form biofilms on dog gallbladder epithelial cells ..........175

Figure 46. Proteinase treatment disrupts biofilms formed by S. Typhimurium wild-type (JSG210) and JSG3538...............................................................................................................176
Chapter 1: Introduction

1.1. Salmonella and Typhoid Fever

1.1.1. The genus Salmonella: Taxonomy, genetics and clinical importance

The genus Salmonella, named after the American microbiologist D.E. Salmon, belongs to the Enterobacteriaceae family which includes the well-studied Escherichia coli, Klebsiella pneumoniae, Yersinia pestis and Shigella dysenteriae. As all members of this family, Salmonella organisms are rod-shaped, gram negative and facultative anaerobes (Fabrega and Vila, 2013). Salmonella taxonomy is derived from the Kauffman-White scheme and is updated by The World Health Organization (WHO) Collaborating Centre for Reference and Research on Salmonella (Grimont and Weill, 2007). The genus contains three species: Salmonella enterica, Salmonella bongori and Salmonella subterranean (Su and Chiu, 2007). S. enterica contains six subspecies: S. enterica subsp. enterica (I), S. enterica subsp. salamae (II), S. enterica subsp. arizonae (IIIa), S. enterica subsp. diarizonae (IIIb), S. enterica subsp. houtenae (IV), and S. enterica subsp. indica (VI). There is only one subspecies under S. bongori (subsp. V). S. enterica subsp. II-VI and S. bongori are commensals of cold-blooded animals and only S. enterica subsp. enterica (I) inhabits warm-blooded animals (Ellermeier and Slauch, 2006; Fabrega and Vila, 2013).
Salmonella isolates are serotyped on the basis of surface antigen identification of O (somatic) and H (flagellar) epitopes, permitting the characterization of 46 O-serogroups and 119 H-antigens. The O-antigen is the outermost component of the lipopolysaccharide (LPS), and it is extremely polymorphic due to variations in the types of sugars present and their arrangements; thus contributing to antigenic diversity on the cell surface. To date, more than 2,500 serovars have been identified; 1,531 of them belong to subspecies I (Cooke et al., 2007). The nomenclature should include S. enterica followed by the serovar (e.g. S. enterica serovar Typhi). However, abbreviated versions are acceptable such as S. Typhimurium instead of S. enterica serovar Typhimurium (Ellermeier and Slauch, 2006).

According to their clinical relevance, Salmonella can be divided in two groups. The first group includes the human-host restricted serovars S. Typhi and S. Paratyphi A, B and C, which are the etiological agents of typhoid fever and paratyphoid fever; respectively (collectively called enteric fever). They are important causes of life-threatening febrile illness especially in impoverished populations with inadequate sanitation. The second group comprises all the serovars that usually causes self-limited gastroenteritis or enterocolitis/diarrheal disease with secondary bacteremia occurring in less than 10% of patients. They are commonly referred as non-typhoidal Salmonella (NTS) with S. Enteritidis and S. Typhimurium as the most predominant serovars causing disease in humans (Baumler et al., 1998; Galanis et al., 2006; Rabsch et al., 2001). Despite the improvement in sanitation, gastroenteritis caused by NTS is still a significant burden not only in the developing world but also in industrialized countries. It is
estimated that 94 million cases of gastroenteritis due to NTS occur worldwide, leading to 155,000 deaths each year (Laupland et al., 2010; Majowicz et al., 2010).

*Salmonella* is widely distributed in nature, the host range of NTS serovars is broad; including poultry, cattle, pigs, dogs, cats, rodents and wild animals where bacteria can persist undetected. *Salmonella* is recognized as the leading cause of foodborne outbreaks and infections in many countries (CDC, 2008; Muhlenberg, 1993). NTS gastroenteritis is commonly due to food poisoning in developed countries and is transmitted via contaminated food including meat, eggs, dairy products; contaminated water; fresh produce such as tomato and lettuce; thereby gaining entry into almost every aspect of the human food chain (Behravesh et al., 2012; Dominguez et al., 2002; Hanning et al., 2009; Tauxe et al., 1997). However, in Sub-Saharan Africa, invasive NTS are among the three most common pathogens causing bacterial bloodstream infections in HIV-adults and children (malaria and malnutrition-associated) with concomitant multi-drug resistance (MDR) that exacerbates the situation. (Feasey et al., 2012; Graham, 2010; Kariuki et al., 2006; Morpeth et al., 2009; Parry and Threlfall, 2008). MDR strains of *S. Typhimurium* have become a major cause of salmonellosis worldwide. In contrast to Sub-Saharan Africa, NTS invasive disease is not common in Asia except in subjects with severe immunosuppression (Dhanoa and Fatt, 2009; Khan et al., 2010).

In the United States (US), it is estimated that annually there are 1.3 million cases of salmonellosis, 15,000 hospitalizations, and 400 deaths with approximately 2.5 billion dollars of lost productivity and medical costs (Hardnett et al., 2004; USDA-ERS, 2007). In addition, NTS infections were the major cause of death among foodborne bacterial
pathogens between 1996 and 2005 (Barton Behravesh et al., 2011). From 2005 to 2010, CDC reported an unusual number of cases in the US as a result of several independent outbreaks. In 2010, a multistate outbreak of human infection across the US was attributed to the consumption of eggs contaminated with S. Enteritidis, which resulted in 1,939 reported illnesses (CDC, 2010).

The core genomes of *E. coli* and *S. enterica* differ by only 10% in their DNA sequences and suggest that the two species derived from a common ancestor about 100 million years ago (Baker and Dougan, 2007). The variation in pathogenesis of the serovars is accountable to horizontal gene acquisition, with *Salmonella* pathogenicity islands (SPIs) as the most remarkable example. The genes acquired play roles in pathogenesis, resistance against antibiotics, and metabolism (Groisman and Ochman, 1996). Horizontal gene transfer could occur by different modes including phage infection, conjugative plasmids, transposition, transformation or most commonly by inserting genes within tRNA genes (Porwollik and McClelland, 2003). At present, 21 SPIs have been identified. *S. Typhimurium* and *S. Typhi* genomes share 11 common SPIs; four are specific to *S. Typhi* (SPI-7, 15, 17, and 18) and only one (SPI-14) to *S. Typhimurium* (Sabbagh et al., 2010). In addition to gaining external genes, *Salmonella* may also tend to lose certain genes to maintain virulence, for instance, *Salmonella* lacks the *lac* operon (Eswarappa et al., 2009).

Comparison of different *S. Typhi* isolates show that they are highly related and have arisen from a single point of origin approximately 30,000–50,000 years ago (Baker and Dougan, 2007). *S. Typhi* genome, compared to that of *S. Typhimurium*, harbors
many inactivated or disrupted genes. This can partly explain the restricted host range of S. Typhi and the different immune responses both serovars induce upon entering their host (Holt et al., 2009; McClelland et al., 2001). NTS strains genomic analyses have also provided some evolutionary aspects of host-adapted microbes. Of the 23 S. Typhimurium genes that are inactivated in S. Typhi, 11 are pseudogenes in NTS isolates (Kingsley et al., 2009). These observations suggest that a similar process of adaption to the human host may be occurring in African NTS as has been observed in S. Typhi.

1.1.2. Typhoid fever

1.1.2.1. Historic background

Typhoid fever is caused by S. Typhi. This organism was described as the etiological agent of typhoid fever in 1880 by Carl Joseph Eberth but descriptions are recognized in Greek and Chinese texts dating back to hundreds of years B.C. (August and Konert, 1993; Cunha, 2004). The disease was called typhoid because of its similarity to typhus. In fact, typhoid derives from the Greek typhos, which translates as “putrid odor”. It is believed that typhoid fever was responsible for the plague of Athens and the death of Pericles and Alexander the Great; events that dramatically changed the fate of the Greek Empire (Papagrigorakis et al., 2006; Wood, 1997). In most recent times, especially at the end of 19th century and beginning of the 20th, typhoid fever was a leading cause of mortality in north American and European cities (Budd, 1918). The improvement of sanitation practices and the availability of antibiotics drastically changed the incidence of typhoid fever in industrialized countries after the mid-20th century (Woodward et al., 2004).
1.1.2.2. Epidemiology

Typhoid fever is a human-restricted systemic disease responsible for approximately 21 million cases each year, resulting in more than 200,000 deaths worldwide (Crump and Mintz, 2010). Typhoid fever is endemic in less developed countries where poor sanitation and limited access to clean water facilitate the spread of the disease (Crump et al., 2004; Parry et al., 2002). The incidence of typhoid fever varies considerably between different regions and is difficult to obtain in endemic areas due to poor reporting and diagnostic inaccuracy. High incidence (>100/100,000 cases/year) is reported in the Indian subcontinent and South East Asia. Medium incidence (10-100 cases per 100,000/year) is reported in Africa, Latin America, the Caribbean and the rest of Asia and Oceania. Low incidence (< than 10/100,000 cases/year) are reported in Europe, Australasia and North America where they are mostly related to travel to endemic areas (Crump et al., 2004). In the US, the incidence is low with approximately 400 cases per year (O’Brien et al., 2001). Globally, children are disproportionately affected. In the last decade, the annual incidence among children of 2 to 5 years of age in India and Pakistan was 450/100,000 with an incidence of bacteremia in children less than 2 years of age of 443/100,000 (Crump and Mintz, 2010; Kariuki, 2008; Ochiai et al., 2008; WHO, 2008b).

Transmission of typhoid fever typically occurs via the fecal-oral route through contaminated food or water (Parry et al., 2002). Urine may also be a vehicle of S. Typhi, but this usually occurs in individuals with chronic schistosomiasis. People with reticuloendothelial abnormalities due to malaria, sickle cell disease and schistosomiasis
are at risk for severe disease. Because gastric acidity is one of the primary host defense mechanisms, the use of anti-acids or proton pump inhibitors (related to gastric achlorhydria and gastric surgery history) are associated with increased risk of typhoid fever (Crum, 2003). Typhoid fever represents a major threat to current society due to the disease severity and life-threatening complications, recurrence of disease through carrier state, widespread emergence of MDR strains and its use as a potential candidate in bioterrorism (Marathe et al., 2012; WHO, 2004). Mortality rates in untreated typhoid fever can be 10-15% and is related to MDR strains (Mastroeni and Grant, 2011).

1.1.2.3. Pathogenesis and clinical manifestations

Typhoid fever is a systemic disease that varies in severity and has a primary and a secondary infection. The incubation period is usually 7 to 14 days, although it has a range of 3 to 60 days depending upon the inoculum and the host defense (Bhan et al., 2005; Linam and Gerber, 2007; Parry et al., 2002). The infectious dose of S. Typhi has a range of $10^3$ to $10^6$ organisms (Hornick et al., 1970a). After ingestion, bacteria that survive the acidic barrier in the stomach, colonize the small intestine (Mastroeni and Grant, 2011). Bacteria cross the intestinal epithelial barrier via three routes: (i) active invasion of enterocytes; (ii) passive invasion into M cells in the Peyer’s patches/lymphoid associated tissue of the distal ileum, which sample antigens from the intestinal lumen; and (iii) direct uptake by CD18$^+$ cells (macropages and/or dendritic cells) that intercalate epithelial cells by extending protrusions into the gut lumen (Jepson and Clark, 2001; Niess et al., 2005; Rescigno et al., 2001; Vazquez-Torres et al., 1999). Invading bacteria from the first two routes are internalized by macrophages and dendritic cells residing in
the lamina propria. *Salmonella*-infected phagocytes migrate to the intestinal lymphoid follicles and the draining mesenteric lymph nodes (MLN) and spread systemically via lymph and blood (Vladoianu et al., 1990; Voedisch et al., 2009; Wain et al., 1998; Zhao et al., 2006) (Fig. 1). The most common sites of secondary infection are the liver, spleen, bone marrow and the gallbladder. The bacteria reach the gallbladder via the vasculature or through the ducts emanating from the liver (Hornick et al., 1970a; Kaur and Jain, 2012; Parry et al., 2002). The bacteria that are excreted in the bile can then reinvade the intestinal wall by the mechanism previously described or are excreted in the feces. The gallbladder is the main reservoir during a chronic infection with *S. Typhi* (Gonzalez-Escobedo et al., 2011; Levine et al., 1982).

*Salmonella* are able to survive and multiply within the macrophages of the MLN, liver, spleen and bone marrow (House et al., 2001a; Richter-Dahlfors et al., 1997; Salcedo et al., 2001). Infection of neutrophils and dendritic cells by *Salmonella* has also been reported in the spleen and MLN (Cheminay et al., 2005; Dunlap et al., 1992; Geddes et al., 2007; Yrlid et al., 2001). Thus, phagocyte carriage of intracellular *Salmonella* is required for establishing a systemic infection (Voedisch et al., 2009). Interestingly, phagocytes typically contain a single bacterium and dendritic cells restrict the growth of *Salmonella* in the MLN (Sheppard et al., 2003; Voedisch et al., 2009). At a threshold level, determined by the number of bacteria, the bacterial virulence and the host immune response; the bacteria are released from their intracellular habitat and spread within each organ and/or into the bloodstream (Grant et al., 2008a). This bacteremic or secondary phase of the disease coincides with the onset of typical typhoid fever symptoms (Naylor, 1983).
S. Typhi bacterial invasion of several host cells and the inflammatory response are responsible for the clinical manifestations. Typical symptoms of typhoid fever are high fever, malaise and abdominal pain (Parry et al., 2002). Before the onset of fever, symptoms may include dull frontal headache and diarrhea or constipation. Diarrhea is most common in children and adults with HIV infection (Mahmud et al., 2008). Initially there is low-grade fever that rises progressively with a high sustained fever in the second week. Fever may persist for up to 4 weeks if untreated (Patel et al., 2010; Pohan, 2004; Thisyakorn et al., 1987). Other non-specific symptoms include myalgia, anorexia and dry cough (Mayer and Neilson, 2010). Clinical features such as a coated tongue, tender abdomen, hepatomegaly, splenomegaly, and a relative bradycardia (temperature-pulse relationship) are common (Mahmud et al., 2008). Rose spots appear during the second week and occur in 5 to 30% of cases. They appear as 2-4 mm blanching maculopapular lesions on chest and abdomen (Kuvandik et al., 2009; Parry et al., 2002; Sanchez-Vargas et al., 2011). Unlike many other gram-negative bacteremias, neutrophilia and septic shock are not typical manifestations (Tsolis et al., 2008).

Complications may present in 10 to 15% of patients in endemic regions and they include gastrointestinal bleeding, intestinal perforation (mostly in the ileum), peritonitis, septicemia and meningitis with the highest incidence found in pediatric and immunocompromised patients. These complications usually appear during the third week, they are life-threatening and require advanced medical care that is often not available in typhoid-endemic regions (Cohen et al., 1987; Gordon, 2008; Kaur and Jain, 2012; Sinha et al., 1999). Relapse may occur 2 to 3 weeks after fever resolution in 5 to 10% of patients. The relapse is usually milder than the original attack (Parry et al.,
Re-infection may also occur and can be distinguished from relapse by molecular typing (Wain et al., 1999). Up to 5% of patients, regardless of treatment, may become chronic asymptomatic carriers (Levine et al., 1982).

The use of animal models has greatly advanced understanding of S. Typhi pathogenesis at intestinal and systemic sites. Because S. Typhi is a human restricted pathogen, in vivo studies of S. Typhi pathogenesis typically involve a murine model of infection using S. Typhimurium. The pathological features of the course of murine systemic infection with S. Typhimurium are similar to those of human infection with S. Typhi (Santos et al., 2001). Typically, susceptible mice lacking a functional copy of the gene Nramp1 (Slc11a1) are used to model acute typhoid fever. Nramp1 (natural resistance-associated macrophage protein one) is a critical factor in controlling the replication of intracellular bacteria (Forbes and Gros, 2001). It exerts this role by stimulating expression of lipocalin-2 which in turn scavenges iron-loaded bacteria siderophores and mediates iron efflux from macrophages (Fritsche et al., 2012).

1.1.2.4. Virulence factors of Salmonella enterica

The complex pathogenesis of systemic typhoid and paratyphoid infections correlates with the presence of a large number of offensive and defensive virulence factors (Groisman and Ochman, 1997). About 90% of the genes in S. Typhi and S. Typhimurium serovars are identical (McClelland et al., 2001). The 10% of genes that differ include virulence factors, which determine their pathogenic potential (Sabbagh et al., 2010). Virulence factors, most notably, type III secretion systems (T3SS), Vi antigen
(S. Typhi only), LPS and other surface polysaccharides; flagella, fimbriae and various factors essential for the intracellular life cycle of S. enterica have been characterized. Many of these factors are regulated by two-component systems (TCS) such as PhoP/Q, PmrA/B, OmpR/EnvZ, SirA/BarA and SsrA/SsrB; by global regulators such as SirA, HilA, CsrA, IHF, Hfq and Fnr; and by the sigma factors RpoS and RpoE (Garai et al., 2012; Groisman, 2001; Marathe et al., 2012; Merighi et al., 2005; Yoon et al., 2011).

Fimbriae or pili are proteinaceous appendages expressed on the bacterial surface. They mediate initial attachment to the host cells, colonization, and biofilm formation; although little is known about their true virulence potential (Ibarra and Steele-Mortimer, 2009). Each Salmonella serovar harbors a unique combination of fimbrial operons. Type 1 fimbriae (fim), plasmid encoded fimbriae (pef), long polar fimbriae (lpf) and thin aggregative fimbriae or curli (csg) have been reported to adhere to epithelial and dendritic cells (Baumler et al., 1996; Boddicker et al., 2002; Humphries et al., 2001). Other adhesins (non-fimbrial) include autotransporters MisL, ShdA and the SPI-4 encoded adhesin SiiE. They have a role in Salmonella intestinal colonization and persistence via binding to various eukaryotic extracellular matrix (Dorsey et al., 2005; Gerlach et al., 2007b; Kingsley et al., 2002; Kingsley et al., 2000). In S. Typhi, type IV B pilus is a major adhesion factor during entry of this pathogen into intestinal epithelial cells (Balakrishna et al., 2009).

The Vi-capsular polysaccharide antigen (Vi antigen) is an important factor for S. Typhi virulence, but notably absent in S. Typhimurium, S. Paratyphi A, and most other Salmonella serovars. Vi-antigen synthesis is controlled by tviA and tviB, which are
located on SPI-7 (Kolyva et al., 1992; Virlogeux et al., 1995). Vi antigen is very immunogenic and its presence increases infectivity of S. Typhi and disease severity. In fact, natural infection is usually associated with the expression of Vi antigen in isolated S. Typhi (Wain et al., 2005). However, Vi negative mutants are still able to cause a typhoid-like illness in human volunteers (Hone et al., 1988; Zhang et al., 2008). Recently it has been hypothesized that the Vi capsule can prevent host-pathogen detection by preventing LPS recognition by pattern recognition receptors (PRRs) (Wilson et al., 2008).

*Salmonella* possesses extremely versatile strategies to infect different target host cells. *Salmonella* is a facultative intracellular pathogen that can be found in both phagocytic and non-phagocytic cells, in which bacteria can survive and replicate (Everest et al., 2001; Parry et al., 2002). The mode of entry as well as the strategy followed to survive inside the target cell varies according to the type of cell and depends on the temporal expression of particular bacterial genes (de Jong et al., 2012). *S. enterica* spp. possesses two well characterized T3SS, also called injectosomes, which are encoded within SPI-1 and SPI-2, respectively. The T3SS is a secretion apparatus that functions like a molecular syringe by delivering effector proteins from the bacterial cytoplasm into the target-cell cytosol (Figueira and Holden, 2012). These effector proteins are suggested to have multiple activities within host cells, manipulating their signaling cascades.
SPI-1 harbors the genes for T3SS1, which is crucial for the invasion of non-phagocytic cells by inducing membrane ruffling which consists in alterations of the actin cytoskeleton that ultimately results in macropinocytosis of Salmonella (Galan, 1999, 2001; Raffatellu et al., 2005). SPI-1 plays an important role in both forms of diseases caused by Salmonella (gastroenteritis and enteric fever) (Hansen-Wester and Hensel, 2001). SPI-1 encoded protein effectors also induce tight junction disruption, fluid accumulation in the intestine and activation of pro-inflammatory responses (Bruno et al., 2009; Garai et al., 2012; Haraga et al., 2008). SPI-1 also induces apoptosis in macrophages but exerts the opposite function in epithelial cells (Hersh et al., 1999; Knodler et al., 2005; van der Velden et al., 2000). Although its pivotal role in invasion of non-phagocytic cells, S. Typhimurium mutants deficient in SPI-1 can disseminate and cause systemic infection from the gastrointestinal tract through direct uptake by CD-18 phagocytic cells (Rescigno et al., 2001; Vazquez-Torres et al., 1999) (Fig. 1).

Following internalization, Salmonella remains within a specialized membrane-bound compartment known as Salmonella containing vacuole (SCV) and translocates effector proteins into the host endomembrane system and cytoplasm (Bakowski et al., 2008) (Fig. 1). This intracellular survival is mediated by T3SS2, encoded on SPI-2. T3SS2 is not essential for gastroenteritis but it is required for systemic infection in the mouse and survival within macrophages (Hensel et al., 1998). T3SS2 protein effectors control the formation and maintenance of the SCV and its juxtanuclear position among other changes that arrest the host endosomal pathway at the late endosome stage (Figueira and Holden, 2012). This confers protection against reactive oxygen species (ROS) and reactive nitrogen species (RNS) inside macrophages, therefore preventing
phagocytic burst (Chakravortty et al., 2002; Gallois et al., 2001; Haraga et al., 2008; Vazquez-Torres et al., 2000). Thus, *Salmonella* has the ability to survive and replicate within SCV where it is able to avoid host antimicrobial mechanisms.

Both T3SS1 and T3SS2 contribute to the development of enterocolitis (Grassl and Finlay, 2008). *Salmonella* exploits this inflammation to outcompete the gut microbiota by using tetrathionate (generated by oxidative burst during inflammation) as a respiratory electron acceptor (Winter et al., 2010). Although the importance of these systems for the virulence of *S. Typhimurium* is clear, limited data are available concerning the role of T3SS in *S. Typhi*. It has been shown that T3SS2 of *S. Typhi* is not required for survival in human macrophages but may be used during infection of other cell types, such as dendritic cells or natural killer cells, leading to the notion that the T3SS2 may be required to modulate the host immune system to establish long-term asymptomatic infection (Forest et al., 2010).
Figure 1. Strategies that allow *Salmonella* spp. to cross the intestinal barrier, survive in intestinal tissues and spread systemically. *Salmonella* spp. cross M (microfold) cells of the follicle associated epithelium mainly in the Peyer’s patches of the ileal portion of the small intestine but possibly also in the colon. In this sub-epithelial location, *Salmonella* spp. might cause macrophage apoptosis through effectors injected using Type III secretory system (T3SS) 1, thereby also triggering inflammation. *Salmonella* spp. also switch to expression of T3SS-2, which allows injection of effector proteins from the endocytic vacuole into the cell cytoplasm, thereby enabling bacteria to modify the vacuole to a *Salmonella*-containing vacuole, which supports bacterial survival and multiplication. This provides bacteria with the capacity to both invade epithelial cells basolaterally and to disseminate systemically. Alternatively, *Salmonella* spp. can also directly enter intestinal cells by the apical pole of the cell or be captured by dendritic cells that emit pseudopods between epithelial cells. The latter process promotes systemic dissemination of *Salmonella* spp.
1.1.2.5. Diagnosis

Diagnosis of typhoid fever is confirmed by the recovery of the pathogen from blood, stool or bone marrow through conventional microbiological methods. Stool cultures are positive in only 30-35% of cases (Vallenas et al., 1985). Excretion in stool usually begins about a week after the onset of illness and continues through convalescence, and sporadically thereafter in carriers (Parry et al., 2002). Blood cultures have low sensitivity as only 40-60% are positive in typhoid fever cases whereas aspirate cultures of the bone marrow have a sensitivity greater than 80% (gold standard method) (Baker et al., 2010; Vallenas et al., 1985). However, blood and bone marrow cultures are not always possible to perform in endemic areas because of resource-limitations. Serological tests have been extensively used for diagnosis. The Widal test identifies the presence of antibodies against Salmonella specific O and H antigens in the serum. The diagnosis based on Widal test is frequently inaccurate as false positives and false negatives results are common (Olopoenia and King, 2000). More sensitive and specific methods include PCR which can detect very small numbers of bacteria within 4-5 days of onset of infection (Hatta and Smits, 2007; Nagarajan et al., 2009; Zhou and Pollard, 2010). There is a need for antigen based rapid diagnostic test kits. Recent advances include IDL® Tubex Test, Typhidot® Test and Multi-Test Dip-S-Ticks (Olsen et al., 2004).
1.1.2.6. Treatment and vaccines

Most commonly used antibiotics for the treatment of typhoid fever are fluoroquinolones such as ciprofloxacin, ofloxacin and pefloxacin and third generation cephalosporins such as ceftriaxone or cefotaxime (Marathe et al., 2012; Zavala Trujillo et al., 1991). Fluoroquinolones reversibly inhibit DNA replication by targeting proteins such as DNA gyrase. Resistance to quinolones is usually mediated by point mutations in the gyrA genes (Randall et al., 2005). Over the past decade, the incidence of antibiotic resistance has risen dramatically in endemic regions with the emergence of strains refractory to virtually every available first line-antibiotic and up to 60% of strains exhibiting MDR in acute and chronic patients (Harish and Menezes, 2011; Kariuki et al., 2010; Pratap et al., 2012; Wain et al., 1997).

Although improvement in safe water supply and sanitation measures should be enough to prevent typhoid fever, this is still very difficult to achieve in endemic areas. Thus, in 2000 and 2008, WHO has explained the significance of the development of new vaccines against typhoid fever (WHO, 2000, 2008a). Currently, there are only two licensed and commercially available vaccines for typhoid fever: Vi-polysaccharide (Vi PS) parenteral vaccine and a live oral attenuated S. Typhi strain (Ty21a). However, these vaccines do not confer 100% protection and they are not effective for children below 2-5 years of age, who are the group with greatest risk (Marathe et al., 2012). Thus, research is still ongoing to obtain vaccines with increased immunogenicity (humoral and cellular) that can also protect infants and young children. The most promising one is Vi-CRM197 glyconjugated vaccine, developed by Novartis, which is a
conjugation of the Vi-PS (S. Typhi) and the O polysaccharide (S. Paratyphi A) to CRM197, a non-toxic mutant of diphtheria toxin (Micoli et al., 2011).

1.1.2.7. Host-pathogen interactions and immunity

Humoral and cell-mediated immune responses are necessary for efficient control of *Salmonella* infection (Mastroeni, 2002). S. Typhi is able to take advantage of different cell types of the immune system to ensure its intracellular replication and dissemination throughout the body. Interaction of *Salmonella* with the epithelium and the underlying resident immune cells leads to pro-inflammatory responses which recruit and activate other immune cells such as neutrophils, macrophages and dendritic cells with the subsequent activation of the adaptive host response mediated by T and B cells (Grassl and Finlay, 2008). Innate secretory antibodies such as IgA also contribute to control of the spread of *Salmonella* (Wijburg et al., 2006).

The host restricts dissemination of NTS by rapid recruitment of neutrophils, leading to localized gastroenteritis (Tsolis et al., 2008). In contrast, in S. Typhi infection, the cell infiltrates are dominated by mononuclear cells and neutrophils are scarce (Kraus et al., 1999; Mukawi, 1978). Thus, typhoid infection leads to a reduction in host inflammatory response which explains the lack of classic gastroenteritis during initial invasion of the intestinal mucosa, as indicated by a longer incubation time. Recent studies indicate that tight regulation of virulence gene expression during the transition from the intestinal lumen into the intestinal mucosa enables this pathogen to evade
detection by the innate immune system and thus being able to disseminate systemically (Wangdi et al., 2012).

Mucosal invasion by *Salmonella* is detected by PRRs including Toll-like receptors (TLRs) and Nod-like receptors (NLRs). PRRs are the first component of the immune system to detect host invasion by pathogens, initiate immune responses, and form the crucial link between innate and adaptive immunity (Kawai and Akira, 2010). PRRs recognize conserved motifs on pathogens termed “pathogen-associated-molecular-patterns” (PAMPs). PAMPs such as curli fimbriae, LPS (lipid A), flagella, and bacterial DNA are recognized by TLR2, TRL4, TRL5, and TRL9; respectively (de Jong et al., 2012; Gewirtz et al., 2001; Tukel et al., 2010; Vazquez-Torres et al., 2004; Wyant et al., 1999). The O-antigen moiety of LPS is recognized by complement (Gewurz et al., 1968; Joiner et al., 1989). The NLRs are situated in the cytosol and can also recognize PAMPs, for instance NLRC4 recognizes bacterial effectors and flagellin which are secreted into the cytoplasm by T3SS (Hersh et al., 1999; Miao et al., 2006).

The interaction of *Salmonella* PAMPs with TLRs and NLRs induces pro-inflammatory responses that lead to the recruitment of neutrophils and macrophages to contain the infection. These include the production of cytokines, most notably interleukin (IL)-6, IL-1β, IL-12, IL-18, tumor necrosis factor (TNF)-α, and interferon-gamma (IFN)-γ (Keuter et al., 1994; Kupz et al., 2012; Mastroeni, 2002; Raffatellu et al., 2006; Thompson et al., 2009). In addition, activation of cell death pathways, such as pyroptosis, is induced early in infection by SPI-1 and promotes inflammation (Fink and Cookson, 2007). It is believed that in NTS, bacteria within macrophages are recognized
by NLRC4, leading to bacteria release through pyroptosis. Some of these bacteria can be exposed to neutrophils, thus avoiding disseminated NTS infection (Bergsbaken et al., 2009; Miao et al., 2010).

S. Typhi invasion of the intestinal mucosa does not trigger neutrophil influx which allows bacteria to disseminate systemically. The presence of the Vi-antigen on the cell surface prevents recognition of Salmonella LPS by TLR4 (Wilson et al., 2008). Vi antigen expression is regulated by TviA (encoded on SPI-7 or viaB locus) in response to osmolarity (Winter et al., 2009). At low-osmolarity conditions (similar to blood and tissue conditions), TviA activates Vi antigen expression but genes encoding T3SS-1 and flagella are repressed (Winter et al., 2008). The tviA gene is not expressed under high-osmolarity conditions (such as the intestinal lumen) and thus invasion is increased (Arricau et al., 1998; Leclerc et al., 1998; Santander et al., 2008; Winter et al., 2010; Zhao et al., 2001). Therefore, TviA protein serves as a regulatory switch affecting the ability of the host to recognize S. Typhi as an intruder at crucial stages of the disease. In the intestinal lumen, S. Typhi is motile and invasive as it approaches the mucosal surface. However, upon invasion of the intestinal epithelium, S. Typhi is exposed to conditions of lower osmolarity, which results in induction of Vi-antigen expression, repression of flagellin and concealing of LPS that now cannot be detected by the innate immune system (Tran et al., 2010; Winter et al., 2010). This may then explain the distinct host response and disease manifestations between typhoid fever and gastroenteritis caused by NTS.
Once in the liver, spleen or MLN; inflammatory macrophages are recruited to the sites of infection, and components of the host immune response such as intercellular adhesion molecule (ICAM1) and TNF-α mediate the confinement of these macrophages to localized foci that become pathological lesions surrounded by apparently normal tissue. Failure to form pathological lesions results in abnormal growth and bacteria dissemination in the infected tissue (Mastroeni and Grant, 2011). It has been shown that dendritic cells of the MLN prohibit dissemination and relapse of *Salmonella* (Griffin et al., 2011; Voedisch et al., 2009). *S. Typhi* may induce apoptosis to escape infected cells and infect new phagocytes or to be engulfed by other phagocytes (cell-to-cell spread) contributing to spreading and organ colonization (Grant et al., 2008b; Mastroeni and Grant, 2011; Richter-Dahlfors et al., 1997; Watson and Holden, 2010). Most studies agree that macrophages play a crucial role in systemic infection but the replication within these cells seems to very heterogeneous. Many intracellular bacteria do not replicate, but appear to be in a dormant-like state (Helaine et al., 2010).

In addition to surviving inside macrophages, *Salmonella* has evolved different strategies to evade the host immune response. Long O-antigen chains confer resistance to complement (Holzer et al., 2009). Anti-microbial peptides produced by Paneth cells of gut epithelia and macrophages can kill extracellular and intracellular *Salmonella*, respectively. To avoid this, *Salmonella* undergo changes in the LPS composition to prevent interactions of the cationic peptides or synthesize proteins to export these peptides outside the bacterial cells (Gunn, 2001; Guo et al., 1997; Nizet, 2006; Raetz et al., 2007). These LPS modifications also block recognition by TLR-4, thus preventing downstream signaling cascades that induce pro-inflammatory responses (Chow et al.,
1999; Poltorak et al., 1998). In addition, resistance mechanisms against antimicrobial proteins produced as a result of Salmonella-induced colitis provide a fitness advantage in the lumen of the inflamed gut (Fischbach et al., 2006; Liu et al., 2012). Finally, Salmonella also evades adaptive immunity by preventing dendritic cells from activating T cells (Tobar et al., 2006).

Transcriptional profiling of peripheral blood from acute, convalescent and recovered typhoid patients has revealed specific neutrophil and lymphocyte gene expression sets associated with each of these stages. (Thompson et al., 2009). In addition, immunoscreening techniques have been performed to identify immunogenic proteins expressed during human infection with S. Typhi in acute, convalescent and chronic patients (Charles et al., 2010; Harris et al., 2006). Thus, identification of immune response parameters specifically associated with each stage of the disease might result in serological tests that can be used to identify active or chronic carrier patients.

1.1.3. Chronic typhoid carriage

1.1.3.1. Historic background

S. Typhi is a human-restricted pathogen, making healthy carriers critical in the infectious cycle. This role was famously illustrated by Mary Mallon, or Typhoid Mary, a cook in New York City in the early 20th century. She is reported to have infected at least 54 people. Other New York City carriers were also identified around this time, many of whom spread the infection to more people than Mary Mallon. Around the same time, in the small town of Folkstone, England; another chronic carrier referred to as “Mr. N The
“Milker” infected more than 200 people over the course of 14 years. In these cases, public health officials ultimately stepped in and requested that carriers remove themselves from food service and while Mr. N and others agreed, Typhoid Mary refused, ultimately leading to her arrest and involuntary lifelong quarantine in 1907 on North Brother Island. Interestingly, her autopsy revealed that her gallbladder was colonized with S. Typhi (Brooks, 1996; Marr, 1999; Mortimer, 1999). This confinement practice continued even after Mary’s death. These cases allowed doctors to begin tracing typhoid epidemics to their human sources and to identify carriers. In 2008, it emerged that 43 female typhoid carriers were quarantined in an asylum in Surrey, England between 1907 and 1992 and some were held for more than 40 years until the asylum closed in 1992, well after the widespread use of antibiotics had begun and increased medical knowledge of the typhoid fever carrier state was gained (Gonzalez-Escobedo et al., 2011).

1.1.3.2. Epidemiology and risk factors

Most of typhoid fever patients recover from the acute phase of typhoid after adequate treatment; however, between 3 to 5% of individuals infected with S. Typhi develop a chronic infection in the gallbladder regardless of treatment (Levine et al., 1982; Merselis et al., 1964). Chronic carriers are defined as individuals shedding S. Typhi in their feces for more than 12 months after acute infection (Sanchez-Vargas et al., 2011). Because S. Typhi is a human-restricted pathogen, these chronic carriers serve as a critical reservoir for further spread of the disease through persistent bacterial shedding in feces and urine (Bhan et al., 2005; Khatri et al., 2009; Vogelsang and Boe, 1948). Chronic S. Typhi infections can persist for decades and although highly
contagious, they are typically asymptomatic, making identification of carriers within a population difficult (Shpargel et al., 1985; Sinnott and Teall, 1987). The situation is further complicated by the fact that approximately 25% of chronic carriers never experience any clinical manifestation of the acute phase of the disease (Parry et al., 2002). In addition, identification of carriers by monitoring of bacteria in their stools is not always successful because shedding is intermittent (Gopinath et al., 2012).

Epidemiological studies have determined that typhoid carriers are a crucial target for disease control because they shed the pathogen in high enough numbers to contribute to the transmission of the pathogen by contaminating water or food sources (Chandrasekaran and Balakrishnan, 2011; Feglo et al., 2004; Gopinath et al., 2012; Senthilkumar and Prabakaran, 2005). Mutational analysis of S. Typhi isolates from different areas of the world revealed that ancestral haplotypes were found in current-day isolates, suggesting these strains persist in asymptomatic carriers (Roumagnac et al., 2006). Thus, identification and treatment of carriers are crucial for disease prevention and control. Methods for carrier detection have used serology against Vi antigen, O-antigen as well as PCR. However, these methods lack specificity and sensitivity, especially in endemic areas (Gupta et al., 2006; House et al., 2001b; Nath et al., 2010b; Yan et al., 2011). As a preventive measure, typhoid carriers should be excluded from handling food until 3 consecutive stool specimens are negative (Crum, 2003). Compounding the problem, recent studies have also reported the incidence of chronic carriers in people infected with NTS (Boisrame-Gastrin et al., 2011; Buchwald and Blaser, 1984; Kariuki et al., 2006).
Among adults, individuals older than 50 years and females are more likely to become carriers (Ames and Robins, 1943; Levine et al., 1982; Merselis et al., 1964). Particularly in high endemicity areas, the carrier state has been highly associated with pre-existing hepatobiliary disease including cholelithiasis (presence of gallstones in the gallbladder), biliary obstruction, intrahepatic cholestasis due to Caroli’s disease, biliary cirrhosis, hepatic haematoma, echinococcal cysts and amoebic abscesses (Cohen et al., 1987; Gosbell et al., 1995; Lai et al., 1992; Levine et al., 1982). Studies conducted in endemic regions have indicated that approximately 90% of chronically infected carriers have gallstones (Karaki and Matsubara, 1984; Schioler et al., 1983).

1.1.3.3. Treatment

In contrast to acute infection, antibiotic treatment has proven poorly effective in the resolution of chronic S. Typhi colonization of the gallbladder. Even prolonged, high-dose antibiotic therapy resolves less than two-thirds of chronic infections, and treatment with ampicillin has been shown to be effective only in patients without gallstones (Dinbar et al., 1969; Lai et al., 1992). These longer treatments with larger doses have also side effects, with reported cases of gastrointestinal bleeding and gastric discomfort (Diridl et al., 1986; Zavala Trujillo et al., 1991). Complete resection of the gallbladder (cholecystectomy) is able to increase this cure rate but it does not guarantee elimination of the carrier state (Freitag, 1964; Ristori et al., 1982). Additional bacteria foci may persist in the biliary tree, MLN, spleen or liver (Erlik and Reitler, 1960; Gaines et al., 1968; Monack et al., 2004; Nath et al., 2010c; Nix et al., 2007; Rovito and Bonanno, 1982). In addition, cholecystectomy is both a costly and invasive procedure. Although
not available to many patients, the most effective treatment available is a combination of surgery and antibiotics (Jonsson, 1977). However, this is becoming increasingly difficult with the increased emergence of MDR in carriers (Chandrasekaran and Balakrishnan, 2011; Pratap et al., 2012).

1.1.3.4. Complications

In addition to the complications related to the acute phase of the disease, especially in the ileum and lymph organs (Everest et al., 2001), typhoid carriage complications include chronic hepatitis, acute or chronic cholecystitis, cholangitis, chronic diarrhea and rarely, pancreatitis (Crum, 2003; Vaishnavi et al., 2005a). Additionally, the typhoid carrier state, both with and without the co-incidence of gallstones, has been indicated as a predisposing factor for the development of gallbladder cancer (Caygill et al., 1994; Dutta et al., 2000; Nath et al., 2008; Shukla et al., 2000). Chronic carriers have an approximately 8-14 fold excess risk of developing gallbladder carcinoma and approximately 150-fold excess risk of developing hepatobiliary carcinoma than non-carriers (Caygill et al., 1994; Nath et al., 1997; Shukla et al., 2000; Welton et al., 1979). It has been hypothesized that bacterial degradation of bile salts, bacterial metabolites and toxins; and chronic cholecystitis (gallbladder inflammation) related to gallstones could promote gallbladder carcinomas (Kumar, 2006; Nath et al., 2010a). This impact on human health, combined with the high incidence of typhoid fever in many parts of the world, and the poor efficacy of current vaccines; highlights the importance of understanding the mechanisms involved in S. Typhi carriage.
1.1.3.5. Animal studies of chronic carriage

Chronic carriage has been studied in different animals including cattle, pigs, chicken and mice. In these models, a subset of infected individuals showed increased shedding of bacteria, and as result they rapidly transmit infection. These individuals are defined as super-shedders and they have shown increased innate pro-inflammatory responses (Calenge et al., 2010; Huang et al., 2011; Lawley et al., 2008). In chronically infected mice with S. Typhimurium, the microbiota influences the bacterial levels in the gastrointestinal tract and plays a role in controlling transmission (Lawley et al., 2008). Antimicrobial treatment, which alters the gut microbiota, induces the super-shedder phenotype and carrier status (Endt et al., 2010). Importantly, a single dose of antibiotic given to chronically infected mice shifted non-shedders mice to a super-shedder phenotype (Lawley et al., 2008). Also in mice, a mutant of SPI-16, which contains genes responsible for O-antigen glycosylation, was outcompeted by wild-type bacteria in the gastrointestinal tract but not in systemic sites, suggesting that O-antigen variation is required for fecal shedding (Bogomolnaya et al., 2008). In human carriers, it has been shown that antibiotic treatment slightly increases the duration but not the quantity of S. Typhimurium shedding (Jafary and Burke, 1970).

The majority of Salmonella spp. pathogenesis studies have been conducted in susceptible BALB/c or C57BL/6 mice, which has provided valuable data on acute-phase infection but has yielded little data relating to chronic infection (Santos et al., 2001). Supported by previous investigations (Carter and Collins, 1974; Sukupolvi et al., 1997a), a 2004 study by Monack et al. proposed the use of the 129x1/SvJ Nramp1+/+ mouse for
studies of chronic infection, demonstrating that S. Typhimurium was detectable in tissues and feces for 1 year following oral infection. In addition to the gallbladder, bacteria typically persisted within macrophages in systemic tissues such as the liver, spleen and MLN (Monack et al., 2004). Furthermore, S. Typhimurium survival and replication in hemophagocytic macrophages may help establish a persistent infection (Nix et al., 2007).

Factors that contribute to S. Typhimurium long-term carriage in mice include components of T3SS1 and T3SS2 such as SseI which is required to maintain a persistent infection in mice. SseI interferes with the migration of macrophages and dendritic cells to lymphoid tissues. This may represent a mechanism for preventing presentation of Salmonella antigens to naïve T cells, thus inhibiting adaptive immunity (Lawley et al., 2006; McLaughlin et al., 2009). However, SseI is not present in S. Typhi. T3SS2 is also important for the transmission of S. Typhimurium from persistently infected mice to naïve cage mates (Lawley et al., 2008). Several fimbrial operons (lpf, bcf, stb, stc, std and sth) and the adhesins SdhA and MisL, also contribute to long-term intestinal carriage and fecal shedding (Dorsey et al., 2005; Kingsley et al., 2000; Weening et al., 2005). Salmonella factors such as PgtE, Mig-14, VirK, RscC and YdeI; which protect against antimicrobial peptides (Brodsky et al., 2005; Detweiler et al., 2003; Erickson and Detweiler, 2006; Lawley et al., 2006); and the genes sodC1, sppJ and hmp that protect against ROS and RNS (Bang et al., 2006; Lawley et al., 2006; van Diepen et al., 2002); also contribute to persistent Salmonella infection. Finally, AceA, an enzyme that enables the use of fatty acids, is required during the persistent infection (Fang et al., 2005).
Finally, humanized mouse models, such as the TLR 11−/− and immunodeficient Rag2−/− γc−/− mice engrafted with human fetal liver hematopoietic stem and progenitor cells, have been reported for studies of S. Typhi. (Mathur et al., 2012a; Song et al., 2010). Such models may be extremely useful in future studies of chronic infection, allowing S. Typhi to be directly studied.

1.1.3.6. Immune response during chronic carriage

Chronic carriers have high levels of circulating serum antibodies against Vi antigen and flagella (House et al., 2001a). Salmonella also induces a strong mucosal antibody response in carriers (Dham and Thompson, 1982). This is however, dispensable for super-shedder formation because IgA knockout mice showed no attenuation in cecal Salmonella loads (Endt et al., 2010). Although Th1 inflammatory response and IFN-γ are associated with Salmonella infection (Kaur and Jain, 2012), their role in carrier state development has not been defined. Convalescing typhoid patients (who have the potential to become carriers) have increased circulating inflammatory cytokines such as IL-6, TNFα and IL-1β (Butler et al., 1993; Keuter et al., 1994). IFN-γ plays a central role in the control of mice persistent infection by affecting the extent of macrophage activation (Monack et al., 2004; Ruby et al., 2012). The limited intracellular replication and the control of T-cell responses by Salmonella, through limited antigen presentation, are likely to be important factors for the establishment of a persistent infection (Albaghdadi et al., 2009; Halici et al., 2008). In fact, regulatory T cells (Tregs) has been showed to modulate Th1 responses, influencing the progression to persistent infection (Johanns et al., 2010).
1.2. The Gallbladder: Anatomy, physiology, infection and inflammation

1.2.1. The Gallbladder and bile

The biliary tract consists of the gallbladder, cystic duct, common bile duct and intrahepatic ducts. The gallbladder is a hollow, pear-shaped organ that stores bile prior to its excretion into the small intestine. Anatomically, it is located in a fossa on the posterior surface of the right hepatic lobe. In human adults, it measures 7-10 cm in length, and 3 cm at its widest part (Martini, 2005; Odze and Goldblum, 2009). The gallbladder is divided into three regions: the fundus, the body and the neck (Fig. 2). Histologically, the gallbladder consists of three layers: mucosa, muscularis, and adventitia or serosa. The gallbladder has no muscularis mucosae or submucosa. The mucosa consists of a single layer of columnar epithelial cells and the underlying lamina propria that contains loose connective tissue, blood vessels and some diffuse lymphatic tissue. In the non-distended state, the gallbladder wall shows temporary mucosal folds that disappear when the gallbladder becomes distended with bile. These mucosal folds resemble the villi in the small intestine but they vary in size and shape and display an irregular arrangement. Between the mucosal folds, are found diverticula or crypts that often form deep indentations in the mucosa. In cross sections, the diverticula or crypts in the lamina propria resemble tubular glands. However, there are no glands in the gallbladder, except in the neck region of the organ. The following layer is the muscularis externa which has numerous collagen and elastic fibers among the randomly oriented smooth muscle bundles. This layer is responsible for the contraction and expulsion of bile. A thick layer of dense connective tissue lies outside the muscularis externa. It is rich in adipose tissue and elastic fibers, and contains large blood and lymphs vessels and
autonomic nerves. The connective tissue of the gallbladder is covered by adventitia (where the gallbladder is attached to the liver) and by a serosa (where the gallbladder surface is exposed to the peritoneal cavity). (Eroschenko, 2008; Henrikson and Mazurkiewicz, 1997) (Fig. 3).

(Figure 2. The gallbladder and biliary tract. The gallbladder is divided into three regions: the fundus, the body and the neck. Bile is produced by the liver and transported from there into the common hepatic duct which then joins the cystic duct (coming from the gallbladder) to form the common bile duct. Release of bile into the duodenum is under the control of the hormone cholecystokinin which causes relaxation of the Sphincter of Oddi, promoting bile secretion.)
The primary functions of the gallbladder are to collect, store, concentrate and expel bile to the small intestine when it is needed for the emulsification of lipids after the ingestion of food (Center, 2009). Bile is continuously produced by the liver hepatocytes and transported from there into the common hepatic duct which then joins the cystic duct (coming from the gallbladder) to form the common bile duct (Fig. 2). Release of bile into the duodenum is under hormonal control. The hormone cholecystokinin, when stimulated by a fatty meal into the proximal duodenum, is released into the bloodstream by enteroendocrine cells located in the intestinal mucosa. Cholecystokinin is carried to the gallbladder where it causes contraction of the smooth muscles of the gallbladder wall, relaxation of the sphincter muscles of the common bile duct (Sphincter of Oddi) and also promotes bile secretion by increased production of hepatic bile (Eroschenko, 2008; Martini, 2005). When the sphincter of Oddi is closed, newly synthesized bile from the liver is forced into storage in the gallbladder. When open, the stored and concentrated bile exits into the duodenum. The gallbladder is not considered essential for proper biliary function because humans do not suffer from mal-digestion or mal-absorption of fat after removal of the gallbladder (Odze and Goldblum, 2009). In addition to aiding in the digestion of fats, bile also facilitates absorption of fat-soluble vitamins in the intestine, induction of mucin secretion, control of electrolyte absorption; and contributes to the elimination of excess cholesterol and waste metabolic products produced in the liver (Begley et al., 2005; Hofmann and Hagey, 2008).

The liver secretes up to 1 liter of bile per day and the gallbladder contains up to 40-70 mL of bile. Bile is a complex fluid containing bile salts, cholesterol, phospholipids, bilirubin, IgA, biliary pigments, mucus and a variety of proteins and electrolytes (Esteller,
Abigail Hofmann and Karonn Hagey, 2008). About two thirds of bile (dry weight) is made of bile salts, a family of molecules with steroid structure which derive from cholesterol (Hofmann and Hagey, 2008; Odze and Goldblum, 2009). Bile is concentrated 5-10 times in the gallbladder. Sodium is actively transported through the gallbladder epithelium into the extracellular connective tissue, creating a strong osmotic pressure that allows passive transfer of water and chloride ions from the lumen into the intercellular spaces of the epithelium (Hofmann and Hagey, 2008; Odze and Goldblum, 2009).

The gallbladder epithelium participates in both innate and adaptive immunity (Harada et al., 2004; Harada et al., 2003; Reynoso-Paz et al., 1999). It expresses all known TLRs and MyD88, which are key factors of the innate immune response (Harada et al., 2003). With respect to the adaptive immune response, gallbladder epithelial cells constitutively express HLA class I and after stimulation with cytokines, they also express many HLA class II antigens (Auth et al., 1993; Rumin et al., 1997). This implies that gallbladder epithelial cells can interact with both CD8+ T cells as well as CD4+ T cells. Moreover, all Ig classes are present in low concentration in bile; having been synthesized by plasma cells and then transcytosed to the apical surface and secreted into bile (Brown and Kloppel, 1989; Reynoso-Paz et al., 1999; Rojas and Apodaca, 2002). Gallbladder epithelial cells also secrete a variety of cytokines and chemokines such as TNF-α, IL-1β, IL-6, CCL5 and Mip-2. This production is enhanced after exposure to antigens such as bacterial LPS (Savard et al., 2002). Thus, the gallbladder epithelium participates in cellular and humoral immunity through antigen presentation, cytokine and chemokine production as well as Ig transport.
Figure 3. Gallbladder histology. The gallbladder comprises the mucosa (columnar epithelium and lamina propria), the muscularis and the serosa. The mucosa consists of a single layer of columnar epithelial cells and the underlying lamina propria that contains loose connective tissue, blood vessels and some diffuse lymphatic tissue. Between the mucosal folds, are found diverticula or crypts that in cross sections like this, resemble tubular glands.
1.2.2. Cholelithiasis

The term cholelithiasis refers to the presence of gallstones or choledoliths in the gallbladder or the bile ducts and is one of the most prevalent medical conditions leading to surgery (Schirmer et al., 2005). Gallstone disease is common with incidence ranging from 10-20% of the world population. Patient factors that predispose for gallstone formation include age, obesity, female gender, unknown genetic determinants and chronic bacterial colonization (Maurer et al., 2009; Paumgartner, 2010; Schirmer et al., 2005). The composition of gallstones determines their classification as cholesterol gallstones (more than 70-80% of cholesterol), pigment gallstones (40-60% of calcium bilirubinate) or mixed gallstones (30-70% of cholesterol) (Kim et al., 2003). Cholesterol gallstones are commonly present in the gallbladder but pigment gallstones can also develop in the bile ducts (Tazuma and Kajiyama, 2001).

Cholesterol gallstone formation depends on a combination of factors including cholesterol super saturation of bile, alteration of gallbladder contractility and hypersecretion of mucin. Bile cholesterol is carried by bile acid micelles and lecithin–cholesterol vesicles. The excessive cholesterol is carried predominantly by lecithin–cholesterol vesicles, and such cholesterol-rich vesicles tend to aggregate and fuse to each other, eventually forming cholesterol monohydrate crystals as an initial and essential step in the cholesterol gallstone formation process. Another transitional form to carry such an excessive cholesterol is phospholipid lamella (discoidal particles), which also plays a role in cholesterol crystal nucleation. The secretion of mucin produced within the gallbladder wall is indirectly stimulated by free fatty acids and lysolecithins.
The excessive mucin form a gel bed on the surface of the gallbladder epithelium, providing a preferential circumstance for cholesterol crystal growth to a macroscopic stone. The gallbladder itself plays a crucial role in the process of cholesterol crystal growth to form a macroscopic stone. Thus, impaired gallbladder emptying leads to bile stasis (lack of bile flow to and from the gallbladder), promoting cholesterol precipitation and, further, providing the time needed for crystal growth (Admirand and Small, 1968; Tazuma et al., 1994) (Fig. 4).

1.2.3. Cholecystitis

Cholecystitis refers to the inflammation of the gallbladder. This is primarily caused by the obstruction of the biliary tract due to the presence of gallstones. Typically, this obstruction causes distention, bile stasis, inflammation and infection of the gallbladder (Capoor et al., 2008; Swidsinski and Lee, 2001). A variety of bacteria have been identified by culture or by PCR in the gallbladder of patients with cholecystitis and cholelithiasis including *E. coli*, *K. pneumoniae*, *Citrobacter freundii*, *Salmonella* spp., *Helicobacter* spp., *Enterobacter* spp, *Enterococcus* spp., *Pseudomonas aeruginosa*, *Bacteroides fragilis*, *Staphylococcus aureus*, *Proteus* spp. and *Acinetobacter* spp. (Capoor et al., 2008; Csendes et al., 1996; Hazrah et al., 2004; Kawai et al., 2002; Leung et al., 1989; Vaishnavi et al., 2005b; Yucebilgili et al., 2009).
Figure 4. The physical-chemical processes involved in formation of cholesterol gallstones. The classic physical-chemical symbols for cholesterol (pink), phospholipid (green), and bile salt (purple) molecules are shown, along with the macromolecular structures they form. Cholesterol, phospholipids, and bile salts combine by hydrophobic interactions to form mixed micelles and cholesterol and phospholipids form unilamellar vesicles. Normally the unilamellar vesicles would be 5–10 times larger than micelles (40 Å in radius), but for illustration purposes they are depicted here nonproportionately. As cholesterol concentration in gallbladder bile increases principally from hepatic hypersecretion of cholesterol, the true supersaturated state forms transiently. Supersaturated bile usually implies that phase separation of excess cholesterol from micelles has occurred, forming unilamellar vesicles with biliary phospholipids (mostly 95% phosphatidylcholine). In the most common nucleation sequence, unilamellar vesicles fuse to form multilamellar vesicles, or liquid crystals, which are visible by low-power polarizing microscopy. From these, plate-like cholesterol monohydrate crystals (solid cholesterol crystals) nucleate heterogeneously, usually in a mucin gel. The dotted arrow indicates how cholesterol can occasionally phase separate directly from supersaturated micelles. The solid resulting cholesterol monohydrate crystals are a polymorph of the classic cholesterol monohydrate plates into which they transform with passage of time. Once the nucleation sequence has occurred and solid cholesterol crystals have formed, the phase sequence is not repeated if bile remains continuously supersaturated. Gallbladder dysmotility and mucin gel formation also contribute to the aggregation of the plate-like cholesterol monohydrate crystals and contribute to their agglomeration and growth into macroscopic cholesterol gallstones.
In the case of *Helicobacter* spp., a prospective study showed a higher frequency of gallstone formation in mice fed a lithogenic (gallstone-inducing) diet that were inoculated with bacteria compared with uninfected controls (Maurer et al., 2005a). However, whether these microorganisms have a primary role in causing cholecystitis or cholelithiasis or are only colonizers of a previously damaged gallbladder, is still questionable. Acalculous cholecystitis (inflammation in the absence of gallstones) is less common (~10% of acute cholecystitis cases) and occurs predominantly in critically ill or injured patients or after complicated surgery (Huffman and Schenker, 2010).

1.3. *Salmonella* interactions with the gallbladder

1.3.1. *Salmonella* and bile signaling

While the main purpose of bile is to aid in digestion, it also has antimicrobial properties. Bile salts can disrupt the phospholipids comprising the bacterial membrane and cause dissociation of integral membrane proteins. Intracellularly, the detergent activity of bile salts causes misfolding and denaturation of proteins as well as chelation of calcium and iron (Begley et al., 2005; Merritt and Donaldson, 2009). Furthermore, bile salts have DNA damaging capacity, stimulate DNA rearrangements, and induce plasmid curing (Garcia-Quintanilla et al., 2006; Merritt and Donaldson, 2009; Prieto et al., 2004, 2006). Thus, bile is involved in the control of commensal microbial growth in the gastrointestinal (GI) tract as well as defense against invading pathogens. However, certain enteric bacteria are resistant to the antibacterial activities of bile salts. In fact, the ability of enteric microbes to survive in bile has been exploited in the form of selection media (e.g. MacConkey agar and *Salmonella*-Shigella agar) (Gunn, 2000). On the other
hand, bile salts regulate the expression of specific bacterial genes, some of them necessary for bile resistance and others involved in pathogenesis (Begley et al., 2005; Gunn, 2000). Bile salts may thus be viewed both as environmental signals used by bacteria to identify bile-containing animal environments and as antibacterial compounds (Gunn, 2000).

_Salmonella_ spp. can thrive in the GI tract, liver and gallbladder; all of which are sites where bile is encountered (Bajor et al., 2010). Persistent colonization of the gallbladder (the site with the highest concentrations of bile) by _Salmonella_ depends on bile resistance. Transcriptomic and proteomic analyses have been performed to detect the global effects of commercial and physiological bile in _Salmonella_ (Antunes et al., 2012; Prouty et al., 2004b). In addition, a genome-wide screen identified 169 _S. Typhi_ genes required for bile resistance (Langridge et al., 2009). The list of bile resistance factors in _Salmonella_ includes envelope barriers such as LPS and its modifications (Hernandez et al., 2012; Murata et al., 2007; Prouty et al., 2002b; Ramos-Morales et al., 2003; Tsai et al., 2012) and the enterobacterial common antigen (ECA) (Ramos-Morales et al., 2003); the outer membrane (Prouty et al., 2002b; Pucciarelli et al., 2002; Tsai et al., 2012), the cytoplasmic membrane (Lopez-Garrido et al., 2010) and efflux pump systems such as AcrAB-TolC that expel bile from the bacterial cytoplasm (Gunn, 2000; Lacroix et al., 1996; Prouty et al., 2002b; Tsai et al., 2012). In addition, multiple antibiotic resistance (mar) genes, the PhoPQ regulons (van Velkinburgh and Gunn, 1999) and DNA repair functions are also important to mitigate bile damage (Prieto et al., 2004, 2006).
It has been suggested that *Salmonella* regulates the length distribution of the O-antigen in its LPS to respond to different conditions. Thus, very long O-antigen might enhance resistance to bile in an ECA-dependent manner, conferring increased fitness of NTS in the inflamed intestine (Crawford et al., 2012; May and Groisman, 2013). Interestingly, it has been shown that *S. Typhi* has increased bile resistance due to periplasmic space remodeling and alterations of the membrane protein TolA (Lahiri et al., 2011). Furthermore, it has been determined that bile not only alters *Salmonella* gene expression but also induces bile-resistant mutations that can be important in gallbladder adaptation, especially during chronic carriage (Hernandez et al., 2012).

In addition to resist the deleterious effects of bile, *Salmonella* can also thrive in this detergent-like fluid, with growth rates comparable to those achieved in rich culture medium (Menendez et al., 2009). Metabolomic studies have assessed the changes elicited in the chemical composition of murine bile after *Salmonella* infection. They suggest that during gallbladder colonization, *Salmonella* may grow in bile by using glycerophospholipids as a source of carbon and energy (Antunes et al., 2011).

Bile has also been demonstrated to down-regulate the expression of SPI-1 genes, which are involved in host cell invasion. However, this is likely to be a spatio-temporal response that does not interfere with invasion after the bacteria have penetrated the mucous layer of the epithelia where a decreased apparent bile concentration would be encountered (Prouty et al., 2004b; Prouty and Gunn, 2000). Bile also slightly down-regulates motility gene expression, but this transcriptional regulation
does not have a dramatic effect on the number of flagella per bacterium or on motility (Crawford et al., 2010a; Prouty et al., 2004b).

Finally, early studies to investigate the ability of *Salmonella* to form biofilms on human gallstones and cholesterol-coated surfaces indicated that formation of a robust biofilm on cholesterol is dependent on the presence of bile (Prouty et al., 2002a). Interestingly, in *S. Typhimurium*, bile also seems to have an effect on several known global gene-regulation pathways independently of traditionally implicated stress or biofilm mediators such as RpoS and CsgD (Crawford et al., 2008; Prouty et al., 2004a).

### 1.3.2. Acute cholecystitis caused by *Salmonella*

Despite the fact that cholecystitis and sonographic gallbladder abnormalities have been reported in acute and chronic typhoid fever patients (Cohen et al., 1987; Mateen et al., 2006; Shetty and Broome, 1998; Vaishnavi et al., 2005b; Vogelsang and Boe, 1948), little is known about the specific interaction of *S. Typhi* with the gallbladder. To begin to examine host-pathogen interactions during acute infection, Menendez *et al.* (Menendez et al., 2009) utilized *S. Typhimurium* infection of *Nramp1* knockout mouse strains. Approximately 10^7 bacteria were inoculated orally and intravenously, and the number and localization of bacteria were assessed up to 120 h after infection. *S. Typhimurium* was found in the gallbladder 48 h after infection, with the highest bacterial burden found at 120 h post-infection in both the gallbladder lumen and tissue. The presence of bacteria in the intestine and shedding in the feces was evident during the entire course of infection. Active invasion by *S. Typhimurium* into gallbladder epithelia followed by
efficient replication and intracellular survival were also observed. *S. Typhimurium* was localized to a sub-nuclear position in gallbladder epithelial cells within a SCV. Interestingly, the bacteria did not translocate to the lamina propria (Menendez et al., 2009) (Fig. 5).

*S. Typhimurium* colonization of the gallbladder induced a localized inflammatory response mediated by neutrophils that led to loss of epithelial integrity, thickening of the mucosa and tissue damage (Fig. 5). Invasion-deficient bacteria were unable to infect gallbladder tissue and to elicit the inflammatory response and pathological damage, although they replicated efficiently in the gallbladder lumen, indicating that only invasive intracellular bacteria are responsible for the inflammatory process. This study corroborated the traditional implication of *S. Typhi* as a cause of acute cholecystitis and its potential role during acute typhoid disease. It is of interest to contemplate the events that must occur to transition from an acute, strong pro-inflammatory response to a relative lack of symptoms and pathology in chronic carriers.
Figure 5. *Salmonella* spp. acute infection of the gallbladder. Following systemic infection, *Salmonella* spp. colonize the gallbladder from the liver. Bacteria can replicate extracellularly in the lumen or can actively invade the gallbladder epithelium in a *Salmonella* pathogenicity island 1 (SPI-1)-dependent manner (step 1). Although the bacteria can replicate inside the epithelial cells, in the *Salmonella* -containing vacuole (SCV), they do not translocate to the lamina propria and mucosa. This intracellular infection leads to a local inflammatory response (step 2) mediated by neutrophils (step 3), with subsequent tissue damage and epithelial sloughing (step 4). This could lead to the release of *Salmonella* spp. cells into the lumen for invasion of new epithelial cells. Based on data from (Menendez et al., 2009).
1.4. *Salmonella* biofilms

1.4.1. Biofilms

Over the past two decades, bacterial biofilms have been increasingly implicated as burdens to food and public safety worldwide, and are broadly defined as structured communities of microorganisms that adhere to each other and to inert or live substrates by a self-produced polymeric matrix (Costerton et al., 1999; Monds and O'Toole, 2009; Watnick and Kolter, 2000). They demonstrate heightened resistance to immune host responses, antibiotics, nutrient stress and disinfectants (Hoiby et al., 2010; Monier and Lindow, 2005; Tabak et al., 2009) which enhances their spread and persistence inside and outside the host, thus being very difficult to eradicate. Biofilm formation occurs in sequential, highly regulated stages that begin with adherence of free-swimming, planktonic bacteria to a surface. Subsequent biofilm maturation is characterized by the production of a self-initiated extracellular matrix composed of exopolysaccharides, proteins and nucleic acids; that encase the community of microorganisms and provide structure and protection (Monds and O'Toole, 2009). Planktonic cells from this sessile, matrix-bound population are continuously shed, which can result in reattachment and fortification of the biofilm or release of the organism into the environment (O'Toole et al., 2000; Pasmore and Costerton, 2003).

Typically considered as a response to stress, biofilms have been implicated in many chronic and acute infections. Approximately 80% of all bacterial infections are related to biofilms (Fux et al., 2005; Hall-Stoodley and Stoodley, 2009). Also,
contamination of processed foods in industrial plants is often due to biofilm formation on both food and food-contact surfaces (Kumar and Anand, 1998). The biofilm state can alter the host-pathogen interaction and is often associated with a reduction of the host inflammatory response that has been referred to as a “silent chronic inflammation” (Cappelli et al., 2005). The molecular genetic mechanisms mediating adherence and microcolony formation during biofilm growth have recently begun to be elucidated for many microorganisms.

1.4.2. Factors mediating *Salmonella* biofilm formation

*Salmonella* spp. are known to form matrix-encased biofilms on abiotic and biotic surfaces including glass, plastic, plant surfaces, animal epithelial cells and gallstones (Barak et al., 2007; Lapidot and Yaron, 2009; Ledeboer and Jones, 2005; Prouty et al., 2002a; Steenackers et al., 2012). Biofilm formation on epithelial cells likely plays a significant role in the establishment and persistence of mucosal infections in appropriate hosts and is a possible cause of intestinal carriage in domestic animals (Althouse et al., 2003; Morgan et al., 2004; Ricke, 2003).

Current work addresses the molecular details pertaining to *Salmonella* spp. biofilm formation including important structural and protective constituents of the extracellular matrix, adherence factors, quorum sensing molecules, outer membrane proteins, environmental signals that induce biofilm formation as well as the role of enzymes controlling cellular c-di-GMP levels. A better understanding of the regulatory mechanisms that mediate *Salmonella* spp. biofilm formation and maintenance promises
to elucidate important targets for the dissolution of these microbial communities and the risks they pose to human health.

The major extra-polymeric substances (EPS) components in *Salmonella* spp. biofilms include cellulose, curli fimbriae, colanic acid, O-antigen (O-ag) capsule, biofilm related proteins and nucleic acids (Johnson et al., 2013; Steenackers et al., 2012). The extracellular matrix components of *Salmonella* biofilms vary considerably with the environmental conditions and the type of surface (Prouty and Gunn, 2003). EPS material within bacterial biofilms is thus heterogeneous and provides both rigidity to the biofilm and protection to bacteria embedded within established biofilms.

1.4.2.1. Flagella

Flagella have been shown to promote surface binding during the initial stages of biofilm development in various microorganisms (O'Toole and Kolter, 1998; Pratt and Kolter, 1998). They mediate adherence of *S. Enteritidis* to cultured epithelial cells (Dibb-Fuller et al., 1999) and *S. Derby* diarrhea isolates to HEp-2 cell monolayers (Budiarti et al., 1991). However, expression of *S. Typhimurium* flagella has been shown to inhibit biofilm formation on polystyrene wells and glass surfaces (Prouty and Gunn, 2003; Teplitski et al., 2006) but directly mediates biofilm development on cholesterol-coated surfaces (Crawford et al., 2010a) suggesting that the contribution of this surface appendage to biofilm formation varies with binding substrates. Interestingly, flagellar-mediated phenotypes including swimming motility and chemotaxis differentially contributed to colonization and biofilm formation depending on environmental cues (Mey
Bile, for example, enhances tumbling frequency while down-regulating motility in *S. enterica* (Prouty et al., 2004). Swarming motility is inversely correlated with *S. Typhimurium* biofilm formation on microtiter plates (Mireles et al., 2001). In addition, the conserved *S. Typhimurium* flagellar regulon gene *flhE*, involved in the flagellar type III secretion specificity switch, is not required for flagella production or swimming, but appeared to play a role in swarming and biofilm formation (Stafford and Hughes, 2007). Flagella were also shown to be important for *Salmonella*-plant interaction in certain serovars under some environmental conditions (Berger et al., 2009).

### 1.4.2.2. Fimbriae

Fimbriae have been shown to mediate adherence during biofilm initiation and cell-cell interactions during biofilm growth for a variety of microorganisms (Davey and O'Toole G, 2000; O'Toole and Kolter, 1998). *S. Typhimurium* genome encodes 13 putative fimbrial operons, some of which are not expressed in vitro (Nuccio et al., 2007). Type 1 fimbriae (encoded by *fimAICDHF* gene cluster) have been shown to be important for biofilm formation on HEp-2 tissue culture cells and the murine and chicken intestinal epithelium (Boddicker et al., 2002; Ledeboer and Jones, 2005), but not on human gallstones incubated with bile (Ledeboer et al., 2006; Prouty et al., 2002a) or to chick gut explants (Allen-Vercoe and Woodward, 1999).

Curli fimbriae (thin aggregative fimbriae) are amyloid-like cell-surface proteins, encoded by *csgBAC-csgDEFG*. They are important in host-colonization, persistence,
invasion and host-immune response (Barnhart and Chapman, 2006; Tukel et al., 2010). Curli are crucial mediators of biofilm formation on plant surfaces and chicken and murine intestinal epithelium (Barak et al., 2005; Ledeboer et al., 2006; Sukupolvi et al., 1997b) but not on glass surfaces (Grantcharova et al., 2010). Other fimbriae such as plasmid encoded fimbriae (Pef), long polar fimbriae (Lpf), bovine colonization factor (Bcf) and Sth are also important for biofilm formation on HEp-2 and chicken intestinal tissue (Ledeboer et al., 2006) Thus, it seems that fimbriae may have separate and complementary functions that are important during genesis and maturation of Salmonella biofilms on eukaryotic cell surfaces. Recently, Type IV pilus from S. Typhi has been correlated to biofilm formation in human isolates tested in vitro (Raza et al., 2011).

1.4.2.3. Cellulose and colanic acid

Cellulose, encoded by the bcsABZC-bcsEFG genes, is an important Salmonella biofilm-associated EPS on glass, epithelial cell surfaces (HEp-2 and chicken intestinal tissue) and plant surfaces (Barak et al., 2007; Ledeboer et al., 2006; Ledeboer and Jones, 2005; Prouty and Gunn, 2003). However, cellulose appears not to be an important constituent of the EPS during gallstone biofilm formation (Prouty and Gunn, 2003). Similarly, cellulose is not a major component of the biofilm matrix of feed industry-isolated S. Agona and S. Typhimurium (Vestby et al., 2009) and for S. Enteritidis biofilms on polystyrene (Malcova et al., 2009).

Colanic acid (encoded by wcaM, wcaA, wza) is a capsular extracellular polysaccharide that plays a role during Salmonella biofilm formation, specifically to
create extensive three-dimensional structures on Hep-2 cells and chicken intestinal epithelium (Ledeboer et al., 2006; Ledeboer and Jones, 2005) but is not required for biofilms on abiotic surfaces, gallstones and alfalfa seeds (Barak et al., 2007; Ledeboer and Jones, 2005; Prouty and Gunn, 2003).

1.4.2.4. O-antigen capsule and other exopolysaccharides

O-ag capsule, consisting of more than 2,300 repeating tetrasaccharide units, is membrane linked by a 2-hydroxymyristic acid residue (Snyder et al., 2006). It is conserved amongst Salmonella spp. and encoded by two divergent operons (yihU-yshA and yihVW) (Gibson et al., 2006). Structurally, the O-ag capsule is similar to LPS O-ag of S. Enteritidis. Despite having similar repeating sugar units, the O-ag capsule and the LPS O-ag differ in size, charge, substitution patterns, and immunoreactivity (Gibson et al., 2006; Snyder et al., 2006; White et al., 2003). This capsule was proven to be involved in desiccation tolerance and enhanced plant colonization (Barak et al., 2007; Gibson et al., 2006). Thus, the O-ag capsule is hypothesized to play an important role in environmental persistence (Gibson et al., 2006). Furthermore, production of O-ag capsule in S. Typhimurium was shown to be transcriptionally induced during murine infection (White et al., 2008), suggesting involvement during systemic infection and the development of typhoid fever. The O-antigen capsule was also shown to be a crucial determinant in biofilms on gallstones and cholesterol surfaces, but the expression of this exopolysaccharide is not necessary for binding of Salmonella to glass or plastic (Crawford et al., 2008).
In addition, de Rezende et al. purified another capsule from the extracellular matrix fraction of the MDR strain S. Typhimurium DT104. This exopolysaccharide differs from O-ag capsule by lacking rhamnose and was shown to be important in biofilm maturation (de Rezende et al., 2005). This capsular polysaccharide is important for biofilm formation in strains that do not express curli or cellulose (Malcova et al., 2008).

**1.4.2.5. Biofilm associated proteins and extracellular DNA**

In addition to polysaccharides, biofilm-associated proteins contribute to EPS production in *S. aureus* and *E. faecalis* biofilms (Latasa et al., 2006). *Salmonella* spp. express a large cell surface protein encoded by *bapA* that was shown to be important for *S. Enteritidis* biofilm formation but not for *S. Typhimurium* (Jonas et al., 2007; Latasa et al., 2005). BapA is coordinately regulated by CsgD at 30°C with curli, cellulose and O-ag capsule, and it is secreted by proteins produced from the genetically linked BapBCD TSS1 (Latasa et al., 2005). SadA, an autotransporter adhesin expressed on the *Salmonella* cell surface in vivo, has also been implicated in biofilm formation (Raghunathan et al., 2011).

Extracellular DNA (eDNA) is a ubiquitous polymer that has been recently shown to be a matrix component of *S. Typhimurium* biofilms. In addition, this eDNA demonstrated chelating properties that induced antimicrobial peptide (AP) resistance in biofilms by activating the PhoPQ/PmrAB systems (Johnson et al., 2013). eDNA-induced AP resistance may allow immune evasion and long-term survival of *Salmonella* biofilms in the environment, on gallstones or possibly during intestinal infection.
1.4.3. Coordinated regulation during *Salmonella* biofilms

*Salmonella* biofilm formation is governed by a complex regulatory system that involves global regulatory DNA and RNA binding proteins, second messenger signaling and global metabolism shift. This ultimately results in the production of EPS components, repression of motility and altered metabolism.

1.4.3.1. CsgD

Atomic force microscopy of *S.* Typhimurium biofilms grown on agar plates or mica slides demonstrated that curli and cellulose were important for EPS production and suggested that expression of surface appendages may be co-regulated during biofilm formation (Jonas et al., 2007). CsgD (AgfD) is a major transcriptional regulator of *Salmonella* biofilm-associated matrix components (Brombacher et al., 2003; Gerstel and Romling, 2003). CsgD coordinately regulates curli, O-ag capsule and BapA expression (directly) and cellulose production (indirectly) (Gibson et al., 2006; Latasa et al., 2005; Romling et al., 1998; Zakikhany et al., 2010). CsgD activates AdrA, a GGDEF-containing protein that alters the cellular levels of c-di-GMP and leads to activation of cellulose expression (Simm et al., 2004; Zogaj et al., 2001). Transcription of *csgD* is itself regulated by at least seven global regulatory proteins including OmpR, RpoS, Crl, MlrA, CpxR, H-NS and IHF. This complex network enables fine-tuning regulation in response to different environmental conditions (Gerstel and Romling, 2003). Interestingly, it has been shown that CsgD expression has a bistable nature depending on phosphorylation. Thus, only unphosphorylated CsgD activates curli and cellulose. This likely enables
adaptation to highly variable micro-environments, thus giving rise to a heterogeneous biofilm population (Grantcharova et al., 2010; Zakikhany et al., 2010).

On the basis of colony morphology, bacteria expressing cellulose and curli fimbriae have a red, dry and rough (rdar) morphotype on Congo red agar plates. This phenotype is dependent upon both curli and cellulose (Romling, 2005). It appears that the primary role of the rdar morphotype is to foster survival of *Salmonella* in the environment, likely via biofilms (Jain and Chen, 2007), thereby aiding in transmission. In addition, it has been proposed that the balance in production between both curli and cellulose appears to depend to a certain extent on LPS and hence on the cell surface (Anriany et al., 2006; White et al., 2003). Finally, a recent study demonstrated that Rcs phosphorelay system controls biofilm formation in *S. Typhimurium* depending on RcsB phosphorylation status. Unphosphorylated RcsB is a positive regulator of *csgD* while the accumulation of phosphorylated RcsB represses *csgD* expression (Latasa et al., 2012).

1.4.3.2. Cyclic di-guanylic acid

The novel second messenger bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) or cyclic di-guanylic acid, has been demonstrated to mediate the transition from motility to sessility during biofilm formation for a variety of microorganisms including *Salmonella*, *Vibrio*, and *Pseudomonas* (Romling and Amikam, 2006; Tischler and Camilli, 2004; Ueda and Wood, 2009). This bacterial signal transduction molecule is synthesized and degraded by two protein domain superfamilies,
diguanylate cyclases (GGDEF domain proteins) and phosphodiesterases (EAL- or HD-GYP domain proteins), respectively (Simm et al., 2004).

Elevated concentrations of c-di-GMP have been shown to activate CsgD at the transcriptional and post-transcriptional levels (Kader et al., 2006) resulting in expression of cellulose, curli fimbriae, BapA, and the O-ag capsule (Gibson et al., 2006; Latasa et al., 2005; Simm et al., 2007; Zogaj et al., 2001). Cellulose biosynthesis requires c-di-GMP availability (Solano et al., 2009) and can occur independently of CsgD (Garcia et al., 2004; Simm et al., 2007; Zogaj et al., 2001). EAL domain proteins STM3611 (yhjH) and STM1827 are involved in down-regulation of c-di-GMP levels leading to reduced cellulose production and biofilm formation and enhanced motility (Simm et al., 2004). S. Typhimurium also regulates GGDEF-EAL domain proteins and the transition from motility to sessility via carbon storage regulator A (CsrA) (Jonas et al., 2010; Teplitski et al., 2006). In addition, high c-di-GMP levels negatively regulate virulence properties of S. Typhimurium during epithelial cell invasion at the mucosal lining and abolishes induction of a pro-inflammatory immune response; suggesting an important role for c-di-GMP during intestinal colonization, likely independent of biofilm formation (Ahmad et al., 2011; Lamprokostopoulou et al., 2010).

Interestingly, the specific binding of Sal4 (a monoclonal IgA antibody) to the O-ag of S. Typhimurium has been shown to induce EPS production and biofilm formation by triggering the c-di-GMP-dependent signaling pathway via YeaJ (GGDEF protein), thereby leading to suppression of bacterial motility and T3SS, thus blocking entry into the intestinal mucosa (Amarasinghe et al., 2013).
1.4.3.3. Small RNAs

Small RNAs (sRNAs) are non-coding RNA molecules (50-250 nucleotides) produced by bacteria which has been recently demonstrated as post-transcriptional regulators of a variety of bacterial functions (Mika and Hengge, 2013; Van Puyvelde et al., 2013). They regulate their mRNA targets by direct base pairing which affects stability, processing and/or translation efficiency. Their function is mostly facilitated by the chaperone Hfq (Waters and Storz, 2009). In fact, a S. Typhimurium hfq mutant, in which the sRNA regulation is disturbed, cannot produce biofilms (Kint et al., 2010). Recently, it has been shown that Hfq dependent sRNAs ArcZ and SdsR positively affect biofilm formation, rdar morphotype and csgD expression (Monteiro et al., 2012) whereas RprA, a sRNA induced by RcsCDB activation, negatively regulates CsgD via an anti-sense mechanism (Latasa et al., 2012). MicA is an RpoE-dependent sRNA involved in outer membrane remodeling which has also been shown to have a role in Salmonella biofilm formation (Kint et al., 2010; Vogel and Papenfort, 2006).

1.4.3.4. Quorum sensing

Quorum sensing (QS) is a mechanism of cell-cell communication that enables bacteria to regulate population behavior by monitoring the local environment with diffusible signaling molecules known as autoinducers (Bassler, 2002). In gram negative cell populations, this autoinduced positive feedback system is mediated by acyl-homoserine lactones (AHLs) and/or autoinducers 2 and 3 (AI-2, AI-3) (Irie and Parsek, 2008). AHLs provide bacteria with a measuring stick for detection of its own species and,
as such, have been shown to mediate the formation of differentiated multicellular microcolonies in *P. aeruginosa* biofilms on both live and inert surfaces (Kjelleberg and Molin, 2002). *Salmonella* do not synthesize AHLs, but can sense those produced by other species using SdiA, a LuxR homolog that detects mixed microbial communities and has been implicated in *E. coli* biofilm formation (Lee et al., 2009; Michael et al., 2001). In *Salmonella*, SdiA regulates the expression of several virulence genes including type 1 fimbriae (Dyszel et al., 2010) but its role in biofilm formation has not been elucidated.

In *S. Typhimurium*, Al-2 molecules are synthesized by the highly conserved LuxS enzyme and are derived from the same compounds as those for *Vibrio harveyi*, a model QS microorganism (Xavier and Bassler, 2003). The *lsr* operon of *Salmonella* encodes a receptor, LsrB that transports Al-2 molecules from the extracellular space into the cell (Taga et al., 2003). This operon is thought to be up-regulated in the intestinal microenvironment (Sonck et al., 2009). Recently, the *rbs* transporter has been suggested as an alternative Al-2 uptake system (Jesudhasan et al., 2010). It has been reported that expression of LuxS and Al-2 is necessary for *Salmonella* virulence (Choi et al., 2007) and mutations in *luxS* inhibited *Salmonella* biofilm formation on polysterene and gallstones (De Keersmaecker et al., 2005; Jesudhasan et al., 2010; Prouty et al., 2002a). Microarray analysis revealed that several motility genes and biofilm-related genes are down-regulated in the *luxS* mutant as compared to the wild-type. Interestingly, the effects of biofilm formation by the *luxS* mutant have been shown to be dependent of the sRNA molecule MicA, rather than on LuxS itself (Kint et al., 2010). However, further research is needed to assess whether this is also the case in other experimental setups.
Finally, the third quorum sensing system of *Salmonella* utilizes the TCS PreA/B which senses AI-3 and the eukaryotic hormones epinephrine and norepinephrine (Clarke et al., 2006; Merighi et al., 2006). A preB mutant was found to be defective in biofilm formation under certain conditions and displays decreased colonization of gastrointestinal tract of swine (Steenackers et al., 2012). New strategies that more accurately mimic the natural microbe environment may be necessary to provide significant additional insights as to how QS pathways mediate biofilm development and maintenance.

1.4.3.5. Global metabolic shift

In addition to the regulatory pathways that are responsible for the structural components of *Salmonella* biofilms, other functional categories of genes are also affected, reflecting a global physiological shift (Hamilton et al., 2009; White et al., 2010). The metabolic state of the bacterial cell is itself responsible for further biofilm development. Different studies described how the amino acid and carbon metabolism affect biofilm formation. For instance, mutants of *trpE* (required for tryptophan biosynthesis), *aroD* and *aroA* (involved in aromatic amino acid synthesis) showed a decreased biofilm phenotype (Hamilton et al., 2009; Malcova et al., 2009). In terms of carbon metabolism, a global metabolic shift toward gluconeogenesis has been noticed during *Salmonella* biofilm formation (White et al., 2010) which is thought to be a result of the metabolic demand of polysaccharide production rather than elaboration of a defined genetic program. Alteration of the outer membrane homeostasis can also drastically influence the biofilm phenotype (Monteiro et al., 2011; Van Puyvelde et al., 2013). Thus,
the local microenvironment that results from being embedded in a self-produced matrix would result in the induction of numerous pathways associated with stress tolerance and nutrient scavenging (White et al., 2010).

1.5. *Salmonella* biofilms on gallstones

The clinical observations of carriers with regard to recalcitrance to antibiotics, absence of symptoms, confinement to an organ with shedding, and organ removal as the most successful therapy are observations consistent with biofilm-related disease (Hall-Stoodley and Stoodley, 2009; Lai et al., 1992; Swidsinski and Lee, 2001). As previously stated, many studies have shown an association between the presence of gallstones and typhoid carriage (Karaki and Matsubara, 1984; Lai et al., 1992; Schioler et al., 1983). Thus, it was hypothesized that biofilm formation on gallstones mediates chronic carriage in the gallbladder (Prouty et al., 2002a).

1.5.1. In vitro biofilm formation on gallstones

Various static and dynamic systems have been employed to examine *Salmonella* spp. biofilms. Early studies of biofilms in chronic *Salmonella* spp. carriage used gallstones removed from patients during cholecystectomy. *S. Typhi*, *S. Typhimurium* and *S. Enteritidis* formed dense matrix-encased biofilms on gallstones over the course of 7-14 days; however, in controls using an alternate substrate of similar size and shape, no such biofilm was formed (Prouty et al., 2002a). As an in vitro surrogate of gallstones, the tube biofilm assay (TBA) was developed for the study of biofilms on cholesterol
This method involves the coating of siliconized microcentrifuge tubes with cholesterol. Bacteria are incubated in these tubes for a period of 24 h, after which the culture is aspirated and non-adherent bacteria are removed by washing with phosphate-buffered saline (PBS). Using this method, the role of bile in the enhancement of Salmonella spp. biofilm formation was confirmed and specific binding to cholesterol was observed (Crawford et al., 2008). Bilirubin, a major component of pigment stones, was also evaluated in the TBA. Salmonella spp. formed biofilms poorly on calcium bilirubinate compared with cholesterol, further indicating the specificity of Salmonella spp. binding to, and subsequent biofilm formation on cholesterol-coated surfaces. The use of the TBA eliminates the dependence on human gallstones and allows for assay standardization, as the cholesterol composition of gallstones is variable (Crawford et al., 2008). However, while the TBA is both economic and reproducible, the flow-through system is probably more representative of the gallbladder environment. This flow-through system consists of media with bile flowing through chambers at a specific flow rate. The chambers contain glass or cholesterol-coated glass coverslips.

1.5.2. Biofilm initiation on gallstones

For successful biofilm formation on gallstones, Salmonella spp. must first access and colonize the gallbladder or biliary tract. The bacteria must then attach to the surface of gallstones as well as persist in the presence of natural host defenses (Stewart et al., 2007). It is thought that bile stasis that can occur in the presence or absence of gallstones contributes to successful colonization (Swidsinski and Lee, 2001).
Several known bacterial biofilm-associated factors have been investigated to determine which are critical for the formation of biofilms on the surface of gallstones and on cholesterol-coated surfaces (Prouty and Gunn, 2003; Prouty et al., 2002a). In S. Typhimurium, the presence of flagellar filaments but not motility (verified by a mutation in the gene _motA_ that lacks the ability to rotate the flagellum) was necessary for biofilm formation on gallstones. In contrast, motility was required for biofilm formation in different assay conditions on glass and polyvinyl chloride (Mireles et al., 2001; Prouty and Gunn, 2003). To build upon this work and examine cholesterol-specific biofilm-required factors, a pool of transposon mutants was examined in the TBA with daily passage of planktonic bacteria (Crawford et al., 2010a). Using this method, 49 mutants deficient in cholesterol binding and subsequent formation of biofilms were obtained. Many of the non-adherent mutants represented transposon insertions in flagellar biosynthetic genes. Specifically, the _FliC_ subunit was demonstrated to be necessary for initial binding to cholesterol-coated surfaces. Loss of _OmpC_ (an outer membrane protein) also negatively affected binding to cholesterol as well as subsequent biofilm formation. Additionally, 18 of the transposon-library mutants showed insertions in the _fimW_ gene. _FimW_ is a negative regulator of the type 1 fimbriae operon and its deletion confers a constitutively expressed type 1 fimbrial phenotype. Further analysis demonstrated that a hyper-fimbriate phenotype negatively affected the initial stages of biofilm formation on cholesterol but not to glass or plastic. Thus, the initial attachment phase of biofilm formation may involve a combination of flagella and outer membrane proteins that can be masked by over-expression of surface fimbriae (Crawford et al., 2010a).
1.5.3. Biofilm maturation on gallstones

After the initial attachment phase, biofilm development typically involves the formation of microcolonies followed by a mature biofilm. Both stages are characterized by the presence of extracellular polymeric substances (EPS) that aid in biofilm structure and cell-cell interaction (Costerton et al., 1999). Different components of EPS have been studied in Salmonella biofilms on gallstones including cellulose, colanic acid, Vi antigen and O-ag capsule (Gibson et al., 2006; Jonas et al., 2007; Ledeboer and Jones, 2005). Deletion of the genes encoding the S. Typhi Vi antigen does not affect biofilm formation (Prouty et al., 2002a; Raza et al., 2011) and Vi-antigen is not present in S. Enteritidis or S. Typhimurium, but these serovars can still form robust biofilms on cholesterol-coated surfaces. Cellulose and colanic acid are important for biofilm formation on abiotic and biotic surfaces (Ledeboer and Jones, 2005). However, although a S. Typhimurium double mutant for cellulose and colanic acid was negatively affected in biofilm formation on plastic and glass, biofilm formation on gallstones was unaffected (Prouty and Gunn, 2003).

Gibson and colleagues identified the polysaccharide O-ag capsule in S. Enteritidis. This capsule has been shown to be important for environmental persistence and attachment to and colonization of plants (Barak et al., 2007; Gibson et al., 2006). Mutation of galE, the gene responsible for the synthesis of galactose - which is used in construction of the LPS outer core and LPS O-ag and is putatively involved in synthesis of the O-ag capsule - has been shown to yield mutants that are unable to form biofilms on gallstones (Prouty et al., 2002a). Conversely, mutations in rfaD, a gene that is
involved in synthesis of the LPS outer core and LPS O-ag alone, exhibit no such defect (Prouty and Gunn, 2003). In addition, mutation of the genes putatively associated with O-ag capsule synthesis negatively affected *Salmonella* spp. biofilm formation on cholesterol-coated surfaces and gallstones. Furthermore, bile has been shown to up-regulate the expression of O-ag capsule genes in a *csgD*-independent manner, further suggesting an important role for the O-ag capsule in *Salmonella* spp. biofilms on gallstone surfaces (Crawford et al., 2008).

### 1.5.4. Mouse model of typhoid carriage

The persistence model of *Salmonella* chronic infection (Monack et al., 2004) was recently adapted for in vivo studies of chronic gallbladder infection. After 6-8 weeks of a lithogenic diet (1% cholesterol and 0.5% cholic acid), mice developed cholesterol gallstones. *S. Typhimurium* infection in mice harboring cholesterol gallstones resulted in enhanced colonization of gallbladder tissue and bile compared with mice lacking gallstones (Crawford et al., 2010b). Likely due to increased bacterial load in the gallbladder, these mice exhibited a 3-log increase in fecal shedding of *S. Typhimurium* compared with similarly infected mice lacking gallstones. Electron microscopy analysis of the gallstones removed from infected mice revealed a dense bacterial biofilm covering more than 50% of the surface. This work strongly supported the hypothesis that biofilms on gallstone surfaces mediate the typhoid carrier state. Furthermore, the greatly increased fecal shedding in these carriers supports the importance of carriers to the spread of typhoid fever. The development of this murine model of cholesterol gallstone-mediated chronic carrier state facilitates further in vivo studies (Crawford et al., 2010b).
1.5.5. Human studies of typhoid carriage

The strong correlation of typhoid carrier state with the presence of gallstones was recently validated in a study conducted in an endemic region. Asymptomatic patients presenting in a hospital from Mexico City for cholecystectomy due to the presence of gallstones, were screened for the presence of S. Typhi (Crawford et al., 2010b). Multiplex PCR and traditional plating assays revealed the presence of S. Typhi in five of the 103 patients with cholelithiasis (~5% of patient samples). While the gallstones from all five patients were positive for S. Typhi, two of the five harbored S. Typhi in the gallbladder epithelial tissues while only one had a positive culture of bile. Electron microscopy analysis revealed that gallstones from three out of four of these patients exhibited 80-90% surface coverage with a dense bacterial biofilm (Fig. 6). The gallstone from the patient who lacked biofilm formation was thought to be a pigment stone, with calcium bilirubinate and not cholesterol as the main constituent. This supports previous in vitro findings that calcium bilirubinate is not the preferred surface for Salmonella spp. biofilms on gallstones. However, gallstones from patients harboring E. coli in the absence of S. Typhi (13% of patient samples collected) exhibited no such biofilms (Fig. 6). E. coli is a common inhabitant of a poorly functioning gallbladder, but was not found to co-infect gallbladders with S. Typhi (Crawford et al., 2010b).

Recent work examining S. Typhi isolates from patient stool samples has revealed correlations between biofilm formation and shedding duration. Interestingly, biofilm formation by these isolates was also associated with increased antibiotic resistance, with the best biofilm producers possessing multiple antibiotic-resistance cassettes (Raza et
al., 2011). Thus, measurement of the ability of fecal bacteria isolates to form biofilms during the early onset of typhoid infection might be informative regarding both the duration and quantity of shedding.

These clinical data correlate with previous in vivo observations and support the hypothesis that chronic carriage of S. Typhi is mediated by biofilm formation on gallstones. In addition, they demonstrate the presence of a subset of healthy carriers in an endemic population and highlight the importance of the development of methods to identify and successfully treat such patients.
Figure 6. Biofilm formation on human gallstones was observed only in patients seeking cholecystectomy that were positive for typhoid carriage. SEM micrographs show bacterial biofilms on gallstones of two asymptomatic typhoid carriers in A and B. (C) Biofilm formation was not detected on the gallstone of one of the serovar Typhi carriers. The unique surface texture and pigment were suggestive of black, calcium bilirubinate gallstones. (D) Absence of biofilm formation on gallbladder stones from a patient positive for *Escherichia coli* and *Shigella flexneri*. 
1.6. Hypothesis and goals

The specific host and pathogen factors and the molecular basis that facilitates typhoid carrier state and host-to-host transmission are poorly understood. The main objective of this work was to determine the mechanisms controlling the *Salmonella* -host interactions in the gallbladder during asymptomatic chronic carriage. The general methodology included in vitro models that closely mimicked the gallbladder and gallstones environment. These in vitro results were validated in the murine model of chronic carriage.

It has been demonstrated that gallstones aid in the development and maintenance of gallbladder carriage in a mouse model and in humans, serving as a substrate to which *S. Typhi* attach and form a protective biofilm. *Salmonella* EPS components that mediate attachment and biofilm development are often uniquely required depending on environmental conditions and the composition of the binding substrate. In fact, most of these components do not seem to be important for biofilm formation on gallstones. Thus, the first objective of this work was to identify specific bacterial factors mediating biofilm formation on gallstones and thus chronic carriage in the gallbladder. These factors may represent new targets for therapeutic/preventive approaches to eliminate the carrier state. We hypothesized that specific genes up-regulated during biofilm formation on cholesterol-coated surfaces are required for biofilm development on these surfaces.
Although human chronic carriers often have biliary-tract disease such as cholelithiasis, this condition is not an absolute requirement for development of the carrier state. Previous studies from endemic areas have suggested the gallbladder epithelium as an additional site of *Salmonella* persistence. Thus, our second objective was to demonstrate the role of the gallbladder epithelium in the development of chronic carriage. We hypothesized that *Salmonella* is able to intracellularly persist in and/or form biofilms on the gallbladder epithelium.

The developed mouse model of typhoid chronic carriage is fundamental to reveal the host-pathogen interactions occurring in the gallbladder. Examination of the ongoing dynamics of the gallbladder response to *Salmonella* colonization in the presence or absence of gallstones can provide specific signatures of chronic carriage in both scenarios. These studies not only have implications in the continuous effort to combat typhoid fever disease but also in the prevention of related malignancies associated with typhoid carriage such as gallbladder cancer. Thus, our third objective was to analyze the histopathological features of the gallbladder and associated hepatopancreatobiliary organs during *Salmonella* carriage. We hypothesized that *Salmonella* induces chronic inflammation of the gallbladder that can lead to the development of pre-malignant lesions in the gallbladder epithelium.

As a result of long-term in vivo chronic carriage, hyper-biofilm forming isolates were isolated from the gallstone of a carrier mouse at nine months post-infection. Interestingly, isolates from the gallbladder tissue and bile from the same mouse did not show the hyper-biofilm phenotype. Considering the significance of these isolates in
explaining specific long-term adaptation to the gallstones environment, an additional objective of our work was to determine the genome sequence of this isolate to identify mutations responsible for the hyper-biofilm phenotype.

In summary, this work was focused in determining the mechanisms used for *Salmonella* and the host to allow chronic carriage in the gallbladder. To our knowledge, this represents the first comprehensive study that uses both in vitro and in vivo models to validate important signatures of *Salmonella* chronic carriage from a host-pathogen perspective. Investigating the mechanisms controlling host–pathogen interactions during *Salmonella* chronic carriage could lead to the identification of specific signatures and/or biomarkers of typhoid carriage. This can be crucial not only to identify chronic carriers but also to develop new approaches for the prevention and treatment of this persistent infection. Ultimately, these studies may contribute to the elimination of *Salmonella* carriage in the gallbladder and the spread of typhoid fever.
Chapter 2: Identification of *Salmonella* Genes Regulated During Biofilm Formation on Cholesterol (Gallstone) Surfaces

2.1 Abstract

*Salmonella* spp. are able to form biofilms on abiotic and biotic surfaces. In vivo studies in our laboratory have shown that *Salmonella* can form biofilms on the surface of cholesterol gallstones in the gallbladder of mice and human carriers. Biofilm formation on gallstones has been demonstrated to be a mechanism of persistence. The purpose of this work was to identify and evaluate *Salmonella* spp. cholesterol-dependent biofilm factors. Differential gene expression analysis between biofilms on glass or cholesterol coated-surfaces and subsequent qRT-PCR revealed that type 1 fimbriae structural genes and a gene encoding a putative outer membrane protein (*ycfR*) were specifically up-regulated in *S. Typhimurium* biofilms grown on cholesterol surfaces. Spatio-temporal expression of *ycfR* and FimA verified their regulation during biofilm development on cholesterol surfaces. Surprisingly, confocal and scanning electron microscopy demonstrated that a mutant of type 1 fimbriae structural genes (∆*fimAICDFH*) and a *ycfR* mutant showed increased biofilm formation on cholesterol-coated surfaces. In vivo experiments using *Nramp1*+/− mice harboring gallstones showed that only the ∆*ycfR* mutant formed extensive biofilms on mouse gallstones at 7 and 21 days post-infection; ∆*fimAICDFH* was not observed on gallstone surfaces after the 7 day post-infection time.
point. These data suggest that in *Salmonella* spp. wild-type, type 1 fimbriae are important for attachment to and/or persistence on gallstones at later points of chronic infection whereas YcfR may represent a specific potential natural inhibitor of initial biofilm formation on gallstones.

### 2.2. Introduction

Typhoid or enteric fever, caused primarily by *S. Typhi*, is a global human specific disease that is responsible for an estimated 21 million new infections annually resulting in more than 200,000 deaths worldwide (Crump et al., 2004). It is an important health problem in developing countries and poses a significant risk to travelers. After ingestion through contaminated water or food, bacteria cross the intestinal epithelial barrier, are phagocytosed by macrophages and spread systemically producing acute disease (Jepson and Clark, 2001; Vazquez-Torres et al., 1999; Vladoianu et al., 1990; Wain et al., 1998). During this systemic infection, *S. Typhi* can reach the gallbladder from the liver and establish an acute infection with inflammation (cholecystitis) or chronically persist in this organ. It is estimated that between 3-5% of typhoid fever patients become chronic carriers with the gallbladder as the primary site of carriage (Levine et al., 1982; Merselis et al., 1964). As clinical evidence, inflammation of the gallbladder and sonographic gallbladder abnormalities have been reported in acute and chronic typhoid fever patients (Cohen et al., 1987; Mateen et al., 2006; Shetty and Broome, 1998; Vaishnavi et al., 2005b; Vogelsang and Boe, 1948).
Because *S. Typhi* is a human specific pathogen, these carriers serve as a critical reservoir for further spread of the disease through bacterial shedding in feces, which is a sporadic and intermittent event (Bhan et al., 2005; Vogelsang and Boe, 1948). Chronic typhoid infections can persist for decades and although highly contagious, they are typically asymptomatic (Shpargel et al., 1985; Sinnott and Teall, 1987). These factors make this carrier state difficult to confirm and an understudied area of human health and research.

Particularly in high areas of endemicity, this carrier state is linked to the presence of gallstones, as approximately 80-90% of chronically infected carriers have this gallbladder abnormality (Karaki and Matsubara, 1984; Lai et al., 1992; Schioler et al., 1983). Gallstones are primarily composed of cholesterol (up to 70-100% cholesterol), although calcium bilirubinate predominates in certain parts of the world (Kim et al., 2003). The increasing incidence of antibiotic resistant bacteria colonizing chronic typhoid patients exacerbates morbidity and mortality (Dinbar et al., 1969; Harish and Menezes, 2011; Lai et al., 1992; Pratap et al., 2012). In addition, chronic carriers have an approximately 8-14-fold increased risk of developing gallbladder carcinoma and approximately 150-fold increased risk of developing hepatobiliary carcinoma than non-carriers (Caygill et al., 1994; Nath et al., 1997; Shukla et al., 2000; Welton et al., 1979). This impact on human health combined with the high incidence of typhoid fever in many parts of the world highlights the importance of understanding the mechanisms involved in typhoid carriage.
To date, removal of the gallbladder (cholecystectomy) is the most common treatment for chronic typhoid carriers and those with gallbladder abnormalities; however, this is both costly and invasive and does not guarantee elimination of the carrier state (Ristori et al., 1982) since additional foci of infection can persist in locations including the biliary tree, MLN or liver (Erlik and Reitler, 1960; Gaines et al., 1968; Monack et al., 2004; Nath et al., 2010c; Rovito and Bonanno, 1982). Therefore, alternative treatments are needed to eradicate the gallbladder carriage state. The known clinical observations of carriers regarding recalcitrance to antibiotics, absence of symptoms, confinement to an organ with shedding, and organ removal as the most successful therapy are observations consistent with biofilm-related disease (Gonzalez-Escobedo et al., 2011; Lai et al., 1992; Swidsinski and Lee, 2001). We have shown that Salmonella can form biofilms on the surface of cholesterol gallstones in the gallbladder of mice and human carriers, and this biofilm formation has been demonstrated to be a mechanism of persistence and chronic colonization in the gallbladder (Crawford et al., 2010b).

Previous studies have showed that initial interactions between Salmonella and gallstones is likely mediated by OmpC and the flagellar subunit FliC, independent of motility (Crawford et al., 2010a). The hallmark of a mature biofilm is the development of a self-initiated extracellular matrix. Reports have shown that Salmonella spp. biofilms contain extracellular polymeric substances, including cellulose, colanic acid, curli fimbriae, O-antigen capsule, biofilm-related proteins and nucleic acids (Gibson et al., 2006; Jain and Chen, 2007; Jonas et al., 2007; Latasa et al., 2005; Ledeboer and Jones, 2005; Romling, 2005; Zogaj et al., 2001). Cellulose and colanic acid are important for Salmonella biofilms on abiotic surfaces, HEp-2 cells, and chicken intestinal tissue
(Ledeboer and Jones, 2005; Prouty and Gunn, 2003), but are not required for biofilms on human gallstones in vitro (Prouty and Gunn, 2003; Prouty et al., 2002a). These data suggest that the contribution of bacterial factors varies depending on binding substrates.

In this study, we identified *Salmonella* genes specifically regulated during biofilm development on cholesterol-coated surfaces using an in vitro flow system that mimics the gallbladder and gallstone environment. Identified genes encode surface (type 1 fimbriae) or outer membrane proteins (YcfR). We demonstrated in vitro and in vivo that these bacterial surface factors affect the development of biofilms on cholesterol gallstones unveiling novel mechanisms to modulate biofilms on this specific substrate. By understanding the basis of this biofilm-mediated carriage, it may be possible to identify effective strategies to prevent or eliminate *Salmonella* carriage and thus the human-to-human spread of typhoid fever.

### 2.3. Materials and Methods

#### 2.3.1 Ethics Statement

Mice were housed and used in strict accordance with guidelines established by The Ohio State University Institutional Animal Care and Use Committee (IACUC), and all efforts were made to minimize animal suffering. The work performed in this study was approved by the OSU IACUC. The Ohio State University Animal Care and Use Program is accredited by The Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The protocol identification number is 2009A0057. All research activities must conform to the statutes of the Animal Welfare Act and the
guidelines of the Public Health Service as issued in the Guide for the Care and Use of Laboratory Animals (revised 1996).

2.3.2 Bacterial strains and growth conditions

S. Typhimurium and S. Typhi behave similarly in biofilm assays previously conducted in our laboratory. Wild-type and derivatives of S. Typhimurium ATCC14028 (JSG210) were used in these studies. The strains used are listed in Table 1. All cultures were grown in Luria-Bertani (LB) broth supplemented with and without 3% ox bile (Sigma-Aldrich, MO) at 37°C with aeration. Antibiotics, when needed, where used at the following concentrations: ampicillin, 100 µg/mL; kanamycin, 45 µg/mL; chloramphenicol, 25 µg/mL.

2.3.3. Biofilm growth on microtiter plates

Glass bottom 12-well plates (14 mm microwell diameter glass No 1.5, MatTek Corp., MA) uncoated or coated by evaporation with 4 mg of cholesterol (diluted in ether, anhydrous; J.T. Baker, NJ) were inoculated with 2x10^8 bacteria in 2 mL of LB bile (3%). The plates were incubated for 24 h or 96 h at 37°C in a GyroMini nutating mixer (LabNet International, Inc., NJ) at 24 rpm. The use of cholesterol-coated-surfaces eliminates the dependence on human gallstones and replicates the gallstone surface (Crawford et al., 2008).
Table 1. Bacterial strains and plasmids used in Chapter 2

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant phenotype</th>
<th>Reference and/or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSG210</td>
<td>Wild-type S. Typhimurium ATCC 14028</td>
<td>ATCC</td>
</tr>
<tr>
<td>JSG3392</td>
<td>$\Delta$fimAICDHF</td>
<td>(Nuccio et al., 2007)</td>
</tr>
<tr>
<td>JSG3119</td>
<td>$\Delta$ycfR</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3393</td>
<td>$\Delta$fimAICDHF$\Delta$ycfR double mutant</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3541</td>
<td>Wild-type S. Typhimurium ATCC 14028 pWSK29</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3529</td>
<td>Wild-type S. Typhimurium ATCC 14028 pWSK129</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3542</td>
<td>$\Delta$fimAICDHF pWSK29</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3546</td>
<td>$\Delta$fimAICDHF pGGE1</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3530</td>
<td>$\Delta$ycfR pWSK129</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3532</td>
<td>$\Delta$ycfR pGGE2</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3526</td>
<td>Wild-type S. Typhimurium ATCC 14028 pGGE3</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pWSK29</td>
<td></td>
<td>(Wang and Kushner, 1991)</td>
</tr>
<tr>
<td>pWSK129</td>
<td></td>
<td>(Wang and Kushner, 1991)</td>
</tr>
<tr>
<td>pFPV25</td>
<td></td>
<td>(Valdivia et al., 1996)</td>
</tr>
<tr>
<td>pAZ24</td>
<td>pET16b [fimAICDHF]</td>
<td>(Guo et al., 2007)</td>
</tr>
<tr>
<td>pGGE1</td>
<td>pWSK29[fimAICDHF]</td>
<td>This study</td>
</tr>
<tr>
<td>pGGE2</td>
<td>pWSK129[ycfR]</td>
<td>This study</td>
</tr>
<tr>
<td>pGGE3</td>
<td>pWSK129[ycfR/GFP]</td>
<td>This study</td>
</tr>
</tbody>
</table>

2.3.4. Flow-through system assays

To closely mimic the gallbladder and gallstones environment, a flow-through system was used for gene expression analysis and biofilm observation by microscopy.

Single-channel chambers (24mm length x 8mm width x 4 mm height) with glass
coverslips (Stovall Life Science Inc., IA) with or without cholesterol coating (8mg) were inoculated with $2 \times 10^8$ of exponentially growing *Salmonella*. Bacteria were allowed to adhere for 2h before starting the flow with LB bile (3%) at 270 µL/min for 24 or 72h.

2.3.5. Crystal violet assays

Biofilms attached to the microtiter wells or chamber coverslips were washed, heat-fixed for 1h at 60°C and stained with 0.25% crystal violet for 5 min. After four washes with 1XPBS, acetic acid at 33% was used to extract the dye. Determination of the amount of dye retained by the biofilms was obtained at an OD$_{570}$ in a SpectraMax® Spectrophotometer with SoftMax®Pro software. Experiments were performed in triplicate. To compensate for background absorbance, values from non-inoculated glass and cholesterol-coated coverslips were averaged and subtracted.

2.3.6. Differential gene expression analysis

A flow-through system (as described above) was used for gene expression analysis. After 24h of incubation with flow, planktonic (from the flow-through) and biofilm (from the flow chamber) cells were collected. After three consecutive washes in 1X PBS, RNA was immediately extracted with a RNeasy® Plus Mini Kit (Qiagen), reverse transcribed to cDNA by using random hexamers and SuperScript III following the manufacturer’s guidelines (Invitrogen, CA) and submitted to Roche NimbleGen, Inc. for microarray analysis (*S. Typhimurium* LT2 array). Two biological replicates were used. Differential gene expression analysis was performed using ArrayStar® v3.0. To validate
the results obtained in the microarray analysis, Quantitative real-time PCR (qRT-PCR) was performed using RNA extracted from biofilm cells (on glass and cholesterol) after 24 and 72h of flow. cDNA (20 ng) was added to IQ SYBR green PCR master mix (Bio-Rad) containing 1 µM of primers specific to fimC, ycfR or rpoB (housekeeping gene) (Table 2). Samples were run in triplicate and repeated three times using the Bio-Rad CFX96 iCycler apparatus. Relative copy numbers were calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

2.3.7. Generation of mutants and cloning procedures

Mutation of the ycfR gene was performed by using the λ-red mutagenesis method (Datsenko and Wanner, 2000) with primers JG2055 and JG2056 (Table 2). To create a complemented strain of ycfR, the ycfR gene was cloned into pWSK129 to create pGGE1 by using the XbaI and KpnI restriction sites and the primers JG2526 and JG2527 (Table 2). To obtain a complemented strain of the fimAI/CDHF mutation, the fimAI/CDHF cluster of genes was excised from the plasmid pAZ24 (Guo et al., 2007) and cloned into pWSK29 by using the restriction sites XbaI and BamHI. To create a reporter strain for ycfR expression, the promoter of the gene was cloned in pFPV25, upstream of a promoterless gfp cassette (Valdivia et al., 1996) by using the restriction sites KpnI and XbaI and the primers JG2512 and JG2513 (Table 2). The pycfR/gfp fragment was then sub-cloned into pWSK129 by using Sacl and HindIII restriction sites to create pGGE3.
Table 2. Oligonucleotide primers used in Chapter 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer sequence (5’-3’)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG2081</td>
<td>ACGGTCCGCTATGTGCTCTATC</td>
<td><em>rpoB</em> qRT-PCR</td>
</tr>
<tr>
<td>JG2082</td>
<td>GAGTTGCGCTGAGGCTATAAC</td>
<td><em>rpoB</em> qRT-PCR</td>
</tr>
<tr>
<td>JG1995</td>
<td>GTTTCTGCAACTGCCGATTCT</td>
<td><em>fimC</em> qRT-PCR</td>
</tr>
<tr>
<td>JG1996</td>
<td>GGAATTTGCATGTCGCTTTTT</td>
<td><em>fimC</em> qRT-PCR</td>
</tr>
<tr>
<td>JG2025</td>
<td>TAAAAACCCCTCATCGCTGCT</td>
<td><em>ycfR</em> qRT-PCR</td>
</tr>
<tr>
<td>JG2026</td>
<td>GGGCGCGTAACAGAGGTAAT</td>
<td><em>ycfR</em> qRT-PCR</td>
</tr>
<tr>
<td>JG2055</td>
<td>ACACCCATTTCATCTGCTAAAGGTCTCACTA TgAAAAGTGTAGGCTGGAGGCTGCTTC</td>
<td>λ Red deletion of <em>ycfR</em></td>
</tr>
<tr>
<td>JG2056</td>
<td>AATCGAGCGAGGATGATTGAGGTTAGCTT (TTACCTATAAGGATGCTT)</td>
<td>λ Red deletion of <em>ycfR</em></td>
</tr>
<tr>
<td>JG2526</td>
<td>GCTCTAGAGGAGGAATTCACCATGAAAAA CGTAAAAACCCCT</td>
<td>Cloning of <em>ycfR</em> into pWSK129</td>
</tr>
<tr>
<td>JG2527</td>
<td>GGG GTA CCT TAC TTA TAG ATT ACC GCC GTA CCG TG</td>
<td>Cloning of <em>ycfR</em> into pWSK129</td>
</tr>
<tr>
<td>JG2512</td>
<td>GGGGTACCGATGCCGTTGTACCTGTTAAA GAG</td>
<td>Cloning <em>ycfR</em> promoter into pFPV25</td>
</tr>
<tr>
<td>JG2513</td>
<td>GCTCTAGAGCGAGATGGAATGGGTGTCGTAAG CAT</td>
<td>Cloning <em>ycfR</em> promoter into pFPV25</td>
</tr>
</tbody>
</table>

2.3.8. Biofilm treatment with DNase I, proteinase and cellulase

Glass bottom 12-well plates (14 mm microwell diameter glass No 1.5, MatTek Corp., MA) with or without 4 mg of cholesterol coating were inoculated with 2x10⁸ bacteria and incubated for 24 h and 96 h at 37°C in a GyroMini nutating mixer (LabNet International, Inc.) at 24 rpm. DNase I (56 units), proteinase (0.53 mg) and cellulase (50 units) solutions were added to the established biofilms and the plates were then incubated for 16 h at 37°C in a GyroMini nutating mixer (LabNet International, Inc.) at 24 rpm. Experiments were repeated twice.
2.3.9. FimA and CsgA detection by immunofluorescence

Glass bottom 12-well plates (14 mm microwell diameter glass No 1.5, MatTek Corp., MA) with or without 4 mg of cholesterol coating were inoculated with $2 \times 10^8$ bacteria and incubated for 24 h and 96 h at 37°C in a GyroMini nutating mixer (LabNet International, Inc.) at 24 rpm. Bacteria were stained with FilmTracer™ calcein red-orange (Invitrogen), fixed with 4% paraformaldehyde in 0.1 M sodium phosphate pH 7.4 for 15 min at room temperature (RT), rinsed with water and blocked in 5% BSA in Tris-buffered NaCl solution with Tween 20 pH 7.6 for 1 h at RT, incubated with pre-absorbed rabbit polyclonal anti-FimA (1:500, provided by A. Baumler) or rabbit polyclonal anti-CsgA for 1 h at RT (1:500, provided by M. Chapman). Incubation with the secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit (1:1000; Invitrogen) was performed also for 1 h at RT. Mounted slides were observed using an Olympus Fluoview FV10.1. Experiments were repeated two times.

2.3.10. Mouse infections

Because S. Typhi is a human-restricted pathogen, in vivo studies of S. Typhi pathogenesis typically involve a mouse model of infection using S. Typhimurium. The pathological features and the course of mouse infection with S. Typhimurium are similar to those of human infection with S. Typhi (Santos et al., 2001). Naturally resistant Nramp1+/+ mice 129X1/SvJ (n=168) (Jackson Lab., ME) were fed with a lithogenic diet (1% cholesterol and 0.5% cholic acid; Sigma) (n=84) or normal chow (Harlan Lab., IN) (n=84). Nramp1 is a macrophage-associated protein that is a critical factor in controlling
the replication of intracellular bacteria (Forbes and Gros, 2001). After 9 weeks, mice were infected intraperitoneally (n=120) with $10^4$ S. Typhimurium or left as uninfected controls (n=42); and sacrificed at 7, 21 and 60 days post-infection (dpi). Spleen, liver, pancreas, gallbladder tissue, bile, gallstones and feces were homogenized and/or diluted for bacterial enumeration using *Salmonella-Shigella* agar (Difco™). Gallstones were washed with 1X PBS and then processed for SEM as described below.

2.3.11. Confocal microscopy

Flow-through chambers were washed once with 1X PBS and incubated with Live-dead® Stain (Invitrogen, CA) for 15 min at RT in dark conditions. Biofilms from strains harboring pGGE3 were stained with FilmTrace™ calcein red-orange (Invitrogen). Chambers were then washed twice with 1X PBS and fixed with 4% paraformaldehyde in 0.1M sodium phosphate pH 7.4 for 15 min at RT, rinsed with water and observed using an Olympus Fluoview FV10.1.

2.3.12. Scanning electron microscopy

Biofilms on glass, cholesterol or mouse gallstones were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer-0.1 M sucrose (pH 7.4), rinsed twice with 0.1 M phosphate buffer, and dehydrated by the addition of solutions of ethanol in a graded series, as follows: 35%, 50%, 70%, 80%, 95%, 100%. Samples were chemically dried with consecutive washes of 25%, 50%, 75%, and 100% hexamethyldisilazane (Ted Pella, CA). Samples were dried overnight in a fume hood, mounted on aluminum stubs,
and sputter-coated with gold for observation using a FEI Nova NanoSEM at the OSU Campus Microscopy and Imaging Facility (CMIF).

2.4. Results

2.4.1. Cholesterol coated-surfaces enhance biofilm formation

Previous assays using micro-centrifuge tubes coated with cholesterol showed that in static cultures this surface mimics gallstones and enhances biofilm formation (Crawford et al., 2008). To validate our model in a flow-through system, the capacity of S. Typhimurium to form a biofilm was assessed on glass, cholesterol-coated glass surfaces and glass coated with powdered human gallstones. From the biofilm assays, biofilms formed on cholesterol and powdered gallstones were significantly more robust than those on glass and were similar to one another (Fig. 7A). SEM observations of biofilms on glass and cholesterol coated-surfaces corroborated the results obtained by crystal violet (Fig. 7B). Thus, flow chambers coated with cholesterol represent a good model for mimicking human gallstones. They also allow standardization of the procedures while decreasing variability that occurs when using intact human gallstones.
Figure 7. Cholesterol coated-surfaces enhance biofilm formation. (A) Biofilm formation of wild-type S. Typhimurium determined by the crystal violet staining method after 24 h of flow-through in the presence of bile. Biofilm formation was significantly enhanced on cholesterol-coated surfaces compared with glass surfaces. Similar results were observed when using gallstone powder as a coating surface. Experiments were performed by triplicate and repeated four times. A Student’s t test was used to compare biofilms on glass with the other surfaces (***, p<0.001). (B) SEM representative images of Salmonella biofilms on glass and cholesterol-coated surfaces that corroborate crystal violet results.
2.4.2. Differential gene expression between planktonic versus biofilm cells (± cholesterol-coated surfaces)

To determine if differential gene regulation occurred during biofilm formation on cholesterol-coated surfaces, we performed microarray analysis on biofilm cells propagated on glass or cholesterol-coated glass, or from planktonic cells. Using a 2-fold change cut-off, comparison of planktonic vs biofilms cells grown on glass showed 1100 genes differentially regulated whereas comparison of planktonic vs biofilm cells grown on cholesterol-coated glass showed 935 genes differentially regulated. Appendix A lists the 256 genes that showed a 5 fold-change in at least one of the comparisons. To summarize the most important findings, csgG (factor involved in the production of curli fimbriae) was found to be up-regulated in biofilms on both surfaces (6-fold increase). Additionally, greater than 20-fold up-regulation of the followings operons was observed in biofilms regardless of the presence of cholesterol: yciGFE-katN, tdcGECBA (propionate and amino acid metabolism), eut operon (ethanolamine utilization), pdu operon (propanediol utilization), yjiHGE, hydrogenase genes and PTS permease genes. Further analysis of these genes was not the focus of this study, but these data are important for future work regarding Salmonella biofilm development. Notably, a comparison between both planktonic populations (grown in chambers with a glass surface or cholesterol-coated glass) did not show any genes that were differentially regulated in either replicate. The microarray data associated with this paper can be found at GEO repository (GSE48604).
2.4.3. Type 1 fimbriae structural genes (*fimAICD*) and *ycfR* were specifically up-regulated during biofilm development on cholesterol-coated surfaces

Because we observed increased biofilm formation on cholesterol-coated surfaces 24h post-inoculation, we hypothesized that there was altered bacterial gene regulation when biofilms were formed on these gallstone-mimicking surfaces. To examine this, differential gene expression analysis was performed using microarrays. Interestingly, only 7 genes of *S. Typhimurium* were differentially expressed (all up-regulated) when comparing biofilms on glass vs. biofilms on cholesterol-coated glass (Fig. 8). However only 5 of these genes were activated in both replicates: *fimC* (4-fold), *fimA* (3.9-fold), *fimI* (3.7-fold), *ycfR* (3.4-fold), *fimD* (2.1-fold). Four of these genes (*fimA*, *fimI*, *fimC*, *fimD*) belong to the type 1 fimbriae operon. The function of *ycfR* is unknown in *Salmonella*, but it is a stress-related gene in *E. coli* K-12 (Zhang et al., 2007). *ycfR* was also up-regulated when comparing planktonic vs. biofilms cells on cholesterol, but this was not the case when comparing planktonic cells with biofilms on glass. These results were validated by qRT-PCR that showed up-regulation of *fimC* and *ycfR* during biofilms on cholesterol coated-glass not only after 24 h of flow (microarray conditions) but also after 72 h of flow (Fig. 9).
Figure 8. Type 1 fimbriae genes (*fimAICD*) and *ycfR* were induced during biofilm development on cholesterol-coated surfaces. Scatter plot of differential gene expression between biofilms on glass (X-axis) with biofilms on cholesterol surfaces (Y-axis). Diagonal green line represents the cutoff (2-fold change). Four genes of the Type I fimbriae operon and *ycfR* were up-regulated in both replicates.

Figure 9. Quantitative Real-Time PCR validated the increased expression of *fimC* and *ycfR* during biofilm formation on cholesterol coated-surfaces. Fold change of *fimC* and *ycfR* expression between biofilms on glass vs. biofilms on cholesterol surfaces at 24 and 72 h post-inoculation in flow-through conditions. Experiments were performed by triplicate and repeated four times. Statistical significance determined using Student’s *t* test (*, *p*<0.05; ns, not significant).
2.4.4. Mutations of type 1 fimbriae and ycfR increased biofilm formation on cholesterol-coated surfaces

To determine the consequence of the up-regulation of Type I fimbriae-encoding genes and ycfR in biofilms formed on cholesterol surfaces, we utilized a mutant lacking the structural genes of type 1 fimbriae (fimAICDHF) (Nuccio et al., 2007) and a ycfR mutant in biofilm assays. Interestingly, biofilm formation on cholesterol surfaces was enhanced when compared with that of the wild-type strain at both 24 h and 96 h post-inoculation, as determined by crystal violet assays (Fig. 10). The complemented strains (ΔfimAICDHFPGGE1 and ΔycfRpGGE2) restored the biofilm capacities to wild-type levels (Fig. 10). Confocal imaging demonstrated that these biofilms had more biomass after 24 and 72 h of flow and were thicker (only after 24h of flow) than wild-type biofilms. The complemented strains restored the biomass to wild-type levels (Fig. 11, Appendix B). Morphologically, the wild-type biofilms looked patchy in comparison with the mutants which showed more coverage of cholesterol-coated surfaces. In the case of the ycfR mutant, the bacterial membrane integrity was compromised because red-stained (Live-Dead stain) bacterial cells were frequently observed (Fig. 11, Appendix B).
Figure 10. Type 1 fimbriae and ycfR mutants showed enhanced biofilm formation on cholesterol-coated surfaces in vitro. Biofilm capacity screening of the wild-type, mutants and complemented strains in glass microtiter plates coated with cholesterol in the presence of bile at 24 and 96 h post-inoculation. All strains harbor the empty vector (pWSK29 or pWSK129) or the respective complementation vector (pGGE1 and pGGE2 for fimAICDHF and ycfR, respectively). Biofilm formation was determined by the crystal violet staining method. Experiments were performed in triplicate and repeated three times. Both ∆fimAICDHF and ∆ycfR showed an increased biofilm formation compared to the wild-type at both at 24 and 96 h. A Student’s t test was used to determine significant differences (*, p<0.05, **, p<0.01, ***, p<0.001).
Figure 11. Biofilms from type 1 fimbriae and ycfR mutants are thicker on cholesterol-coated surfaces in vitro. Representative confocal images of biofilms on cholesterol coated-surfaces produced by the wild-type, mutants and complemented strains after 24 h of flow in the presence of bile. Biofilms were stained with live/dead stain (Invitrogen), fixed with 4% paraformaldehyde and observed by confocal microscopy. All strains harbor the empty vector (pWSK29 or pWSK129) or the respective complementation vector (pGGE1 and pGGE2 for fimAICDHF and ycfR, respectively). All images have a magnification of 40x and the y-projection of the image is shown at the right side. All mutants showed thicker biofilms, the average thickness (n=15) is shown in the right corner. ∆ycfR also showed increased cell damage/death (increased red staining). The complemented strains demonstrated a reduction of biofilm thickness and biomass.
2.4.5. Expression of FimA and \textit{ycfR} increased during biofilm maturation in vitro

Type I fimbrial gene expression was increased in biofilms propagated on cholesterol-coated surfaces in comparison with glass surfaces. To elucidate if FimA subunits are actually expressed on the surface of bacterial biofilms on cholesterol, we examined FimA by immunofluorescence microscopy. FimA was expressed in biofilms formed by the wild-type at both 24 and 96 h. At the latest time point, FimA expression was dramatically increased (Fig. 12). These data corroborate the observed transcriptional up-regulation of fimbrial genes in biofilms on cholesterol-coated surfaces. The \textit{ycfR} mutant showed higher expression of FimA in comparison with the wild-type in biofilms on cholesterol coated-surfaces at 24 and 96 h (Fig. 12), while no FimA expression was detected in a \textit{fimAICDHF} mutant. A \textit{csgA} mutant not only formed robust biofilms but also showed similar FimA expression as the wild-type.

Because we lack the resource of anti-YcfR antibodies, spatio-temporal expression of \textit{ycfR} in biofilms was monitored by a wild-type strain harboring a low copy plasmid containing a \textit{ycfR::gfp} fusion. The expression of \textit{ycfR} increased over time with the greatest expression observed at the last time point (72 h) post-inoculation (Fig. 13). This shows that \textit{ycfR} induction is not only a characteristic of early (24 h) biofilms. In addition, \textit{ycfR} expression in the biofilm was not observed to be restricted to a particular site in the biofilm architecture.
Figure 12. FimA expression during biofilm formation on cholesterol-coated surfaces. Representative confocal images of biofilms on cholesterol-coated surfaces showing FimA expression by the wild-type and mutants. After 24 and 96 h of flow in the presence of bile, biofilms were stained with filmTracer™ calcein red-orange biofilm stain (Invitrogen), fixed and incubated with primary antibody to FimA. Secondary antibody is Alexa Fluor® 488 donkey anti-rabbit IgG (Invitrogen; green). All images have a magnification of 40x and the y-projection of the image is shown at the right side.
Figure 13. Expression of \textit{ycfR} increases during biofilm maturation. Representative confocal images of biofilms produced by the wild-type \textit{S. Typhimurium} harboring or not the plasmid pGGE3 (\textit{ycfR}::\textit{gfp}). Biofilms were propagated with flow in the presence of bile for 24 and 72h. \textit{ycfR} expression increased along with biofilm maturation. Biofilms were stained with filmTracer\textsuperscript{TM} calcein red-orange biofilm stain (Invitrogen) to observe non-GFP expressing bacteria. All images have a magnification of 40x and the y-projection of the image is shown at the right side.

2.4.6. Increased curli expression is observed in biofilms of \textit{fimAICDHF} and \textit{ycfR} mutants on cholesterol-coated surfaces

To investigate the increased biofilm formation of type 1 fimbriae and \textit{ycfR} mutants on cholesterol-coated surfaces, we tested commonly reported components of biofilms (DNA, proteins, cellulose) by enzymatic treatment of established biofilms with DNase I (56 units), proteinase (0.53mg) and cellulase (50 units). Only proteinase treatments significantly affected the biofilm formation of type 1 fimbriae and \textit{ycfR} mutants (Fig. 14), indicating the phenotype could be due to proteins expressed on the
surface. Viability assays confirmed that the enzymes did not simply kill the bacteria, indicating that any changes were due solely to effects on the biofilm itself. Considering that curli fimbriae are proteins widely reported to be a component of the EPS of Salmonella biofilms, we examined these surface proteins. Immunofluorescence staining with CsgA antibodies demonstrated that both type 1 fimbriae and ycfR mutants have increased expression of CsgA in vitro (Fig. 15).

2.4.7. Type 1 fimbriae and ycfR mutants colonized Nramp1+/+ mice during early and chronic time points regardless of the presence of gallstones

In order to assess the behavior of type 1 fimbriae and ycfR mutants in vivo, we tested 4 different strains (wild-type, ΔfimAICDHF, ΔycfR and ΔfimAICDHFΔycfR) in our mouse model of chronic infection (129X1/SvJ). These mice (Nramp1+/+) are resistant to Salmonella infection and have been previously used for modeling Salmonella chronic infection and persistence in a host (Crawford et al., 2010b; Monack et al., 2004). As showed previously at our laboratory, the presence of gallstones enhanced colonization of the gallbladder by wild-type S. Typhimurium (Crawford et al., 2010b). Interestingly, both fimAICDHF and ycfR mutants showed more bacteria colonizing the gallbladder and bile, especially at earlier time points (e.g. 7 dpi) but this was not statistically significant compared with the wild-type. Also, the presence of gallstones did not significantly affect colonization in feces, liver, bile or gallbladder (Fig. 16). Thus, type 1 fimbriae and ycfR mutants do not have a virulence defect in our model of chronic infection.
Figure 14. Proteinase treatment of established biofilms drastically affected biofilm formation of the wild-type, type 1 fimbriae and ycfR mutants. Inhibitory properties of DNase I, proteinase and cellulase on 24 h biofilms formed by the wild-type, mutants and complemented strains on cholesterol surfaces (plus bile). Enzymatic treatment of established biofilms was performed for 16 h at 37°C. All strains harbor the empty vector (pWSK29 or pWSK129) or the respective complementation vector (pGGE1 and pGGE2 for fimA/C/D/H/F and ycfR, respectively). Biofilm formation was determined by the crystal violet staining method. Experiments were performed by triplicate and repeated twice. Means between conditions with no treatment and with the respective enzyme treatment were compared by a Student’s t test (*, p<0.05; **, p<0.01; ***, p<0.001).
Figure 15. CsgA expression during biofilm formation on cholesterol-coated surfaces. Representative confocal images of biofilms on cholesterol-coated surfaces showing CsgA expression by the wild-type and mutants. After 24 and 96 h of flow in the presence of bile, biofilms were stained with filmTracer™ calcein red-orange biofilm stain (Invitrogen), fixed and incubated with primary antibody to CsgA. Secondary antibody is Alexa Fluor® 488 donkey anti-rabbit IgG (Invitrogen; green). All images have a magnification of 40x and the y-projection of the image is shown at the right side.
Figure 16. Type 1 fimbriae and ycfR mutants colonized *Nramp1*+/+ mice during early and chronic time points regardless of the presence of gallstones. CFU enumeration of wild-type S. Typhimurium and mutants in the feces, liver and gallbladder at 7, 21 and 60 dpi of *Nramp1*+/+ mice. Although the ycfR and type 1 fimbriae mutants were recovered in higher numbers than the wild-type in the gallbladder at 7 dpi, this difference was not statistically significant by using Student's *t* test (*p* <0.5; nd, not detected.)
2.4.8. In vivo, type 1 fimbriae and ycfR mutants showed different biofilm capacities depending on the time of infection

In vitro observations of increased biofilm formation by type 1 fimbriae and ycfR mutants on cholesterol-coated surfaces were examined in the mouse model of typhoid chronic infection. Although fimAICDHF and ycfR mutants (including a double mutant) colonized the gallstones at early time points (7 dpi), no fimAICDHF mutant bacteria were recovered from the gallstones at 21 and 60 dpi (Fig. 17A). This phenotype is independent of the ability to colonize the gallbladder and bile because bacteria were present at these locations at these later time points (Fig. 16). Although the ycfR mutant also colonized the gallstones at early time points (7 dpi), it was recovered from mouse gallstones in higher numbers than the wild-type through 21 dpi and present, though in significantly lower numbers than the wild-type, at 60 dpi (Fig. 17A). The double mutant interestingly mimicked the ycfR mutant at 21 dpi (increased bacteria) but the fimAICDHF mutant at 60 dpi (no bacteria recovered).

These results were corroborated by SEM imaging of biofilms on mouse gallstones. All strains were observed to form robust biofilms on the surface of cholesterol gallstones at 7 dpi (Fig. 17C). However, at 21 dpi, the type 1 fimbriae mutant did not form any microcolonies or biofilms on gallstones (Fig. 17D). Thus, inconsistent with the in vitro phenotype, type 1 fimbriae mutant did not develop robust biofilms in vivo on cholesterol surfaces for extended times post-infection. At 21 dpi, the ycfR mutant formed more robust biofilms compared with the wild-type (Fig. 17D). Thus, in vivo, the ycfR mutant corroborates its in vitro phenotype of increased biofilm formation on cholesterol
surfaces, but only up to 21 dpi. Morphological differences were noted depending on the strain and the time of infection. While the wild-type biofilms showed compact bacteria-bacteria association, bacteria in biofilms formed by the mutants were less tightly associated (Fig. 17C, D). In the case of the ycfR mutant and the double mutant of type 1 fimbriae and ycfR, a cocobacillus shape was evident in biofilms formed at 7 dpi and defined bacilli in biofilms formed at 21 dpi (Fig. 17C, D).
Figure 17. In vivo colonization and biofilm formation on mouse gallstones of S. Typhimurium wild-type and mutants. (A) CFU enumeration of wild-type S. Typhimurium and mutants on mouse gallstones after 7, 21 and 60 dpi. The type 1 fimbriae mutant was only recovered 7 dpi whereas the ycfR mutant was recovered in every time point (increased at 21 dpi and decreased at 60 dpi relative to wild-type). The double mutant mimicked the ycfR mutant at 21 dpi and the fimAICDHF mutant at 60 dpi. A Student’s t test was used to determine significant differences (*, p<0.05). nd, not detected. (B) SEM micrograph of an uninfected gallstone. (C) SEM micrographs of wild-type and mutant biofilms on gallstones at 7 dpi. No difference in the biofilm forming ability was observed among the strains, though some morphological differences are noted. (D) SEM micrographs of biofilms on gallstones at 21 dpi. The ycfR and the double mutant clearly showed increased biofilm formation whereas the type 1 fimbriae mutant was not found attached to the surface of gallstones. Morphological differences are noted among the strains.
2.5. Discussion

The role of gallstones in maintenance of *Salmonella* chronic carriage has been demonstrated in both the mouse and in humans, as *Salmonella* readily forms biofilms on human gallstones (Crawford et al., 2010b; Prouty and Gunn, 2003; Prouty et al., 2002a). In this study, we identified specific factors regulated during biofilm formation on cholesterol gallstones, and examined the role of the identified genes in vitro and in vivo. Our flow-through assays closely mimicked the gallbladder and gallstone environment by using LB bile (3%) and cholesterol, respectively. We demonstrated that cholesterol-coated surfaces enhanced biofilm formation, thus representing a good model for the in vivo interactions of *Salmonella* with cholesterol gallstones in the gallbladder of chronic carriers.

Microarray data revealed limited genes differentially regulated during biofilm formation on cholesterol in comparison with non-cholesterol coated glass surfaces. Four of these genes belong to the type 1 fimbriae operon and all are part of the *fimAICDHF* cluster that encodes the fimbrial shaft. Type 1 fimbriae are proteinaceous filamentous structures 7-nm thick and up to 3 µm in length. They are present on the surface of many members of the *Enterobacteriaceae* (Duguid et al., 1966; Thorns, 1995). These appendages have been associated with attachment and biofilm formation on abiotic surfaces, HeLa, HEp-2, dendritic cells and small intestine and bladder epithelial cells (Baumler et al., 1996; Boddicker et al., 2002; Guo et al., 2007; Hancox et al., 1997; Ledeboer and Jones, 2005; Pratt and Kolter, 1998; Thankavel et al., 1999). They also have been associated with colonization of the gut mucosa in vivo (Althouse et al., 2003;
van der Velden et al., 1998). Here, we showed that deletion of genes encoding the fimbrial shaft had different phenotypes in vitro and in vivo. In vitro, a fimAICDHF mutant demonstrated increased biofilm formation both on glass and cholesterol-coated surfaces. This increased biofilm formation may be reflective of a compensatory mechanism, as Salmonella possess several different fimbriae types that may be increased in expression when type 1 fimbriae is absent (Ledeboer et al., 2006; Weening et al., 2005). Indeed, we showed that this mutant had increased expression of the curli fimbriae main subunit CsgA during biofilm formation on cholesterol-coated surfaces, perhaps partially explaining the phenotype on these surfaces in vitro. These findings could also explain previous in vitro results that showed a negative role of type 1 fimbriae in biofilms formed on cholesterol-coated surfaces. In one of these studies, over-expression of type 1 fimbriae inhibited the initial stages of Salmonella biofilm formation on cholesterol-coated surfaces but not to glass or plastic (Crawford et al., 2010a). In addition, type 1 fimbriae were not required for biofilms in vitro on human gallstones incubated with bile (Prouty et al., 2002a). This compensatory mechanism could also explain the increased biofilm formation of the wild-type and mutants treated with cellulase. Thus, the absence of a particular EPS component could trigger activation or dys-regulation of biofilm-related pathways.

In vivo, however, the role of type 1 fimbriae in biofilm formation on gallstones was different depending on the time post-infection. We observed type 1 fimbriae mutants adhering to the surface of mouse gallstones relatively early during colonization (7 dpi); however, we did not observe any bacteria attached to gallstones during later times post-infection (21 and 60 dpi.). Interestingly, bacteria were still observed in the liver and
gallbladder, implying that the phenotype is not due to bacteria clearance. Thus, it is likely that during early infection, other fimbriae such as curli can compensate the role of type 1 fimbriae in initial attachment to gallstones. Later, however, type 1 fimbriae appear necessary for permanent adherence/persistence. This is supported by the consistent expression of FimA (the main subunit of the fimbrial shaft) observed during biofilm development in vitro. Together, this suggests that during initial colonization in the gallbladder, *Salmonella* finely modulates the expression of different fimbriae depending on the environment it encounters.

YcfR is a 85 amino acid putative outer membrane protein that has been reported to be involved in resistance to multiple stresses in *E. coli*. Its expression in *E. coli* is induced in the presence of heavy metals, drastic pH changes, heat shock, chlorine and hydrogen peroxide (Egler et al., 2005; Maurer et al., 2005b; Richmond et al., 1999; Wang et al., 2009; Zheng et al., 2001). Also in *E. coli*, ycfR was 12-fold activated in biofilm cells compared to planktonic cells (Ren et al., 2004). YcfR has been shown to have a negative role in *E. coli* biofilm formation only in LB with glucose, likely by decreasing bacterial aggregation and cell surface adhesion as a result of decreased hydrophobicity and increased intracellular indole concentrations (Zhang et al., 2007). Others have reported that YcfR lowers the permeability of the outer membrane to copper (Mermod et al., 2012). The ycfR nucleotide sequence is 81% identical between *Salmonella* and *E. coli*. We observed that ycfR was specifically induced during *S. Typhimurium* biofilm formation on cholesterol-coated surfaces but not on glass. Mutation of ycfR resulted in increased biofilm formation on cholesterol-coated glass and on gallstones in vivo (only up to 21 dpi) suggesting that ycfR is highly expressed perhaps to
inhibit biofilm formation on this specific surface. We showed that ycfR expression increased as the biofilms matured, further suggesting a role in biofilm regulation. The increased biofilm formation of this mutant may be partially explained by its increased expression of both FimA and CsgA during biofilm formation on cholesterol-coated surfaces. In addition, the absence of ycfR dramatically altered the bacterial membrane integrity (propidium iodide stained also alive cells) likely by increasing cell permeability.

Thus, considering the effect of the ycfR mutation in E. coli (induction of acid, osmotic, oxidative, and heat stress response genes), we believe that during biofilm formation on cholesterol gallstones, ycfR is induced as a response to the stress generated during biofilm formation on cholesterol due to bacterial membrane damage that has been reported to occur (Snoussi et al., 2012; Webb et al., 2003). The stress response during biofilm formation is a well-reported phenomena in E. coli and Salmonella (Beloin et al., 2004; Mangalappalli-Illathu and Korber, 2006; White et al., 2010). The stress and ycfR induction can alter the cell surface hydrophobic properties resulting in inhibition of biofilm maturation/stability. On glass or epithelial cells, the initiating stress may not the same and thus ycfR may not need in these circumstances. Thus, YcfR represents an intrinsic specific inhibitor of the biofilm process on cholesterol gallstones but not to other surfaces such glass, perhaps due to the hydrophobic nature of cholesterol.

In conclusion, Type I fimbriae genes and ycfR were specifically up-regulated in biofilms formed on cholesterol-coated surfaces. Although mutants of these genes showed increased biofilm formation in vitro, only ycfR mutant showed this phenotype in
vivo but only up to 21dpi. In contrast, type 1 fimbriae mutant did not persistently attach to the surface of gallstones in vivo. Thus, type 1 fimbriae seem important for maintaining chronic carriage on gallstones.
Chapter 3: The Gallbladder Epithelium as a Niche for Chronic \textit{Salmonella} Carriage

3.1. Abstract

Although typhoid fever has been intensively studied, chronic typhoid carriage still represents a problem for the transmission and persistence of the disease in areas of endemicity. This chronic state is highly associated with the presence of gallstones in the gallbladder of infected carriers upon which \textit{Salmonella} can form robust biofilms. However, we hypothesize that in addition to gallstones, the gallbladder epithelium aids in the establishment/maintenance of chronic carriage. In this work, we present evidence of the role of the gallbladder epithelium in chronic carriage by a mechanism involving invasion, intracellular persistence and biofilm formation. \textit{Salmonella} was able to adhere to and invade polarized gallbladder epithelial cells apically in the absence and presence of bile in a SPI-1-dependent manner. Intracellular replication of \textit{Salmonella} was also evident at 12 and 24 hours post-invasion. A flow-through system revealed that \textit{Salmonella} is able to adhere to and form extensive bacterial foci on gallbladder epithelial cell as soon as 12 hours post-inoculation. In vivo experiments using a chronic mouse model of typhoid carriage showed invasion and damage of the gallbladder epithelium and lamina propria up to 2 months after \textit{Salmonella} infection, with an abundant presence of macrophages, a relative absence of neutrophils, and extrusion of infected epithelial
cells. Additionally, microcolonies of *Salmonella* cells were evident on the surface of the mouse gallbladder epithelia up to 21 days post-infection. These data reveal a second potential mechanism, intracellular persistence and/or bacterial aggregation in/on the gallbladder epithelium with luminal cell extrusion, for *Salmonella* maintenance in the gallbladder.

### 3.2. Introduction

Typhoid fever, caused primarily by *S. Typhi*, is a human-specific disease that is responsible for an estimated 21 million new infections per year, resulting in approximately 200,000 deaths annually worldwide (Crump et al., 2004). After contaminated water or food has been ingested, bacteria cross the intestinal epithelial barrier, are phagocytosed by macrophages, and spread systematically, producing acute disease (Vazquez-Torres et al., 1999). However, 3 to 5% of the population infected with *S. Typhi* becomes chronic carriers, with the gallbladder being a site of persistence (Levine et al., 1982; Merselis et al., 1964). These carriers serve as a critical reservoir for further spread of the disease through bacterial shedding in feces (Vogelsang and Boe, 1948). Chronic infections can persist for decades, and although highly contagious, they are typically asymptomatic (Dongol et al., 2012; Shpargel et al., 1985; Sinnott and Teall, 1987). In addition, chronic carriers have an approximately 8-fold greater risk of developing gallbladder carcinoma than non-carriers (Caygill et al., 1994; Shukla et al., 2000). This impact on human health combined with the high incidence of typhoid fever in many parts of the world highlights the importance of understanding the mechanisms involved in the typhoid carriage.
Clinical observations of carriers with respect to the difficulty of eradicating the bacterium with antibiotics and its confinement to an organ with shedding from this site, coupled with evidence for long-term invasion of the immune response, are consistent with a biofilm-related disease (Gonzalez-Escobedo et al., 2011). Biofilms are communities of microorganisms that adhere to each other and to inert or live substrates. They are typically encased in an extracellular matrix and are associated with many chronic and acute human infections (Costerton et al., 1999; Monds and O'Toole, 2009). The carrier state is highly related to the presence of gallstones (Dongol et al., 2012; Karaki and Matsubara, 1984; Schioler et al., 1983). Indeed, studies in our laboratory have shown that Salmonella can form biofilms on the surface of cholesterol gallstones in vitro and in vivo in the gallbladder of mice and human carriers (Crawford et al., 2010b; Prouty et al., 2002a). These reservoirs of bacteria have been demonstrated to be a mechanism of persistence and chronic colonization. To date, removal of the gallbladder (cholecystectomy) is the most common treatment for chronic typhoid carriers and gallbladder abnormalities; however, this treatment is both costly and invasive. Therefore, alternative treatments are needed to eradicate this carriage state.

Despite the fact that gallstones are associated with chronic carriage of Salmonella, patients without gallstones have also been reported as chronic carriers (Sharma et al., 2007; Vaishnavi et al., 2005b). Previous studies have shown that Salmonella can invade mouse gallbladder epithelial cells (Menendez et al., 2009) and can also form biofilms on the surface of various epithelial cells, including HEp-2 cells and chicken intestinal tissue (Ledeboer and Jones, 2005). However, it is unknown if
Salmonella is able to persist in or on the gallbladder epithelium during long-term carriage. Here, we hypothesized that Salmonella also persists in the gallbladder by invading gallbladder epithelial cells and/or forming biofilms on the surface of gallbladder epithelia. The elucidation of these mechanisms is crucial for the development of new therapies to eradicate typhoid carriage, thus blocking the transmission of typhoid fever.

In this study, we not only demonstrate that Salmonella can persist in and on the gallbladder epithelium by intracellular residence and by forming epithelial cell surface biofilms, but we also show that the gallbladder epithelium responds to chronic Salmonella infection in a manner that differs from acute models of infection.

3.3. Materials and Methods

3.3.1. Bacterial strains and growth conditions

Wild-type and derivatives of S. Typhimurium ATCC14028 (JSG210) were used in these studies. The entire list of strains used is given in Table 3. All cultures were grown in Luria-Bertani (LB) broth at 37°C with aeration at 225 rpm. Antibiotics, when needed, were used at the following concentrations: chloramphenicol, 25 µg/mL; kanamycin, 45 µg/mL; ampicillin, 100 µg/mL. Construction of complementation strains was not successful for SPI-1, csgA and yciE mutants. However, to verify their phenotypes were not the result of an unlinked gene, mutations were instead back-transduced by P22HTint into a wild-type background.
Table 3. Bacterial strains and plasmids used in Chapter 3

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>JSG210</td>
<td>Wild-type S. Typhimurium ATCC14028</td>
<td>ATCC</td>
</tr>
<tr>
<td>JSG3391</td>
<td>ΔSPI-1</td>
<td>Gift of Dr. Steve Libby</td>
</tr>
<tr>
<td>JSG3392</td>
<td>ΔfimAICDHF</td>
<td>(Nuccio et al., 2007)</td>
</tr>
<tr>
<td>JSG3132</td>
<td>ΔcsgA</td>
<td>(Santiviago et al., 2009)</td>
</tr>
<tr>
<td>JSG3125</td>
<td>ΔyciE</td>
<td>(Santiviago et al., 2009)</td>
</tr>
<tr>
<td>JSG1149</td>
<td>Wild-type S. Typhimurium ATCC14028 pFPV25.1</td>
<td>(Prouty et al., 2002a)</td>
</tr>
<tr>
<td>JSG3535</td>
<td>ΔSPI-1 pFPV25.1</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3534</td>
<td>ΔfimAICDHF pFPV25.1</td>
<td>This study</td>
</tr>
<tr>
<td>JSG3658</td>
<td>ΔcsgA pFPV25.1</td>
<td>This study</td>
</tr>
<tr>
<td>JSG 3536</td>
<td>ΔyciE pFPV25.1</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>pFPV25.1</td>
<td>(Valdivia et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>GFP constitutive vector</td>
<td></td>
</tr>
</tbody>
</table>

3.3.2. Growth of gallbladder epithelial cells

All tissue culture assays were performed with dog gallbladder epithelial cells (DGEC) (donation of Sum P. Lee lab, University of Washington). Cells were grown at 37°C and 5% CO₂ using Dulbecco’s modified Eagle’s medium with a high concentration of glucose (DMEM-high glucose) (Invitrogen, CA) supplemented with 10% fetal bovine serum (FBS), 100 µg/mL of streptomycin, 100 IU/mL of penicillin and 1x of non-essential amino acids.
3.3.3. Attachment, invasion and intracellular survival assays

DGEC were seeded into 24mm collagen-coated Transwell®-COL inserts (Corning, MA). Polarization and differentiation of the cells were verified by trans-epithelial electrical resistance (TEER) (> 700 Ω cm$^{-2}$) using a Millicell-Electric Resistance System (ERS) (Millipore, MA) and by observation under transmission electron microscopy (TEM). This process generally took 8-10 days. One day before infection, the medium was exchanged with serum-free medium with or without 0.3% ox bile (Sigma, MO). This bile concentration was determined based on tolerance assays in which the TEER and viability of DGEC were not altered. Infections of epithelial cells were performed apically for 2 h at a multiplicity of infection (MOI) of 100. Cells were then incubated with serum-free medium with or without gentamicin (50 µg/mL) for 30 min, washed two times with 1X PBS, and then lysed with 0.1% Triton-X100 for 10 min at 37°C and 5% CO$_2$. Lysates were then plated on LB medium for bacterial enumeration. The assays were performed in triplicate and repeated four times. The number of attached bacteria was obtained by subtraction of total bacteria recovered from wells not treated from the number in wells treated with gentamicin. Intracellular survival assays were performed in triplicate and repeated two times, and included 6, 12, and 24 h post-infection time points. For these assays, gentamicin (10 µg/µL) was used throughout the course of the experiment.
3.3.4. Monitoring of attachment and invasion by microscopy

DGEC were grown as above, and infections were performed using a MOI of 100 or 10 for 2 and 9 h time points, respectively. The 2 h post-inoculation time point allows observation of bacteria initially interacting/invading the epithelium whereas, the 9 h time point is primarily for observation of biofilm/microcolony formation on the epithelium. Cells were then fixed and observed by confocal microscopy, scanning electron microscopy (SEM), and TEM according to the protocols described below.

3.3.5. Flow-through system assays

Flow-through plastic chambers (Stovall Life Science Inc., IA) coated with poly-L-lysine (Sigma) were inoculated with 2x10^6 DGEC and allowed to differentiate and form a confluent monolayer at 37°C and 5% CO₂ (4 days total). DGEC were then inoculated with S. Typhimurium harboring pFPV25.1 (constitutively expressing GFP, Table 3) at a MOI of 100 and allowed to adhere for 1 h. A flow-through system was created whereby DMEM-high glucose supplemented with 10% FBS, 100 µL/mL ampicillin, and 1X non-essential amino acids with or without 0.3% bile was applied to the infected DGEC for 12 h at 37°C and 5% CO₂ at a flow rate of 270 µL/min. Cells were then observed with confocal microscopy or SEM as described below. Biofilm and planktonic cells were also quantified by CFU enumeration. Experiments were performed three times.
3.3.6. Mouse infections

Considering that typhoid fever is a human-specific disease, modeling of the disease in mice is performed by using S. Typhimurium since it produces a typhoid-fever like disease. Female 129X/SvJ mice (Nramp1+/+) (Jackson laboratories, ME) were fed a normal diet (Harlan laboratories, IN) or a normal diet supplemented with 1% cholesterol (Sigma) and 0.5% cholic acid (Sigma) for 9 weeks. Mice fed with the cholesterol diet developed gallstones in their gallbladders. Mice were then inoculated intraperitoneally with approximately 10^4 bacteria and sacrificed at 7, 14, 21 and 60 dpi. Feces, liver, gallbladder, bile and gallstones were collected, homogenized and/or diluted for bacteria enumeration and plating on Salmonella-Shigella agar (Becton Dickenson, NJ). The gallbladder tissues of some mice were also analyzed by histology, immunohistochemistry (IHC) and electron microscopy. Data were gathered and combined from two independent experiments.

3.3.7. Immunohistochemistry

Mouse tissues were fixed with 10% buffered formalin phosphate (Fisher Scientific, MA) for 72 h. Tissues were processed by routine methods, paraffin embedded, cut in sections (thickness, 4 µM), and stained for IHC using the avidin-biotin complex (ABC) method of the OSU Comparative Pathology and Mouse Phenotyping Shared Resource. The primary antibodies included the macrophage marker anti-F4/80 (1/200 dilution), the neutrophil marker anti-Ly6G (1/200 dilution), and anti-Salmonella LPS (1/500 dilution). All antibodies were from Novus Biologicals (Littleton, CO). Briefly,
sections were treated with target retrieval solution (for use with F4/80 and Ly6G antibodies) or proteinase K (for use with *Salmonella* antibodies) for 5 min. Sections were treated with hydrogen peroxide for 10 min, rinsed, blocked with serum-free protein for 10 min, and incubated with primary antibody for 30 min. They were again rinsed and then incubated with mouse-adsorbed biotinylated rabbit anti-mouse antibody (1/200 dilution for F4/80 and Ly6G; 1/1,000 dilution for anti-*Salmonella* antibody) for 30 min. Samples were incubated with Vector RTU ABC Elite complex for 30 min, rinsed, incubated with chromogen (diaminobenzidine [DAB]) for 5 min, counterstained with hematoxylin, rinsed, treated with 1% ammonium hydroxide, dehydrated in ethanol, cleared in xylene and mounted on coverslips. Histological scoring of inflammatory cell recruitment was performed as follows: 0, absent; 1, mild; 2, mild/moderate; 3, moderate; 4, moderate to marked; and 5, marked.

### 3.3.8. Confocal microscopy

DGEC (grown on Transwells or flow chambers) were stained before infection with 5 M Cell Tracker™ Red CMPTX (Invitrogen) according to the manufacturer’s directions. After the respective post-infection time points, DGEC were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, for 15 min at RT, rinsed, and observed using an Olympus Fluoview FV10.1 instrument.
3.3.9. Electron microscopy

For SEM observations, samples were fixed overnight at 4°C with 2.5% glutaraldehyde in 0.1 M phosphate buffer-0.1 M sucrose (pH 7.4), rinsed twice with 0.1 M phosphate buffer, and dehydrated by addition of solutions of ethanol in a graded series, as follows: 35%, 50%, 70%, 80%, 95%, 100%. Samples were chemically dried with consecutive washes of 25%, 50%, 75%, and 100% hexamethyldisilazane (Ted Pella, CA). Samples were then dried overnight in a fume hood, mounted on aluminum stubs, and sputter-coated with gold for observation using a FEI Nova NanoSEM at the OSU Campus Microscopy and Imaging Facility (CMIF).

For TEM observations, transwells or mouse tissues were fixed overnight at 4°C with 1.5% paraformaldehyde-1.5% glutaraldehyde in 0.1 M cacodylate buffer, rinsed, post-fixed for 1 h with 1% osmium tetroxide in 0.1 M cacodylate buffer, rinsed again, and en bloc stained in 2% uranyl acetate with 10% ethanol. Consecutive washes with increasing concentrations of ethanol were performed as described above before the application of propylene oxide. Samples were then embedded in Eponate resin and polymerized at 60°C for 16 to 24 h. Sectioning was performed at 80nm on a Reichert Ultracut E ultramicrotome before observation using a FEI Technai G2 Spirit transmission electron microscope (CMIF).
3.4. Results

3.4.1. S. Typhimurium bacteria attach to the surface of polarized DGEC resembling microcolonies

To model the in vivo host-pathogen interface and examine the initial interaction of *Salmonella* with the gallbladder epithelium, bacteria were added to polarized monolayers of DGEC. *Salmonella* was able to attach to the surface of DEGC regardless of exposure to bile (Fig. 18A). Interestingly, during this attachment, bacteria-bacteria interactions were observed as early as 2 h post-infection. These early interactions resemble the formation of microcolonies, widely reported as the initial process of biofilm formation (Fig. 18B).

3.4.2. S. Typhimurium invades polarized gallbladder epithelial cells in vitro

*Salmonella* can invade and replicate within epithelial cells, typically utilizing a T3SS1 to gain entry into these non-phagocytic cells. S. Typhimurium was able to apically invade in vitro polarized gallbladder epithelial cells that had or had not been previously exposed to 0.3% ox bile. However, the exposure of DGEC to bile before infection significantly decreased bacteria invasion (Fig. 18A). TEM imaging demonstrated that epithelial cells exposed to bile possessed mucus granules on their apical sides that were absent in DGEC untreated with bile (Fig. 19). S. Typhimurium was intracellularly contained in a SCV evident at 2h post-infection (Fig. 19). Invasion of DGEC was SPI-1 dependent since a ∆SPI-1 strain was significantly impaired in the
ability to invade these cells (Fig. 20). This was also supported by the SEM observation of membrane ruffling after 2 h of infection (Fig. 21) which is a mechanism known to be mediated by SPI-1 (Finlay et al., 1991).

3.4.3. S. Typhimurium can replicate intracellularly in DGEC regardless of previous exposure of the epithelium to bile

Replication of bacteria that had invaded the epithelium would aid in creating an intracellular niche. Intracellular survival assays showed that S. Typhimurium replicates inside DGEC, thus representing a good in vitro model to study host-pathogen interactions in the gallbladder. Up to 12 h post-infection, intracellular replication was significantly different between DGEC that were untreated and those that were exposed to bile, with bile-exposed DGEC harboring fewer bacteria. However, this difference was not significant at 24 h post-infection (Fig. 22A). Monitoring of the intracellular behavior of S. Typhimurium by TEM revealed that at 9 h post-infection, *Salmonella* bacteria were observed actively replicating inside a SCV. In accordance with the CFU results, the bacterial burden appeared higher in the absence of bile. Interestingly, when these cells were highly loaded with bacteria, some epithelial cells were observed to be translocated to the extracellular environment, in agreement with a process observed by other investigators (Knodler et al., 2010) (Fig. 22B). Impaired microvillus architecture was also evident.
Figure 18. *S. Typhimurium* attaches and invades into/on polarized DGEC in vitro.

(A) Apical attachment and invasion (mean plus standard deviation) of *S. Typhimurium* bacteria on/into DGEC previously exposed or not exposed to 0.3% bile. Invasion after 2.5 h is shown as percentage of the inoculum. Exposure of the epithelial cells to bile significantly decreased bacterial invasion but not attachment. Means between conditions with (+) and without (-) bile treatment were compared by a Student’s *t* test (**, *p* < 0.01; ns, not significant). Experiments were performed in triplicate and repeated four times. (B) *S. Typhimurium* attaches to the surface of polarized DGEC, resembling microcolonies. Representative SEM images of uninfected and infected DGEC in the absence and presence of 0.3% bile are shown. Note the differentiation when cells were exposed to bile. Cells were fixed and processed at 2 h post-infection.
Figure 19. Exposure of DGEC with bile decreases invasion by S. Typhimurium. Representative TEM images showing uninfected or S. Typhimurium-infected DGEC in the absence or presence of bile are shown. Solid arrows, *Salmonella*-containing vacuoles; dashed arrows, mucus granules that were only present in DGEC exposed to bile. Cells were fixed and processed at 2 h post-infection.
Figure 20. SPI-1, curli fimbriae and yciE but not type 1 fimbriae mutants decrease invasion into DGEC in vitro. Invasion relative to the wild-type (wt) level (mean plus standard deviation) of *S.* Typhimurium mutants into DGEC exposed to 0.3% bile at 2.5 h post-infection. Means of wild-type and each mutant values were compared by a Student’s *t* test (**, *p* < 0.01, ***, *p* < 0.001). Assays were performed in triplicate on two separate occasions.

Figure 21. Representative SEM image of *S.* Typhimurium invading DGEC in the absence of bile. Note the membrane ruffling which is a SPI-1 mediated mechanism for *Salmonella* invasion into epithelial cells.
Figure 22. *S. Typhimurium* can replicate intracellularly in DGEC. (A) Intracellular replication of *S. Typhimurium* in DGEC cultured with or without bile measured as CFU/mL. There is statistical significance between no bile and bile treatments at all time points except 24 h post-infection (*, p<0.05, by a Student's t test). ns, not detected. (B) Representative TEM images showing infected DGEC 9 h post-infection with *S. Typhimurium* in the absence or presence of bile. In the absence of bile, bacteria were seen replicating inside SCV in higher numbers than in cells exposed to bile. Cell/cell debris being released to the extracellular environment was also observed.
3.4.4. *S.* Typhimurium forms extensive cellular aggregations/biofilms on the surface of DGEC

In addition to bacterial invasion of the gallbladder epithelium, the growth and biofilm formation on the surface of the epithelium would aid in chronic colonization. Flow-through experiments verified the ability of *Salmonella* to adhere to and form extensive bacterial foci on the surface of DGEC at 12 h post-inoculation (Fig. 23). Extra-polymeric substance production comprising the biofilm matrix was evident, as observed by electron microscopy. Biofilm formation occurred regardless of the presence of bile in the tissue culture medium during the length of the experiment. However, CFU enumeration revealed that in the presence of bile, the number of bacteria recovered from biofilms was increased ($8 \times 10^8 \pm 2.1 \times 10^7$ CFU/mL) compared to the number from biofilms on DGEC in the absence of bile ($8.5 \times 10^8 \pm 1.4 \times 10^8$ CFU/mL). This difference was statistically significant by Student’s *t* test ($p<0.05$).

![Figure 23. *S.* Typhimurium forms extensive cellular aggregations/biofilms on the surface of DGEC. Representative SEM images of *S.* Typhimurium biofilms on DGEC 12 h post-inoculation in a flow chamber in the absence or presence of 0.3 % bile are shown. The presence of bile modestly increased biofilm formation.](image-url)
3.4.5. Curli and yciE affect invasion into and biofilm formation on DGEC

Considering the observed phenotypes, we tested bacterial factors already reported or highly suggested to affect *Salmonella* invasion and biofilm formation. A ∆SPI-1 mutant was used as an invasion-deficient strain, and strains lacking type 1 fimbriae structural subunits (∆fimAICDHF strain), the curli fimbriae main subunit (∆csgA strain), and the stress-related gene yciE (shown to be the highest up-regulated gene during biofilm formation on glass and cholesterol surfaces, Appendix A) were tested as potential strains defective in attachment/biofilm formation. Experiments were performed with DGEC previously exposed to 0.3% bile.

In addition to their effect on invasion, a ∆SPI-1 strain also showed impaired attachment compared with that of the wild-type (Fig. 24). However, ∆SPI-1 bacteria could form bacterial foci after 9 h of infection (Fig. 25). Thus, impaired invasion did not result in increased microcolony/biofilm formation on DGEC. The ∆fimAICDHF strain did not show a significant difference in its invasion or biofilm formation abilities compared with the wild-type (Fig. 20 and 25) but the attachment at 2 h post-inoculation was increased (Fig. 24). However, the ∆csgA and ∆yciE strains showed no difference in initial attachment (Fig. 24) but demonstrated a significant decrease in invasion into DGEC and impaired microcolony formation on DGEC (Fig. 20 and 25). Thus, SPI-1 and type I fimbriae are not required for biofilm formation on DGEC, but csgA and yciE are important for both invasion and biofilm formation.
Figure 24. SPI-1 is also important for attachment to the surface of DGEC. Representative images are shown of DGEC uninfected or infected with wild-type S. Typhimurium and mutants at 2 h post-infection in the presence of bile. All bacterial strains harbor the plasmid pFPV25.1 constitutively expressing GFP. DGEC are stained with Cell Tracker™ Red CMPTX. All images have a magnification of 60x.
Figure 25. Curli and yciE affect biofilm formation on DGEC. Representative images are shown of DGEC uninfected or infected with wild-type S. Typhimurium and mutants at 9 h post-infection in the presence of bile. All bacterial strains harbor the plasmid pFPV25.1 constitutively expressing GFP. DGEC are stained with Cell Tracker™ Red CMPTX. All images have a magnification of 60x.
3.4.6. In vivo assays recapitulate the invasion, intracellular survival, extrusion, and microcolony formation shown in vitro

By using wild-type (Nramp1<sup>+/+</sup>, where Nramp1 is a natural-resistance associated macrophage protein) mice with gallstones, we have developed a mouse model of chronic gallbladder infection that more appropriately mimics human infection than the commonly used acute infection mouse models (Crawford et al., 2010b). In the current study, infecting bacteria could be recovered from feces, liver, spleen, pancreas, gallbladder tissue, and gallbladder bile. Bacterial loads in the liver, gallbladder tissue, and gallbladder bile were always higher in mice harboring gallstones, especially at later time points such as 21 and 60 dpi (Fig. 26A). Bacteria were also isolated from gallstones at all time points, with higher numbers at later time points such as 21 and 60 dpi (Fig. 26B) but this difference was not significant (analysis of variance test) from amounts at earlier time points (7 and 14 dpi).

In vivo experiments using our chronic model of typhoid carriage corroborated the in vitro findings of invasion and intracellular replication of <i>Salmonella</i> into gallbladder epithelial cells (Fig. 27). In addition to the enumeration of <i>Salmonella</i> in mouse tissues, we were interested in monitoring the precise localization of <i>Salmonella</i> in the gallbladder as well as the gallbladder response during the transition from acute to chronic infection (e.g., 7 to 14 dpi) and during chronic stage (e.g., 21 to 60 dpi) in the absence and presence of gallstones. For this purpose, we monitored <i>Salmonella</i> infection at 7, 14, 21, and 60 dpi by TEM and IHC. TEM imaging showed damage of the gallbladder epithelium in infected mice up to 21 dpi, clearly affecting the microvilli and the integrity of the tight
junctions (Fig. 27). The presence of gallstones *per se* also caused these phenotypes, but to a lesser extent. In contrast to in vitro observations, the SCV were always seen containing only one bacterium. Macrophages were actively recruited and seen engulfing *Salmonella* bacteria (Fig. 27). IHC detection of *Salmonella* also showed the bacterium colonizing the lamina propria (Fig. 28).

As observed in vitro, at 21 dpi, extrusion of infected epithelial cells was also evident, further suggesting an alternative mechanism for gallbladder persistence and transmission (Fig. 27). At 60 dpi, we were still able to observe SCV in the gallbladder epithelium, which corroborates the intracellular persistence of *Salmonella* in this niche (Fig. 27). Interestingly, the gallbladder epithelia tended to recover at 60 dpi which was evident by recovery of tight junction and microvillus normal architecture (Fig. 27). In the absence of gallstones, this was concomitant with low bacterial burden (Fig. 26 and 28). In contrast, in the presence of gallstones, recovery of the epithelium was also evident but along with a moderate bacterial burden (Fig. 26 and 28).

Bacteria were seen extracellularly attached to the surface of the gallbladder epithelium forming microcolonies at 21 dpi. This phenotype was not seen earlier, and it was present only in mice harboring gallstones (Fig. 29). Bacteria were still seen at the surface of the epithelium after 2 months of infection although they were sometimes difficult to observe because of extensive mucus production due to the presence of gallstones and/or the inflammatory response.
Figure 26. The presence of gallstones enhances *Salmonella* colonization at 60 days post-infection. (A) *S. Typhimurium* enumeration in different mouse sites at 7, 14, 21, and 60 days post-infection (dpi). Although all sites showed more bacteria in mice harboring gallstones, the difference was only significant by Student’s t test at 60 dpi in the liver, gallbladder tissue and bile (*, p<0.05). (B) *S. Typhimurium* enumeration on gallstones at different dpi. Bacteria were recovered in higher numbers from gallstones at later time points (21 and 60 dpi.), but this difference was not significant (analysis of variance test) from amounts at earlier time points (7 and 14 dpi). Data were gathered from two different experiments. ns, not significant. nd, not detected.
Figure 27. In vivo assays recapitulate invasion, intracellular survival and extrusion. Representatives TEM images of the mouse gallbladder epithelium in the absence and the presence of gallstones are shown. Damage of the gallbladder epithelium is evident at all days post-infection (dpi) including in uninfected mice with gallstones. SCV are indicated with solid arrows and were only observed containing one bacterium. Dashed arrows indicate cholesterol crystals. Macrophages (M) were sometimes observed harboring SCV. Neutrophils (N) were actively seen engulfing cholesterol crystals. The release of epithelial cells was commonly seen, especially at 21 dpi. Regeneration of tight junctions was evident especially by 60 dpi. However, *Salmonella* was still seen intracellularly in mice harboring gallstones.
Figure 28. In the absence of gallstones, inflammation of the gallbladder is mostly macrophage related during infection. Images show immunohistochemical analysis of representative sections of the gallbladder epithelium from uninfected or infected mice at 7, 14, and 60 dpi by using anti-Salmonella LPS, anti-F4/80 (macrophage marker), and anti-Ly6G (neutrophil marker) antibodies. Brown areas are positives. The recruitment of macrophages was the hallmark throughout the course of infection.
Figure 29. *S. Typhimurium* can form microcolonies on the surface of the mouse gallbladder epithelium. Representatives SEM images of uninfected/infected mouse gallbladder epithelium in the absence or presence of gallstones are shown. *Salmonella* microcolonies were observed on the epithelium only of mice harboring gallstones and only at 21 dpi.
3.4.7. Inflammation of the gallbladder is primarily macrophage related during chronic salmonellosis

The decreased *Salmonella* colonization in the gallbladder of mice without gallstones led us to the hypothesis that an effective immune response is different in mice with and without gallstones. Thus, as an initial indicator, we monitored by IHC the presence of macrophages and neutrophils. Examination of mouse gallbladder tissues showed that in the absence of gallstones, macrophages and not neutrophils comprise the primary cellular infiltrate throughout the course of infection (Fig. 28 and Table 4). These patterns seem to be specific for gallbladder tissue since histopathological analysis of liver sections revealed that neutrophils and macrophages were actively present at all time points, with histological scoring (median) for macrophage recruitment as follows: at 7 days (3), 14 days (3), 21 days (2), 60 days (3). Scoring for neutrophil recruitment was as follows: at 7 days (3), 14 days (2), 21 days (2) and 60 days (2). These data provide evidence that in the absence of gallstones, the immune response against chronic *Salmonella* in the gallbladder is mostly macrophage related.

In the presence of gallstones, macrophages and neutrophils were also observed at all time points including in both infected and uninfected mice (likely due solely to the presence of gallstones). Although the recruitment of macrophages was observed at all time points, neutrophils were highly recruited at 21 and 60 dpi. (Figure 30, Table 4). In addition, histopathology analysis also showed epithelial hyperplasia and hyalinosis. These data provide evidence that when gallstones are present, chronic inflammation is
mediated by both macrophages and neutrophils, which along with epithelial changes (e.g. hyperplasia) may contribute to *Salmonella* carriage.

**Table 4. Histological scoring of macrophage and neutrophil recruitment in mouse gallbladder tissue at different days post-infection**

<table>
<thead>
<tr>
<th>Time point (dpi)</th>
<th>- Gallstones</th>
<th>+ Gallstones</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Macrophages</td>
<td>Neutrophils</td>
</tr>
<tr>
<td>Uninfected</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>21</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*The absence (-) or presence (+) of gallstones is indicated.*
Figure 30. In the presence of gallstones, inflammation of the gallbladder is both macrophage and neutrophil related. Images show immunohistochemical analysis of representative sections of the gallbladder epithelium from uninfected or infected mice at 7, 14, and 60 dpi by using anti-*Salmonella* LPS, anti-F4/80 (macrophage marker), and anti-Ly6G (neutrophil marker) antibodies. Brown areas are positives. Hyperplasia is evident in most of the sections.
3.5. Discussion

These studies describe the in vitro and in vivo interactions of S. Typhimurium with gallbladder epithelial cells. These assays uncovered two potential (non-gallstone-associated) mechanisms by which Salmonella can establish a persistent infection in the gallbladder during chronic carriage: biofilm formation on the epithelium and intracellular persistence within the epithelium, coupled with cell extrusion (Fig. 31). We demonstrated that in vitro, Salmonella infection of polarized gallbladder epithelial cells corroborates in vivo mouse model findings and thus represents a good model for host-pathogen interaction studies. These DGEC have been intensively used for physiological studies, which supported their use as a model for human gallbladder epithelial cells (Choi et al., 1999; Klinkspoor et al., 1995; Klinkspoor et al., 1998; Kuver et al., 1994; Malik et al., 1997; Yoshida et al., 2000). The percentage of S. Typhimurium invasion obtained in this study is relatively lower than results of other studies with polarized epithelial cells such as the dog kidney epithelial cell line (MDCK) and T84 cells, where approximately 4 to 5% of the inoculum could invade after 25 min of infection for MDCK cells or after 1 to 2 h of infection for T84 cells (Bishop et al., 2008; Raffatellu et al., 2005; Wagner et al., 2011).

It is noteworthy that DGEC were exposed to bile during at least part of all assays to mimic closely the gallbladder environment. It is known that bile exposure increases mucus production, which contributes to bile sterility (Begley et al., 2005; Klinkspoor et al., 1998). We observed that DGEC exposed to bile had increased number of apical mucus granules, which could explain the decreased invasion we obtained when these cells were pre-treated with bile. It has also been shown that LPS increases mucus
production (Zen et al., 2002). Thus, mucus induction represents an innate response of the gallbladder for initial protection against pathogens. Regarding the pathogen, bile is an environmental signal that alters expression of many Salmonella genes (Prouty et al., 2004a; Prouty et al., 2004b), including those that decrease invasion of epithelial cells by down-regulating SPI-1 gene expression (Prouty and Gunn, 2000). However, bile was removed from the DGEI prior to bacterial addition in invasion assays, so SPI-1 regulation is likely not directly responsible for the observed invasion defects seen in Fig. 18. Bile it is also known to increase biofilm formation (Crawford et al., 2008). This was also observed in our in vitro studies of biofilm formation on DGEI, although not dramatically, where cells exposed to bile had more bacteria attached to their surface after 12 h of flow-through.

Corroborating in vitro observations, the mouse model demonstrated that Salmonella was able to attach to, invade, and replicate in the gallbladder epithelium. We demonstrated by TEM and IHC analysis that Salmonella is able to persist in the gallbladder epithelium up to 2 months post-infection. The presence of gallstones increased the bacterial loads in the liver, gallbladder, and bile. This is in accordance with previous findings in our laboratory (Crawford et al., 2010b) where we monitored Salmonella carriage for a shorter time period. Although we did not detect Salmonella by CFU counts in the absence of gallstones at 60 dpi, we were able to detect the bacteria in the epithelial layer by TEM and IHC. It is unclear why there was this discrepancy. In addition, mice without gallstones still possessed bacteria in the liver, thus putting the gallbladder at risk for transient or permanent colonization by Salmonella. In support of
this observation, it was common to recover bacteria from bile but not from the
gallbladder tissue in the same mouse.

We also observed *Salmonella* -containing cells being extruded from the
epithelium both in vitro and in vivo, further implicating the gallbladder epithelium as a
facilitator of the innate defense response. This phenomenon has been reported to be a
pyroptosis-related, a caspase-1 event triggered by flagellin of intracellular *Salmonella*.
Signaling events result in pro-inflammatory cell death releasing bacteria to the
extracellular environment (de Jong et al., 2012; Knodler et al., 2010). We believe that the
liberation of *Salmonella* to the gallbladder lumen may play an important role in chronic
carriage and dissemination by releasing bacteria that can re-colonize the gallbladder
epithelium or gallstones or can be shed into the intestine for release.

Biofilm formation on gallstone surfaces has been shown to be the primary
mechanism of *Salmonella* persistence in the gallbladder (Crawford et al., 2010b). Here,
we showed both in vitro and in vivo that *Salmonella* is also able to form cell aggregates
that appear to be biofilms on the gallbladder epithelium. It is unknown if in vivo these
bacteria come from the gallstone surfaces or from the intracellular niche to colonize the
epithelium, but biofilm formation on the gallbladder wall might represent an adaptation
occurring during the chronic state of the disease. In support of this, there are
epidemiological data showing that human chronic typhoid carriers from areas of
endemicity possess bacterial aggregates attached to the surface of the gallbladder
epithelium (S. Baker, personal communication). In the absence of gallstones in vivo,
however, epithelial surface biofilms do not seem to be the primary mechanism of
persistence, as we only detected biofilms on the epithelial surface of infected mice harboring gallstones in our model. This suggests that in the absence of gallstones, invasion of the gallbladder epithelium may be the primary mechanism of persistence. In the presence of gallstones, all three mechanisms (gallstone biofilms, epithelial cell biofilms and cell invasion with cell sloughing) may be active (Fig. 31).

Considering the potential role of invasion and biofilm formation in perpetuating *Salmonella* carriage in the gallbladder, we studied bacterial factors that could be involved in these phenotypes. We hypothesized that invasion-deficient mutants may build up on the epithelial cell surface, resulting in an increase in microcolonies/biofilms. We found that this was not the case because an invasion-deficient mutant (∆SPI-1) attached and formed microcolonies on the surface of the epithelium in vitro (although in a lesser extent than the wild-type). The decreased attachment of ∆SPI-1 strain could be explained by the coordinated regulation of SPI-1 and SPI-4. SPI-4 encodes the giant adhesin SiiE which is secreted by T3SS1 and has been shown to mediate attachment to epithelial cells (Gerlach et al., 2007a). However, SPI-1 mutants still firmly attach to the surface of the mouse cecum epithelia in resistant mouse strains (Sivula et al., 2008).

In the case of type 1 fimbriae, they are reported to be important for biofilm formation on Hep-2 tissue culture cells and in vivo on mouse and chicken intestinal epithelial cells (Boddicker et al., 2002; Dwyer et al., 2011; Ledeboer et al., 2006). In contrast to this, it has been previously noted that different fimbrial types are required to colonize specific organs and that type 1 fimbriae are not required for persistence in mouse intestines (Norris et al., 1998; Weening et al., 2005). Here, we show that genes
encoding the structural components of the fimbriae shaft (fimAICDHF) are not required for attachment/biofilm on or invasion into gallbladder epithelial cells. In addition, results in our laboratory have also showed that a hyperfimbriate strain does not demonstrate increased attachment to gallstone surfaces (Crawford et al., 2010a). We have also shown, in our model of chronic carriage, that type 1 fimbriae mutants colonize the mouse gallbladder in a manner similar to the wild-type strain (Figure 16). Thus, while this could be a niche specific phenotype, our evidence points to the lack of a role for type 1 fimbriae in gallbladder epithelial cell biofilms.

Curli fibers are widely reported to be an important component of the extra-polymeric substance that comprises the Salmonella biofilm matrix upon interaction with surfaces, including epithelial cells (Austin et al., 1998; Jain and Chen, 2007; Jonas et al., 2007; Ledeboer et al., 2006; Romling et al., 1998; Sukupolvi et al., 1997b). Although our results show that strains lacking csgA (encoding the main subunit of curli) are still able to attach to DGEC, they were not able to form microcolonies/biofilms on the surface of the gallbladder epithelium. This phenotype is similar to the csgA mutant phenotype observed on chicken intestinal tissue (Ledeboer and Jones, 2005). It is also in accordance with studies on plant surfaces where csgA and csgB genes were not required for initial attachment but for extra-polymeric substance production that characterizes mature biofilms (Barak et al., 2005).

The yciE gene belongs to the operon yciGFE-katN, which is RpoS dependent, and it is up-regulated in the presence of bile (Beraud et al., 2010; Ibanez-Ruiz et al., 2000; Prouty et al., 2004b; Robbe-Saule et al., 2001). In addition, this gene was found to
be ~50-fold up-regulated in biofilms on glass and cholesterol surfaces in a bile-containing medium (Appendix A). The precise physiological role of most of the genes within this operon is unknown, but since $yciE$ encodes a putative cytoplasmic protein and is up-regulated during biofilm formation in the presence of bile, we hypothesized that this gene might be important for DGEC attachment/biofilms and/or invasion. In fact, a $yciE$ mutant was highly impaired in not only biofilm formation but also invasion. This suggests that $yciE$ may be important in allowing bacteria to endure a bile-containing environment, and thus its absence could impair colonization of the gallbladder epithelium. Supporting this, we have previously shown that RpoS is necessary for biofilm formation on glass and gallstone surfaces (Prouty and Gunn, 2003).

During gallbladder colonization, *Salmonella* has to overcome the gallbladder innate immune response, including the observed recruitment of macrophages and neutrophils in our in vivo model. In acute models of typhoid fever, it has been demonstrated that neutrophils are actively recruited to the gallbladder lumen as a result of *Salmonella* invasion (Menendez et al., 2009). Here, in our model of chronic carriage, we show that the less bactericidal macrophages, not neutrophils, comprise the primary infiltrating cells in the gallbladder tissue, which may facilitate the development of chronic carriage. However, in the presence of gallstones, in addition to macrophages, neutrophils were also highly recruited at 21 and 60 dpi, which suggests an exacerbated inflammatory environment that, along with hyperplasia of the gallbladder epithelium, can also contribute to *Salmonella* persistence.
Figure 31. Current model of typhoid chronic carriage. a. Electron micrograph of S. Typhi in a biofilm on the surface of a human gallstone. b. S. Typhi probably gains access to the gallbladder during the acute phase of infection and initially attaches to gallstone surfaces through a specific interaction between flagellin and cholesterol. This initial binding could be facilitated by the outer-membrane protein C (OmpC). Subsequent attachment of bacteria is aided by the presence of, but not motility mediated by, flagella. On cholesterol, biofilm formation is dependent on the presence of exopolysaccharide (green), probably including the O antigen capsule. Detachment of bacteria from the biofilm would allow entry into the intestine via bile, followed by shedding in the feces. C. An alternative strategy by which S. Typhi persists in the gallbladder is through invasion of gallbladder epithelial cells. In this model, invasive bacteria replicate intracellularly, and shedding could occur wherein gallbladder epithelial cells containing S. Typhi would be extruded to the lumen, and released bacteria could infect new cells or be shed into the intestine via bile. In addition, S. Typhi could also form biofilms on the surface of the gallbladder epithelium.
Based on these observations, we propose a model for chronic carriage in the absence or presence of gallstones. In the absence of gallstones and during acute stages of infection, *Salmonella* reaches the gallbladder from the liver and encounters mucus, slgA, and other innate immune components present in bile and the gallbladder epithelium that likely decrease bacterial load. Bile will limit *Salmonella* invasion into the epithelium, but the bacteria may escape bile when it is within the mucus layer surrounding the epithelium. Some bacteria will invade and, once inside the gallbladder epithelium, actively replicate and in the long-term remain intracellular with a relatively low bacterial load, decreased active inflammation, and intact epithelia. Some cells with replicating bacteria will likely be extruded via pyroptosis. Other bacteria reaching the gallbladder lumen may attach to the surface or be cleared by phagocytic cells or shed into the intestine. Although biofilm formation on the gallbladder epithelium does not appear to be a frequent phenomenon in our in vivo model, evidence from human carriers does suggest that bacterial foci on the epithelial cell surface occur (S. Baker, personal communication).

In addition to the factors mentioned above, in the presence of gallstones *Salmonella* encounters an already inflamed gallbladder (mediated by both neutrophils and macrophages). Also as a result of the presence of gallstones, there is increased epithelial proliferation (hyperplasia), mucus production, and altered bile composition (Chang et al., 1999; Finzi et al., 2006; Mathur et al., 2012b). This environment may benefit *Salmonella* not only in colonization of the compromised gallbladder epithelium but also in attachment to gallstone surfaces, resulting in prolonged carriage of bacteria in all gallbladder niches.
They are still many questions to answer, including how *Salmonella* is able to persist in the gallbladder in the presence of an immune response while the host remains primarily asymptomatic. It is likely that both the bacterium and host respond to the milieu and one another, resulting in a permissive environment responsible for this chronic infection. The findings of future studies related to this unique host-pathogen interface in the gallbladder will provide more insights about how the chronic carriage occurs and persists as well as new treatment/preventive strategies.
Chapter 4: Histopathological Analysis of *Salmonella* Chronic Carriage in the Hepatopancreatobiliary System

4.1. Abstract

*Salmonella* Typhi asymptomatic chronic carriage represents a challenge for the diagnosis and prevention of typhoid fever in endemic areas. Such carriers are thought to be reservoirs for further spread of the disease. Gallbladder carriage has been demonstrated to be mediated by biofilm formation on gallstones and by intracellular persistence in the gallbladder tissue of mice. In addition, both gallstones and chronic carriage have been associated with chronic inflammation and the development of gallbladder carcinoma. However, the pathogenic relationship between typhoid carriage and the development of pre-malignant and/or malignant lesions in the hepatopancreatobiliary system as well as the host-pathogen interactions occurring during chronic carriage remains unclear. In this study, we monitored *Salmonella* hepatobiliary colonization and localization, and the histopathological features of chronic carriage up to 1 year post-infection. We demonstrated by immunohistochemistry that chronic carriage in the gallbladder occurred in a subset of the mice population. In the liver, *Salmonella* colonization was especially observed in mice harboring gallstones. Histopathological analysis demonstrated that chronic cholecystitis and hepatitis ranging
from mild to severe were present in infected mice regardless of the presence of gallstones. At most time points, the mere presence of gallstones caused more gallbladder inflammation than chronic salmonellosis, whereas in the liver and pancreas, the pattern was exactly the opposite. Biliary epithelial hyperplasia was observed in the gallbladder of mice with gallstones (uninfected or infected). However, only *Salmonella* chronic inflammation induced pre-malignant lesions (atypical hyperplasia and metaplasia) of the gallbladder and pancreas epithelium. Atypical hyperplasia/dysplasia was only observed in the gallbladder of infected mice at 3 months post-infection. Mucinous metaplasia was observed in the pancreas of infected mice at 3 and 6 months post-infection. These histopathological features reveal for the first time the inflammation patterns and epithelial changes occurring in the chronically infected mouse hepatobiliary system in the absence or presence of gallstones. This study has implications regarding the role of *Salmonella* chronic infection and inflammation in the development of pre-malignant lesions in the epithelium of the gallbladder and pancreas that could lead to oncogenesis.

4.2. Introduction

Typhoid or enteric fever, caused primarily by *S. Typhi*, is a human systemic disease that is responsible for an estimated of 21 million new infections per year resulting in approximately 200,000 deaths worldwide (Crump et al., 2004). It is an important health problem in developing countries and poses a significant risk to travelers. After ingestion through contaminated water or food, bacteria cross the intestinal epithelial barrier, migrate into the MLN, replicate in the reticulo-endothelial
system and spread systemically producing significant inflammation and acute disease (Crum, 2003; Jepson and Clark, 2001; Vazquez-Torres et al., 1999; Vladoianu et al., 1990; Wain et al., 1998). During this systemic infection, S. Typhi can reach the gallbladder from the liver and establish an acute infection with inflammation (cholecystitis) or chronically persist in this organ. As clinical evidence, inflammation of the gallbladder and bile ducts as well as sonographic gallbladder abnormalities have been reported in acute and chronic typhoid fever patients (Cohen et al., 1987; Mateen et al., 2006; Shetty and Broome, 1998; Vaishnavi et al., 2005b; Vogelsang and Boe, 1948).

Although hepatomegaly is encountered in approximately 30-50% of typhoid patients with or without clinical manifestations (Crum, 2003; Ramachandran et al., 1974), severe hepatic involvement concomitant with acute hepatitis is seen in 1-26% of typhoid fever patients (Pramoolsinsap and Viranuvatti, 1998) and the mortality rate due to typhoid hepatitis is reported to be between 20-33% (Khosla, 1990; Rovito and Bonanno, 1982).

It is estimated that between 3-5% of typhoid fever patients become chronic carriers with the gallbladder being the primary site of carriage (Cohen et al., 1987; Levine et al., 1982; Merselis et al., 1964). Because S. Typhi is a human specific pathogen, these carriers serve as a critical reservoir for further spread of the disease through bacterial shedding in feces, which is a sporadic and intermittent event (Bhan et al., 2005; Vogelsang and Boe, 1948). Chronic infections can persist for decades and although highly contagious, they are typically asymptomatic, making identification of carriers within a population difficult (Shpargel et al., 1985; Sinnott and Teall, 1987).
Particularly in areas of high endemicity, the carrier state has been highly associated with pre-existing hepatobiliary disease including cholelithiasis (presence of gallstones in the gallbladder), biliary obstruction, intrahepatic cholestasis due to Caroli’s disease, biliary cirrhosis, hepatic haematoma, echinococcal cysts and amoebic abscesses (Cohen et al., 1987; Gosbell et al., 1995). Approximately 80-90% of chronically infected carriers have gallstones (Karaki and Matsubara, 1984; Lai et al., 1992; Schioler et al., 1983; Scott, 1971; Vaishnavi et al., 2005a). We have shown that *Salmonella* can form biofilms on the surface of cholesterol gallstones in the gallbladder of mice and human carriers, and this biofilm formation has been demonstrated to be a mechanism of persistence and chronic colonization in the gallbladder (Crawford et al., 2010b). The biofilm state can alter the host-pathogen interaction and is often associated with a reduction of the host inflammatory response that has been referred to as a “silent chronic inflammation” (Cappelli et al., 2005).

In addition to the complications related to the acute phase of the disease, especially in the ileum and lymph organs (Everest et al., 2001), typhoid carriage complications include chronic hepatitis, acute or chronic cholecystitis, cholangitis, chronic diarrhea and rarely, pancreatitis (Crum, 2003; Vaishnavi et al., 2005a). However, the development of gallbladder cancer is the most severe complication associated with chronic carriage. Gallbladder cancer is the fifth most common malignancy of the GI tract and the most common and aggressive type among the biliary tract malignancies (Miller and Jarnagin, 2008). Unfortunately, because of the delayed clinical presentation relative to pathologic progression, most of the gallbladder carcinomas are in an advanced stage when diagnosed and metastasis to the liver and regional lymph nodes are common.
Lazcano-Ponce et al., 2001). Although infrequent in most Western countries, such cancers are highly prevalent in Chile, India and Pakistan (Miller and Jarnagin, 2008; Randi et al., 2006) where there is a coincident increase in gallstone disease and typhoid fever (Dutta et al., 2000; Randi et al., 2006).

Chronic carriers have an approximately 8-14 fold increased risk of developing gallbladder carcinoma and approximately 150-fold increased risk of developing hepatobiliary carcinoma than non-carriers (Caygill et al., 1994; Chang et al., 1992; Csendes et al., 1975; Dutta et al., 2000; el-Zayadi et al., 1991; Nath et al., 1997; Nath et al., 2008; Shukla et al., 2000). Moreover, among patients with gallstones, the chronic typhoid carrier state was shown to be the primary independent risk factor for the development of gallbladder cancer (Dutta et al., 2000). Mortality in typhoid carriers as a result of hepatobiliary carcinomas has been reported to be between 3-6% (Welton et al., 1979). It has been hypothesized that bacterial degradation of bile salts and chronic cholecystitis related to gallstones promotes gallbladder carcinomas (Kumar, 2006). In addition to gallbladder carcinomas, typhoid carrier patients have showed increased risk of pancreas carcinoma (Caygill et al., 1994; Vaishnavi et al., 2005a; Vladoianu et al., 1990).

Despite the many complications that result from chronic carriage, the host and the bacterial contributions that create the environment that allows this chronic infection and subsequent cancers are unknown. We hypothesized that chronic inflammation as a result of Salmonella infection in the hepatopancreatobiliary system leads to premalignant transformation of the gallbladder and pancreas epithelium including
hyperplasia, metaplasia and dysplasia. Hyperplasia is characterized by an increase in the number of cells and it can be physiological or pathological. Metaplasia is the reversible substitution of one type cell for another whereas dysplasia is the alteration of the shape, size and organization of the tissue (Haschek et al., 2010). These epithelial changes, especially metaplasia and dysplasia can be associated with chronic inflammation and they could progress to neoplasia (tumor) development (Haschek et al., 2010). It is believed that metaplasia serves as the precursor of carcinogenetic transformation, progressing into dysplasia, and culminating in invasive gallbladder carcinoma (Monga, 2011).

Understanding the factors that allow bacterial colonization and carriage, as well as the establishment of a mouse model to examine the contribution of carriage to oncogenesis, could lead to the development of new therapies for patients suffering from Salmonella-mediated chronic gallbladder disease, decreasing the incidence of typhoid fever and gallbladder cancer. In this study, we demonstrate that Salmonella can not only persist in the gallbladder and liver of chronic infected mice but also cause a chronic inflammation that can vary from mild to severe. In addition, chronically infected mice showed epithelial changes such as atypical hyperplasia/dysplasia and metaplasia in the gallbladder and pancreas as early as 3 months post-infection. Although gallstone disease and subsequent chronic inflammation are known risk factors for the development of human gallbladder and pancreas cancer (Randi et al., 2006; Sosnik and Sosnik, 2005; Tazuma and Kajiyama, 2001), this is the first prospective study that describes the inflammation patterns and epithelial changes occurring during Salmonella chronic carriage in the absence or presence of gallstones.
4.3. Materials and Methods

4.3.1. Mice infections and bacteria enumeration

Because S. Typhi is a human-restricted pathogen, in vivo studies of S. Typhi pathogenesis typically involve a mouse model of infection using S. Typhimurium. The pathological features of the course of mouse infection with S. Typhimurium are similar to those of human infection with S. Typhi (Santos et al., 2001). We used a murine model of typhoid chronic infection using six-eight week old 129X1/SvJ mice (Jackson Lab., ME) that are fed a lithogenic diet (1% cholesterol and 0.5% cholic acid; Sigma) for six-nine weeks resulting in gallbladder cholesterol gallstones. We previously developed this animal model to study Salmonella chronic infections in the gallbladder that corroborated parallel human studies (Crawford et al., 2010b). S. Typhimurium has been demonstrated to persist in the tissues of this mouse strain up to 1 year post-infection (Monack et al., 2004), but does not typically result in a lethal infection. This is due in part to the presence of a wild-type copy of the gene encoding the natural resistance-associated macrophage protein 1 (Nramp1 or Slc11a1). NRAMP1 is a crucial factor in controlling the replication of intracellular bacteria (Forbes and Gros, 2001).

Female mice (n=160) were fed with a lithogenic diet (n=80) or normal mouse food (n=80) for nine weeks and then inoculated intraperitoneally with $10^4$ S. Typhimurium or left uninfected as controls. Mice were sacrificed at 3, 6, 9 and 12 months post-infection (mpi). Thus, each time point comprised four groups (n=10 per group): uninfected mice fed a normal diet (group 1), uninfected mice fed a lithogenic diet (group 2),
Salmonella infected mice fed a normal diet (group 3) and Salmonella infected mice fed a lithogenic diet (group 4). Spleen and feces from all mice of each group (n=10) and liver, pancreas, gallbladder tissue, bile, gallstones from three mice of each group were homogenized and/or diluted for bacterial enumeration on Salmonella -Shigella agar (Difco™).

4.3.2. Histopathology of the hepatopancreatobiliary system of chronically infected mice

Gallbladder, bile ducts, pancreas and liver tissues (from the 7 mice that remained from each group) were fixed with 10% neutral buffered formalin phosphate (Fisher Scientific, MA) for 72 h. Fixed tissues from groups 1 and 2 (n=3 each) and groups 3 and 4 (n=5 each) were randomly selected for further evaluation. Tissues were processed by routine methods and embedded in paraffin wax. Sections (4 µm) were stained with hematoxylin and eosin (HE), and evaluated with an Olympus BX45 light microscope with attached DP25 digital camera (B & B Microscopes Limited, Pittsburg, PA) by a veterinary pathologist certified by the American College of Veterinary Pathologists (ACVP). Inflammatory lesions were scored according to a modified grading scheme (Fadl et al., 2005) (Table 5).

4.3.3. Immunohistochemistry

Paraffined sections from above were deparaffined with xylene, cut in sections and stained for IHC using the avidin-biotin complex (ABC) method of the OSU
Comparative Pathology and Mouse Phenotyping Shared Resource. Briefly, sections were treated with proteinase K for 5 min, rinsed, treated with hydrogen peroxide for 10 min, rinsed again and blocked with serum-free protein for 10 min, incubated for 30 min with anti-*Salmonella* LPS antibody (1/500 dilution) (Novus Biologicals, CO), rinsed again and then incubated with mouse-adsorbed biotinylated rabbit anti-mouse antibody (1/1,000 dilution) for 30 min. Samples were incubated with Vector RTU ABC Elite complex for 30 min, rinsed, incubated with chromogen (DAB) for 5 min, counterstained with hematoxylin, rinsed, treated with 1% ammonium hydroxide, dehydrated in ethanol, cleared in xylene and mounted on coverslips.

**Table 5. Histopathological grading system for hepatopancreatobiliary lesions.**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Focal to multifocal portal/lobular/perivascular lymphohistiocytic + neutrophilic infiltrates; no necrosis</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Multifocal to widespread portal/lobular lymphohistiocytic ± neutrophilic inflammation with necrosis of individual hepatocytes</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Multifocal to widespread portal/lobular lymphohistiocytic ± neutrophilic inflammation with focally extensive hepatocellular necrosis</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Multifocal to widespread portal/lobular lymphohistiocytic ± neutrophilic inflammation with multifocal coalescing areas of hepatocellular necrosis</td>
</tr>
<tr>
<td>Gallbladder</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Focal-multifocal serosal/adventitial neutrophilic ± lymphohistiocytic inflammation.</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Widespread transmural neutrophilic ± lymphohistiocytic inflammation.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Necrosuppurative inflammation of lumen ± wall; with or without transmural neutrophilic ± lymphohistiocytic inflammation.</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Focal-multifocal lymphocytic aggregates limited to the interstitium/around blood vessels or within adjacent adipose</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Focal-multifocal neutrophilic ± lymphohistiocytic inflammation within the interstitium or duct; with or without lymphocytic aggregates in the interstitium/around blood vessels/within adipose</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Necrosuppurative inflammation obliterating ducts/vessels; with or without lymphocytic aggregates in the interstitium/around blood vessels/within adipose</td>
</tr>
</tbody>
</table>

*modified from Fadl et al.*
4.4. Results

4.4.1. *Salmonella* spp. was recovered at all time points from the feces and spleen of mice without gallstones, but inconsistently observed in other tissues/fluids

In previous work by our laboratory, we consistently observed *Salmonella* in all of the tissues/fluids examined in this study up to at least 21 dpi (Crawford et al., 2010b). However, *Salmonella* long-term colonization in the hepatopancreatobililiary system in the absence or presence of gallstones has not been previously determined. In order to observe the bacterial burden up to 1 year of infection, bacterial enumeration in feces and in the spleen was performed for all mice. *Salmonella* was recovered from the feces of all infected mice at all time points. The spleen was colonized at all time points except in infected mice with gallstones at 9 and 12 mpi (Fig. 32). CFU enumeration from the rest of the organs was performed in only three mice per group. In the liver, bacteria were recovered in some time points but only from mice harboring gallstones (Fig. 32). *Salmonella* was infrequently recovered from the pancreas, gallbladder, bile and gallstones (Fig. 32). It is important to note that the CFU limit of detection was 100 CFU/mL.
4.4.2. *Salmonella* spp. was detected by immunohistochemistry in the liver, gallbladder and pancreas of some infected mice

CFU enumeration has been shown not to be the optimal method for *Salmonella* detection during chronic carriage (Gonzalez-Escobedo and Gunn, 2013). Thus, to better determine the number and location of *Salmonella* in the hepatopancreatobiliary system, we detected *Salmonella* by IHC using anti-*Salmonella* LPS antibody. In the gallbladder, *Salmonella* LPS staining was detected in some of the infected mice without gallstones at 6 mpi (75% of mice tested), 9 mpi (25%) and 12 mpi (25%) whereas in infected mice with gallstones it was detected at 3 mpi (50%), 6 mpi (25%) 9 mpi (25%) and 12 mpi (25%). The localization of *Salmonella* LPS staining in the gallbladder was confined to the lumen, mucosa or muscularis (Fig. 33). In the liver, *Salmonella* LPS staining was detected in infected mice without gallstones at 3 mpi and 6 mpi (100%), 9 mpi (75%) and 12 mpi (50%) whereas in infected mice with gallstones it was detected at 3mpi (100%) and at 6 mpi, 9mpi and 12mpi (75%). In the liver, *Salmonella* LPS staining was observed intracellularly (in macrophages and hepatocytes). Interestingly, in the hepatocytes, *Salmonella* LPS was mostly detected eccentrically shifted to one side of the cytoplasm, presumably closer to the bile canaliculi invaginations (Fig. 34). In the pancreas, *Salmonella* LPS staining was also detected in infected mice without gallstones at 3 mpi (50%), 6 mpi (100%), 9 mpi (50%) and 12 mpi (75%) and in infected mice with gallstones at 3 mpi (50%), 6 mpi (100%), 9 mpi (50%) and 12 mpi (25%). *Salmonella* LPS staining was mostly detected in the interstitium of the exocrine pancreas (Fig. 35). The uninfected controls did not show positive reaction with the antibody.
Figure 32. *Salmonella* detection in organs and bodily fluids of chronically infected mice up to 1 year post-infection. Bacterial CFU enumeration of *Salmonella* at various times post-infection in the absence or presence of gallstones. Limit of detection was 100 CFU/mL. No statistically significant difference was observed between infected mice with or without gallstones at any time point (Student's *t* test). nd, not detected.
Figure 33. *Salmonella* LPS staining was frequently detected in the gallbladder of infected mice regardless of the presence of gallstones. Representative IHC images of mouse gallbladder tissue at 6 and 12 months post-infection using an anti-*Salmonella* LPS antibody. Such bacterial staining occurs at time points in which no CFU were detected from the gallbladder of cage mates. Black arrows show *Salmonella* LPS staining in the muscularis (Panel C and D), in the epithelium (Panel E) or in the lumen (Panel F).
Figure 34. *Salmonella* LPS staining was detected in the liver of infected mice regardless of the presence of gallstones. Representative IHC images of mouse liver sections at 3 or 9 months post-infection using an anti-*Salmonella* LPS antibody. Panel C shows a typhoid nodule. Black arrows indicate the presence of *Salmonella* inside macrophages or Kupffer cells (Panels C and F) or intracellularly in the hepatocytes (Panels D, E).
Figure 35. *Salmonella* LPS staining was detected in the pancreas of infected mice regardless of the presence of gallstones. Representative IHC images of mouse pancreas sections at 3 and 9 months post-infection using an anti-*Salmonella* LPS antibody. *Salmonella* was detected in the interstitium around acini or ductular cells (Panels D, E and F) and rarely intracellularly in acinar cells (Panel C).
4.4.3. Chronic cholecystitis and hepatitis are the hallmark during *Salmonella* carriage

Although chronic inflammation in the liver and gallbladder has been reported in typhoid carriers, the histopathological features of carriage in the absence or presence of gallstones has not been previously assessed. Thus, we monitored the inflammation patterns of the hepatopancreatobiliary system during chronic carriage by histopathology. In the gallbladder, inflammation was widespread and transmural with lymphocytes, macrophages (histiocytes), plasma cells and fewer neutrophils. In general, gallstones in the absence of bacteria showed more inflammation than bacteria in the absence of gallstones. However, at 9 mpi, three infected mice without gallstones showed necro-suppurative inflammation with ulceration of the biliary epithelium (Fig. 36). Mice with gallstones (± *Salmonella*) did not show any significant difference in their inflammation scores, suggesting that, when gallstones are present, *Salmonella* did not exacerbate the inflammation levels. Additionally, inflammation levels between infected mice ± gallstones were statistically significant at 6 mpi whereas inflammation levels between uninfected mice with gallstones and infected mice without gallstones were statistically significant at 6 and 12 mpi, with inflammation higher in the former. This suggests that at least in these time points of chronic carriage, *Salmonella* do not intensify the inflammatory response in the gallbladder above the level of inflammation resulting from the mere presence of gallstones.

In the liver, the highest inflammation scores were present in infected mice without gallstones at 6 mpi and in infected mice with gallstones at 3 mpi (Fig. 37A). Inflammatory
cells were distributed randomly within the lobule and/or in portal areas. Inflammation was often associated with necrosis of individual hepatocytes or large confluent areas of hepatocellular necrosis (Fig. 37B). In contrast to the gallbladder, uninfected mice without gallstones only showed mild degrees of inflammation. Classical histiocytic (macrophage-mediated) inflammation consistent with typhoid nodules was noted in the liver lobules of many infected mice regardless of the presence of gallstones (Fig. 34C).

In the pancreas, the inflammation patterns were similar to the liver with the highest inflammation also observed in infected mice without gallstones at 6mpi and in infected mice with gallstones at 3mpi (Fig. 38A). However, after 6 mpi, the inflammation was milder in all infected mice. Inflammatory cells were mostly observed within the interstitium of the exocrine pancreas between acini. In more severe cases, inflammation obliterated large ducts and was characterized by necrotic debris (centrally), lymphoplasmacytic aggregates and mucous metaplasia at the periphery (Fig. 38B).

Chronic vascular thrombosis with dystrophic mineralization was only observed in infected mice (with or without gallstones). In the gallbladder, these vascular changes predominantly occurred in infected mice with gallstones at 3 mpi (80%). In the liver, chronic thrombosis was only observed at 9 mpi in infected mice without gallstones (40%) and in infected mice with gallstones (100%) (Fig. 39). In the pancreas, chronic arteritis was also only observed in infected mice but in less than 40% of mice per time point.
Figure 36. Chronic cholecystitis occurs as a result of both gallstone disease and *Salmonella carriage*. (A) Inflammation grades in the gallbladder at all time points post-infection (± bacteria, ± gallstones). Means of each time point values were compared by a Student’s *t* test. No statistical significant difference was observed between values of each time point (*p*<0.5) (B) Representative images of each group at 3 and 9 mpi. B1 has an inflammation score of 0; B2, B3, B5 and B6 have a inflammation score of 2; whereas B4 has a score of 3. Gallstones and/or cholesterol crystals are shown (noted as *). Hyperplasia was only evident in mice harboring gallstones and with concurrent epithelial hyalinosis (B2, B5 and B6). Black arrows indicate scattered inflammatory cells within the lamina propria and muscularis, they were mainly composed of lymphocytes, plasma cells and fewer neutrophils.
Figure 37. Chronic hepatitis is exacerbated in infected mice at 3 and 6 months post-infection. (A) Inflammation grades in the liver at all time points post-infection (± bacteria, ± gallstones). Means of each time point values were compared by a Student’s t test (*, p<0.05; ***, p<0.001). (B) Representative images of each group at 3 or 6 mpi. Inflammation scores are as follows: B1 (0); B2 (1); B3 and B6 (2); B4 and B5 (4). Infected mice have scattered inflammatory cells distributed randomly within the lobule (B3-B5) and/or in portal areas (B6) composed of neutrophils, lymphocytes, plasma cells and histiocytes. Inflammation was often associated with necrosis of individual hepatocytes (B3) or large confluent areas of hepatocellular necrosis (B4-B5) (noted as N).
Figure 38. Chronic pancreatitis is exacerbated in infected mice at 3 and 6 months post-infection. (A) Inflammation grades in the liver at all time points post-infection (± bacteria, ± gallstones). Means of each time point values were compared by a Student’s t test (*, p<0.05; ***, p<0.001). (B) Representative images of each group at 3 and 9 mpi. Inflammation scores are as follows: B1 (0); B2 (1); B3 (2); B4, B5 and B6 (3). Inflammatory cells composed of scattered neutrophils, lymphocytes and plasma cells were occasionally distributed within the interstitium of the exocrine pancreas between acini (circle). In more severe cases, inflammation was centered upon and typically obliterated large ducts (B4-B6) and was characterized by necrotic debris (*) centrally, and lymphoplasmacytic aggregates and mucous metaplasia (black arrows) peripherally.
Figure 39. Dystrophic mineralization of venous fibrin thrombi was only present in the gallbladder and liver of infected mice with gallstones. Representative images of H&E stained mouse liver (A, C) and gallbladder adjacent to the liver (B). Note the mineralized thrombi in Panels B and C surrounded by portal aggregates of lymphocytes, plasma cells and neutrophils.

4.4.4. Biliary epithelial hyperplasia was observed as a result of gallstone disease and Salmonella carriage

In addition to inflammation, we also monitored hyperplastic changes in the hepatopancreatobiliary organs. Biliary epithelial hyperplasia concurrent with hyalinosis was observed in the liver and gallbladder. In the gallbladder, biliary hyperplasia was only observed in mice with gallstones (uninfected or infected) in more than 80% of the mice (Fig 36B). In the liver, hyperplasia was only observed in infected mice with gallstones at 9 mpi (80%). Pancreatic tissue did not show signs of hyperplasia or hyalinosis.
4.4.5. Atypical hyperplasia/dysplasia was only observed in the gallbladder of infected mice regardless of the presence of gallstones

Pre-malignant lesions such as dysplasia can predict possible complications such as neoplasia of the gallbladder epithelium which has been associated with chronic *Salmonella* carriage. Atypical hyperplasia in the absence of marked inflammation and/or epithelial hyalinosis was observed in the gallbladder of infected mice at 3 mpi, but not at later time points (Fig. 40). None of these pre-malignant changes were observed in the uninfected animals (with or without gallstones).

![Atypical hyperplasia](image)

**Figure 40. Atypical hyperplasia was observed in the gallbladder of infected mice regardless of the presence of gallstones.** Representative images of H&E stained mouse gallbladder, 3 months post-inoculation. Black arrows indicated tall columnar biliary epithelial cells with large crowded nuclei and loss of polarity.
4.4.6. Pancreatic mucinous metaplasia was only evident in infected mice regardless of the presence of gallstones

In addition to dysplasia, other pre-malignant lesions, such as metaplasia, were also monitored in *Salmonella* chronic carriage. Pancreas histopathology revealed mucinous metaplasia (with columnar epithelium overtly producing mucus). This mucinous metaplasia was associated with exocrine pancreatic acini at the periphery of marked inflammation. Neither the gallbladder nor the liver showed metaplasia at any time point (Fig. 41).

Figure 41. Pancreatic mucinous metaplasia was evident in infected mice regardless of the presence of gallstones. Representative images of H&E stained mouse pancreatic tissue, 6 months post-inoculation. Black arrows indicate mucinous metaplasia associated with exocrine pancreatic acini at the periphery of marked inflammation.
4.5. Discussion

Although there have been many recent advances in clinical and molecular research regarding *Salmonella* spp., including work in endemic regions, chronic typhoid carriage remains an understudied area. In this study, we monitored *Salmonella* chronic colonization and the histopathological changes occurring in the hepatopancreatobiliary system up to 1 year post-infection. This is the first prospective study that compares *Salmonella* chronic carriage features among infected individuals with or without gallstones. Cholelithiasis is a predisposing factor for *Salmonella* carriage and chronic inflammation, but it is also a risk factor for the development of gallbladder cancer (Pilgrim et al., 2013; Randi et al., 2006; Rustagi and Dasanu, 2012; Tazuma and Kajiyama, 2001). Due to the likely multifactorial etiology of gallbladder cancer, the specific signatures during progression from typhoid carriage to pre-malignant lesions in the gallbladder and other hepatopancreatobiliary organs need to be distinguished from other causes and from non-malignant conditions.

Chronic colonization of 129X1/SvJ mice has been previously assessed up to 1 year post-infection (Monack et al., 2004). Here, we also evaluated *Salmonella* chronic colonization in this mouse model, but with the addition of gallstones. Bacterial enumeration showed that feces and spleen were always colonized by *Salmonella* in the absence of gallstones. Interestingly, we did not recover *Salmonella* after 9 mpi from the spleen of infected mice harboring gallstones. It is unclear why this was not observed, but may be related to the immune response during prolonged infection.
In the liver, *Salmonella* was recovered at 3, 9 and 12 mpi but only in mice harboring gallstones. Colonization was minimal in the pancreas, gallbladder and bile. However, it is important to note that only three mice per time point were used for enumeration (CFU) in liver, pancreas, gallbladder, bile and gallstones. It is possible that other mice in the group could have been colonized or that the *Salmonella* burden in these organs was below the limit of detection (100 CFU/mL). In fact, IHC showed that *Salmonella* (alive or dead) could be detected in the liver, gallbladder and pancreas tissues at time points that were negative by CFU analysis. In the gallbladder, *Salmonella* LPS staining was mostly detected in the muscularis. This is at odds to other studies performed during acute stages where *Salmonella* localization was restricted to the mucosa (Knodler et al., 2010; Menendez et al., 2009). In the liver, we detected *Salmonella* LPS staining both intracellularly in macrophages or Kupffer cells and in the hepatocytes. Previous studies have demonstrated that *Salmonella* primarily replicates in the liver of mouse macrophages at early stages of infection (Nnalue et al., 1992; Richter-Dahlfors et al., 1997). Thus, it is possible that hepatocytes can be colonized later during chronic stages of infection. In the pancreas, localization of *Salmonella* LPS staining was mostly in the interstitium, also suggesting *Salmonella* residing inside macrophages, although bacteria could have also been phagocytosed and cleared.

In previous studies, we determined that acute hepatitis and cholecystitis (neutrophilic and histiocytic) occurred up to 2 mpi (Gonzalez-Escobedo and Gunn, 2013). Sonographic findings including mucosal irregularities, edema with distention and thickening of the gallbladder, suggesting cholecystitis; have been reported in acute typhoid fever patients without gallstones (Shetty and Broome, 1998). In fact, histiocytic
inflammation and granuloma formation occurs in the gallbladder and liver during typhoid fever (Vaishnavi et al., 2005a). In this study, chronic cholecystitis and hepatitis (mostly lymphohistiocytic and plasmacytic) were the predominant observation during Salmonella carriage up to 1 year post-infection, which has also been reported in human typhoid carriers (Axelrod et al., 1971; Gosbell et al., 1995; Roa et al., 1999; Scott, 1971). Ulceration of the epithelium was a frequent observation in infected mice without gallstones at 9 mpi. In opposition to this finding, mucosal ulceration of the gallbladder as a result of chronic typhoid has been reported, but is rare, in patients from endemic areas (Mateen et al., 2006).

Corroborating previous studies using mouse models fed a lithogenic diet (Ichikawa et al., 2009; Lavoie et al., 2012; Rege and Prystowsky, 1998), the mere presence of gallstones caused chronic cholecystitis and in general this caused more inflammation than the presence of Salmonella in the absence of gallstones suggesting that in most cases Salmonella chronic colonization does not induce strong infiltration of immune cells. However, we observed severe inflammation (necrosuppurative) in some mice that were infected but without gallstones, implying that this is a heterogenous process. Interestingly, the levels of inflammation were not exacerbated in the gallbladder of infected mice with gallstones, suggesting that Salmonella does not dramatically increase pre-existing inflammation.

In the liver and pancreas, the inflammation patterns were different than in the gallbladder because here, the presence of Salmonella alone caused more inflammation than the presence of gallstones alone (especially at 3 and 6 mpi.). This is an expected
observation considering that gallstones are typically present in the gallbladder and bile ducts, and only infrequently found in the pancreatic duct.

Chronic hepatitis (mostly lymphohistiocytic), in some cases with necrosuppurative inflammation (hepatic abscesses) and hepatocellular necrosis were observed in chronically-infected mice. Reported histopathological findings in the liver of typhoid fever patients are characterized by non-specific reactive hepatitis which includes focal necrosis in lobules, lymphohistiocytic infiltration and biliary canaliculi injury (de Brito et al., 1977; Drozd and Levadnaia, 1983; El-Newihi et al., 1996; Hornick et al., 1970b; Pramoolsinsap and Viranuvatti, 1998; Ramachandran et al., 1974). Acute hepatitis is reported to commonly occur in typhoid fever but is mostly asymptomatic (El-Newihi et al., 1996; Khan et al., 1998; Khan et al., 1999; Pramoolsinsap and Viranuvatti, 1998). Liver abscesses are considered rare complications in typhoid hepatitis (Kumar et al., 1989).

Although pre-existing hepatobiliary disease such as cholelithiasis have been described as a predisposing factor for Salmonella hepatic infection (Cohen et al., 1987), in this study we did not observe higher inflammation patterns in mice with gallstones. However, hepatic abscesses in chronic carries have been previously reported but mostly in patients with pre-existing hepatobiliary disease (Cohen et al., 1987; Erlik and Reitler, 1960; Gosbell et al., 1995; Rovito and Bonanno, 1982). One of the pathological observations commonly seen in the liver of infected mice was the presence of focal/multifocal necrosis and typhoid nodules, which are reported pathological features of typhoid fever along with infrequent focal granulomas in the liver and spleen (Khosla,
Typhoid nodules are primarily aggregates of altered macrophages that phagocytose bacteria, erythrocytes and degenerated lymphocytes, sometimes with central necrosis. They can also contain plasma cells and lymphocytes and atypically, neutrophils (Khosla, 2008; Ramachandran et al., 1974). Since these nodules are mainly comprised of altered macrophages, this may indicate that histiocytic inflammation occurred early during infection (before 3 mpi in this model). In addition, in the liver we also observed mineralized chronic thrombi. Venous thrombosis is considered a result of severe cases of typhoid fever. Related to this observation, disseminated intravascular coagulation, which is a feature of extreme hepatic dysfunction, has been reported in patients with typhoid hepatitis (Pramoolsinsap and Viranuvatti, 1998) and it has been proposed as the cause of systemic manifestations in typhoid fever (Crum, 2003).

Acute and chronic pancreatitis, in some cases with abscesses, has been reported in typhoid fever patients (Hermans et al., 1990; Kadappu et al., 2002; Khan et al., 2009; Parenti et al., 1996; Vaishnavi et al., 2005a). In this study, we observed lymphohistiocytic and necrosuppurative inflammation in the pancreas of infected mice at 3 and 6 mpi. In comparison with the gallbladder and liver, chronic inflammation in the pancreas was milder after 6 mpi.

Hyperplastic changes in the epithelium of the gallbladder and bile ducts of the liver were noted only in mice harboring gallstones, independent of Salmonella infection. This increased cell proliferation as a result of cholelithiasis has been previously reported (Chang et al., 1999; Mathur et al., 2012b). However, typically this was seen concurrently
with hyalinosis in affected tissues and/or inflammation. Hyalinosis is a common lesion in some strains of mice whereby epithelial cells in the nasal cavity, lung, glandular stomach, gallbladder, bile and pancreatic ducts, and ureter accumulate an eosinophilic cytoplasm that may also be associated with spicular eosinophilic crystals. It is thought that crystals form due to local increased concentrations of the chitinase-like proteins Ym-1/Ym-2, secondary to neutrophil degranulation during repeated episodes of inflammation. The crystals are resistant to degradation, and the inflammation and crystals are sufficient in and of themselves to induce epithelial hyperplasia (Harbord et al., 2002; Ward et al., 2001). Biliary hyperplasia in the liver was only consistently observed in infected mice with gallstones at 9 mpi. Such an epithelial change in the liver has not been reported before.

Dysplasia is a pre-malignant lesion that can progress to neoplasia. In fact, more than 80% of invasive gallbladder carcinomas have adjacent areas of dysplasia (Monga, 2011). Atypical hyperplasia/dysplasia was only observed in the gallbladder of infected mice regardless of the presence of gallstones. In fact, this is the first time that atypical hyperplasia/dysplasia is shown as a result of Salmonella chronic infection. It is widely reported that gallstones are the major risk factor for developing gallbladder cancer (Goldin and Roa, 2009). We hypothesized that chronic inflammation of the gallbladder or bile ducts as a result of Salmonella infection may also lead to pre-malignant lesions or carcinogenesis. However, considering that these proliferative changes are more likely to be a physiological response to inflammation and that we did not see atypical hyperplasia/dysplasia after 3 mpi, we cannot predict if these changes will ultimately progress to neoplasia in these mice. However, this could be due to scarce long term (>3
months) *Salmonella* colonization of the gallbladder in our model under the chosen experimental conditions. Thus, we believe that in human chronic carriers, with permanent bacterial colonization in the gallbladder, chronic inflammation and dysplasia could occur and progress to neoplasia.

Chronic inflammation with oxidative stress caused by persistent bacterial infection and their production of bacterial toxins and metabolites has been associated with biliary carcinogenesis (Hornick et al., 1970a). At the molecular level, it has been proposed that chronic inflammation of the gallbladder leads to an allele-specific mutation with loss of p53 gene heterozygosity and overexpression of p53 protein (Wee et al., 1994; Wistuba et al., 1995). This mutation is considered to result in malignant transformation of the gallbladder mucosa because p53 is a recognized tumor suppressor (Rai et al., 2011). In addition, cyclooxygenase 2 (COX-2), which is also associated with gallbladder carcinomas, is also induced during gallbladder inflammation (Legan, 2010; Tazuma and Kajiyama, 2001). Thus, chronic inflammation can lead to molecular disturbances in the cell cycle of the gallbladder mucosa that contribute to gallbladder cancer development (Sosnik and Sosnik, 2005).

In addition to the epithelial changes observed in the gallbladder, acinar-ductal mucinous metaplasia was only observed in the pancreas of infected mice. Acinar-ductal mucinous metaplasia occurred in the terminal ducts adjacent to acini; morphologically, it is commonly identified as mucinous metaplastic epithelia mixed with acinar cells in the pancreatic lobules. This type of metaplasia is commonly seen in chronic pancreatitis and in the pancreatic parenchyma adjacent to ductal carcinoma (Cao et al., 2010). The
severe inflammation observed in the pancreas of infected mice seemed to originate within the ducts but then obliterated the ducts and extended into the acini. Mucinous metaplasia is likely an attempt to regenerate the duct but the acinar cells are also irritated from the inflammation. Although *Salmonella* was always present in the liver during chronic disease, this persistence did not induce epithelial changes such as atypical hyperplasia/dysplasia and metaplasia that were observed in the gallbladder and pancreas, respectively.

In conclusion, chronic inflammation in organs of the hepatopancreatobiliary system was a predominant observation in our model of chronic carriage. Inflammation patterns were different in the gallbladder in comparison with the liver and pancreas. In the gallbladder, the presence of gallstones caused more robust and permanent inflammation than the mere presence of *Salmonella*. However, in the liver and pancreas, the presence of *Salmonella* caused more inflammation. Interestingly, in all organs, infected mice with gallstones did not show increased inflammation in comparison with the other groups (except in the pancreas at 3mpi.) This inflammation, in some cases very severe, likely explains the epithelial changes observed in the gallbladder (atypical hyperplasia) and in the pancreas (mucinous metaplasia). Although the mere presence of gallstones did not cause these epithelial changes, the low level of *Salmonella* colonization of these organs months post-infection could explain the absence of more dysplastic changes that could better correlate chronic carriage with oncogenesis. In the future, we will attempt to alter the model system to permit longer chronic hepatopancreatobiliary colonization, allowing for histological studies that likely better correlate with changes that occur during long-term human chronic typhoid carriage.
5.1. Identification of a hyper-biofilm forming strain

In the course of the long-term in vivo study of chronic carriage from Chapter 4 we recovered S. Typhimurium from different mice sites. When bacteria were recovered from gallstones, colony isolates were further investigated in their biofilm forming capacities by performing crystal violet assays. Interestingly, different isolates recovered from the gallstones of a carrier mouse 9 months post-infection (mpi) exhibited a hyper-biofilm phenotype on cholesterol-coated surfaces (Fig. 42). A pool of these isolates, henceforth called JSG3538, was further investigated. Interestingly, isolates from the gallbladder tissue and bile from the same mouse did not show the hyper-biofilm phenotype (Fig. 43). This hyper-biofilm phenotype persisted over 10 laboratory passages, suggesting mutation(s) as the basis for the phenotype (Fig. 43).
Figure 42. **Biofilm capacity of ten different isolates recovered from mouse gallstones 9 months post-infection.** Biofilms were grown for 24 h at 37°C on cholesterol surfaces and crystal violet assays were performed. Analysis of variance test with Dunnett post-comparison was used to detect statistical significance. (**, p<0.01; ***, p<0.001). ns, not significant.

Figure 43. **Hyper-biofilm formation by JSG3538 did not occur in other sites of the same mouse.** Biofilm capacity of isolates recovered from the different sites of a chronically infected mouse at 9 months post-infection compared with the initial wild-type inoculated strain (JSG210). Biofilms were grown for 24 h at 37°C on cholesterol surfaces and crystal violet assays were performed. The hyper-biofilm phenotype was specific to the gallstone environment (JSG3538) and was not lost after 10 laboratory passages in LB medium. Analysis of variance test was used to compare the wild-type with each isolate (*, p<0.05, ***, p<0.001). ns, not significant.
5.2. Methods and Results

5.2.1. Biofilm formation of JSG3538 on various surfaces

For SEM observations, S. Typhimurium wild-type (JSG210) and the hyper-biofilm isolate were inoculated into glass-bottom 12-well plates (MatTek Corp., MA) uncoated or coated with cholesterol. Samples were incubated in LB bile (3%) at 37° for 96 h, fixed with 2.5% glutaraldehyde and treated as described in Chapter 2, page 79. For biofilm formation assessment on the gallbladder epithelium, stained DGEC (Cell Tracker Red CMPTX) were infected with bacteria harboring the pFPV25.1 (MOI:10) and incubated at 37°C and 5% CO₂ for 9 h, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, for 15 min at RT; rinsed, and observed using an Olympus Fluoview FV10.1 microscope.

S. Typhimurium wild-type formed robust biofilms on glass, cholesterol and gallbladder epithelial cells surfaces (Fig. 44 and 45). As previously demonstrated, cholesterol enhanced biofilm formation (Fig. 44). However, JSG3538 strain showed drastic biofilm phenotypes depending on the surface. On glass and gallbladder epithelial cells, JSG3538 did not form biofilms but on cholesterol-coated surfaces it formed very robust biofilms (Fig. 44 and 45). Interestingly, the morphology and architecture of biofilms formed by JSG3528 was different than the wild-type (Fig. 44). These results suggest the hyper-biofilm phenotype depends on the type of surface and it is likely not only the result of increased biomass.
Figure 44. **Hyper-biofilm formation by JSG3538 does not occur on every surface.** SEM images of the wild-type and JSG3538 biofilms on glass and cholesterol surfaces. JSG3538 displayed no biofilms on a glass-only surface. On a cholesterol-coated surface, however, the hyperbiofilm isolate displayed a thicker, more robust biofilm with a different morphology than that of the wild-type.

Figure 45. **JSG3538 does not form biofilms on dog gallbladder epithelial cells.** Representative confocal images of DGEC, infected with wild-type S. Typhimurium and JSG3538 at 9 h post-infection in the presence of bile are shown. All bacterial strains harbor the plasmid pFPV25.1 constitutively expressing GFP. DGEC are stained with Cell Tracker™ Red CMPTX. All images have a magnification of 60x.
5.2.2. Biofilm treatment with DNase I, proteinase and cellulase

S. Typhimurium wild-type and JSG3538 were inoculated into glass-bottom wells coated with cholesterol. Samples were incubated in LB medium at 37° for 24 h to allow biofilm formation. DNase I (56 units), proteinase (0.53mg), and cellulase (50 units) were added to the appropriate wells and the plates were incubated again at 37° for 16 h. Biofilm capacity was determined by crystal violet assays. Viability assays confirmed that the enzymes did not simply kill the bacteria, indicating that any changes were due solely to effects on the biofilm itself.

Figure 46. Proteinase treatment disrupts biofilms formed by S. Typhimurium wild-type (JSG210) and JSG3538. Biofilm disruption properties of DNase I, proteinase and cellulase were assessed after 16 h of enzymatic treatment. Only proteinase largely decreased both the wild-type and JSG3538 biofilms. Means between conditions with no treatment (-) and with the respective enzyme treatment were compared by a Student’s t test (*, p<.05; ***, p<.001). ns, not significant.
5.2.3. Sequencing

The hyper-biofilm phenotype of JSG3538 was not lost after 10 laboratory passages, suggesting the phenotype is due to genetic alterations, most likely mutation(s). In order to identify the specific mutation(s) responsible for the hyper-biofilm phenotype, the genome sequence of the parental (ATCC14028s [JSG210]) and JSG3538 was determined using the Ion TorrentTM technology. Briefly, the genomic DNA was sonicated to produce 200 base pair fragments. After end repair, barcode adaptors were ligated to the fragments. The fragments were size selected and then amplified to obtain a library. The template preparation used the Ion OneTouch™ system followed by bead enrichment. Sequences were analyzed by the Ion Torrent and CLCBio software. Comparison of the parental strain with JSG3538 allowed the identification of 14 single nucleotide polymorphisms (SNPs) in the genome of JSG3538. These SNPs had a frequency higher than 80% and resulted in missense (12 SNPs) and nonsense mutations (2 SNPs). The list of genes containing the SNPs is listed in Table 6. Missense mutations were present in *envZ* and *fliB*, thus encoding a truncated protein.
Table 6. List of genes containing single nucleotide polymorphisms in JSG3538.

The genes are listed based on their known functions, unnamed genes are listed according to their position in the genome.

<table>
<thead>
<tr>
<th>Function/Annotation</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two component system</td>
<td>rcsB, envZ*</td>
</tr>
<tr>
<td>Flagella assembly</td>
<td>fliB*, flgl, fliG, fliT</td>
</tr>
<tr>
<td>O-antigen synthesis</td>
<td>rfbG</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>malT, melR, ugpE, STM14_0844</td>
</tr>
<tr>
<td>DNA Rearrangements</td>
<td>STM14_3340, STM14_0647</td>
</tr>
<tr>
<td>Hypothetical proteins</td>
<td>STM14_1618</td>
</tr>
</tbody>
</table>

*indicates that the amino acid change resulted in nonsense mutation.

5.3. Discussion

We have isolated and characterized a strain recovered from mouse gallstones which exhibits a hyper-biofilm phenotype, specifically on cholesterol surfaces. Typical biofilm constituents such as cellulose and eDNA did not significantly alter the hyper-biofilm strain and the wild-type whereas proteinase had similar negative effects on biofilm forming ability in both strains. Genomic sequencing of JSG3538 allowed the identification of 14 SNPs causing missense or nonsense mutations. SNPs were present in genes encoding the TCS proteins EnvZ (sensor kinase) and RcsB (response regulator), flagellar assembly proteins and O-antigen synthesis. Further investigation of the genes containing SNPs, particularly rscB, envZ, rfbG and the four flagellar affected genes is ongoing. The identified mutations will be recreated by both removal of the gene
of interest and site-directed mutagenesis to reproduce the exact mutation both in the wild-type strain and in the JSG3538 strain. The generated strains will then be screened for biofilm forming capacities both in vitro and in vivo.

The hyper-biofilm phenotype of JSG3538 not only provides insights about the effectiveness of our chronic model of infection but also strongly suggests that this phenotype is the result of a highly adapted sub-population of bacteria that can chronically colonize the gallstone surfaces. Thus, further investigation will demonstrate if this phenotype is the result of directed genetic alteration in response to the gallbladder and gallstone environment. During long-term carriage in the gallbladder, *Salmonella* may be under the selective pressures of the detergent-like bile salts, the gallbladder immune response and other antimicrobial substances. *S. Typhi* is able to endure this environment by forming biofilms on the surface of gallstones (Crawford et al., 2010b). Thus, it is possible that adaptation to the gallbladder environment during long-term carriage leads to mutations that result in hyper-biofilm strains, specifically on gallstone surfaces.

Results of previous studies provide evidence for this specific adaptation. Interestingly, bile has been shown to be a DNA damaging agent that increases the frequency of nucleotide substitutions, frameshifts and chromosomal rearrangements (Prieto et al., 2004, 2006). In addition, exposure of *Salmonella* to bile results in gene expression alterations and in mutational adaptation of a subset of exposed cultures (Hernandez et al., 2012). Thus, future experiments will determine (both in vitro and in...
vivo) if long-term exposure to bile and/or cholesterol induces mutations that render
Salmonella highly adherent to gallstones.

The appearance of mutations over time have been reported in other biofilm-
associated diseases such as cystic fibrosis where P. aeruginosa mucoid conversion
(over-production of EPS alginate) is due to mucA22 mutation as a response to ROS
(Mathee et al., 1999). A recent study suggested that chromosome rearrangements in S.
Typhi can also occur within the host over time (Matthews et al., 2011).

Of special interest, rcsB and envZ mutations are likely to play a crucial role in the
JSG3538 hyper-biofilm phenotype because they are members of TCS and they have
been reported to have a direct or indirect role in Salmonella biofilm formation. RcsB
belongs to the RcsCDBA phosphorelay system where RcsC is the sensor kinase, RcsD
is an intermediate phosphotransfer, RcsB is the transcriptional regulator and RcsA is the
transcriptional co-regulator (Clarke, 2010). Activation of the Rcs system results in a
drastic induction of genes for colanic acid capsule synthesis and repression of flagella
and virulence (Majdalani and Gottesman, 2005; Wang et al., 2007). This system can be
activated by high osmolarity, cell envelop stress, antimicrobial peptides or iron challenge
(Mariscotti and Garcia-del Portillo, 2009). Recently, a rcsB mutant and the
phosphorylated form of RcsB have been shown to abolish S. Typhimurium biofilm
formation by inhibiting CsgD via the sRNA RprA (Latasa et al., 2012). The rcsB mutation
found in JSG3538 causes the amino acid change K149E. This position affects the first
aminoacid of the helix-turn-helix domain and not the response regulator domain. Thus,
considering the reported role of rcsB in biofilm formation and the position of rcsB
mutation in JSG3538 affecting the DNA binding domain, it is possible that this mutation cause constitutive activation of csgD (directly or indirectly via RprA).

In addition, it has been shown that activation of the Rcs system attenuates virulence in mice and impairs growth of *Salmonella* in bile by increasing the length of O-antigen of LPS (Dominguez-Bernal et al., 2004; May and Groisman, 2013; Mouslim et al., 2004). Although the specific signals that control the Rcs system are unknown, it is activated by particular conditions that perturb the cell envelope (Majdalani and Gottesman, 2005) including certain cationic antimicrobial peptides (Farris et al., 2010). Thus, in an environment containing antimicrobial peptides and bile, enhanced resistance to bile might require a limit on the activity of the Rcs system. Taken together, this further suggests that a repression of these phenotypes due to change in RcsB K149E can enhance the adaptation to bile during chronic mice infection in JSG3538.

EnvZ is a membrane bound sensor kinase that is part of the EnvZ/OmpR TCS. In response to environmental changes such as osmolarity and pH, EnvZ change the OmpR phosphorylation pattern. OmpR binds to different areas of the csgD promoter resulting in transcriptional activation or repression depending on the environmental conditions (Gerstel et al., 2006; Gerstel and Romling, 2003). In JSG3538, envZ is encoding a truncated protein, lacking the histidine kinase domain, thus affecting its phosphorylation properties. This can positively affect biofilm formation by affecting OmpR phosphorylation and subsequent regulation of CsgD.
Concluding remarks

The results of this work demonstrate that *Salmonella* is able to persist in the gallbladder of chronically infected mice both in the presence or absence of gallstones. In both scenarios, biofilm formation seems to mediate the establishment of *Salmonella* chronic carriage in the gallbladder. While gallstone biofilms have been detected many months post-infection, the host cell-associated persistence in or on the gallbladder epithelium was demonstrated but only up to two months post-infection, suggesting that biofilm formation on gallstones is the main mechanism of persistence in the gallbladder. Outside of the gallbladder, it is possible that other locations such as other organs/organ systems or specialized host cells may be identified as alternative niches. In fact, the mesenteric lymph nodes have been shown to be a niche for *Salmonella* persistence in the 129X1/SvJ mice (Monack et al., 2004).

We both identified and characterized several factors involved in persistence on gallstones and the gallbladder epithelium. Type 1 fimbriae and curli fimbriae are important for biofilm formation on gallstones and the gallbladder epithelium, respectively. However, other genes encoding membrane-associated proteins such as *ycfR* may modulate the enhanced biofilm formation on gallstones. Global stress genes such as *yciE* could help to control biofilm formation in a variety of surfaces. Additional screenings
during long-term chronic carriage would provide additional targets for treatments that would result in the dissolution of chronic carriage.

Histopathological analysis of chronically infected mice suggests that *Salmonella* is a primary contributor to chronic infection as a promoter of gallbladder damage in the absence of gallstones. In the presence of gallstones, *Salmonella* does not exacerbate the already inflamed gallbladder. The development of pre-malignant lesions such as atypical hyperplasia/dysplasia only in chronically infected mice and not in uninfected mice harboring gallstones has crucial implications for the role of *Salmonella* carriage in gallbladder oncogenesis.

Although these studies represent major advances to our understanding of typhoid chronic carriage in the gallbladder, many questions remain. Importantly, we do not yet understand how *Salmonella* is able to persist in the gallbladder (with and without gallstones) in the presence of an immune response. Clearly an immune response is generated, but it is not sufficient to clear the bacteria from the gallbladder in all individuals. Thus, it is possible that the host response to acute *Salmonella* infection of the gallbladder is insufficient to inhibit bacterial colonization, consequently creating a permissive environment for bacterial persistence, adaptation and establishment of chronic carriage. Both host genetic variations and bacterial subversion of the host response may be involved in establishing carriage. For instance, preliminary results in our laboratory suggest that IL-10 is a factor that facilitates gallbladder colonization.
From the bacterial perspective, further examination of the specific SNPs detected in the hyper-biofilm forming strain JSG3528 is ongoing and promises to give insights about S. Typhi adaptation throughout the establishment of chronic infection. Thus, this may represent gallbladder-driven adaptive mutations that demonstrate the role of the gallbladder and gallstone environment in inducing biofilm formation and persistent colonization. In addition, *Salmonella* may utilize multiple ways to evade and modulate host innate and adaptive immune response in the gallbladder. For instance, it is possible that bacterial factors influence or manipulate the Th1/Th2 balance or suppress innate or adaptive immune responses. Thus, the examination of the immunological factors involved in the interaction between S. Typhi, gallbladder epithelium and gallstones needs to be investigated to better understand the role of the host in permitting asymptomatic chronic carriage.

Consequently, the establishment of *Salmonella* chronic carriage seems to reside in the balance between the capacity of the bacteria to overcome the host responses and the strength of the immune system to clear the bacteria. Therefore, both host and bacteria contributions should be simultaneously studied during long-term carriage. High-throughput technologies such as RNA-sequencing, immune profiling and metabolomics may reveal how *Salmonella* manipulates the immune system in order to maintain a permissive environment for long-term persistence. Thus, gallbladder tissue, bile and gallstones of mice and human chronic carriers should be assessed to reveal these molecular *Salmonella* –host interactions. A recent new animal model suitable for *S. Typhi* infection (TLR-11<sup>−/−</sup>) has been reported and promises to be an effective model to
study the host-pathogen interactions occurring during typhoid chronic carriage (Mathur et al., 2012a).

Investigation of cholesterol biofilm inhibitors such as those that target EPS production, flagella-cholesterol interaction, kinases required for biofilm formation; and the gallstone itself could lead to the development of promising therapies or preventive approaches (instead of gallbladder removal) to eliminate biofilm formation on gallstones and thus likely chronic carriage, especially in areas of high endemicity. Novel non-toxic biofilm-inhibitory compounds have been identified in our laboratory by high-throughput screenings and they will be further studied in vivo. Finally, our results also suggest that intrinsic bacterial factors (e.g. YcfR) that inhibit biofilm formation on specific surfaces such as gallstones not only may represent promising candidates for biofilm dissolution but also would provide a different therapeutic perspective.

At the epidemiological level, comprehensive studies in endemic areas can provide information about the incidence of chronic typhoid carriers, the incidence of gallstones in these patients and potential non-gallbladder sites of S. Typhi persistence. Such information is lacking and needs to be performed, as it would provide additional insight into the impact of the chronic carrier state regarding both transmission and efficient prevention strategies. Similarly, prospective studies should be performed using animal models to examine strategies of gallstone biofilm prevention. Finally, partnerships with investigators in endemic areas should not only provide a better platform to fully understand and combat acute and chronic typhoid fever but also to implement translational science in these areas.
In conclusion, better understanding of the roles of the host and pathogen interface in the gallbladder during chronic carriage, coupled with the potential of new therapeutic strategies may prevent persistence and host-to-host transmission which are key characteristics of asymptomatic typhoid chronic carriage. All this may ultimately reduce the global burden of typhoid fever and gallbladder cancer.


CDC, 2010. Investigation update: multistate outbreak of human Salmonella Enteritidis infections associated with shell eggs. CDC, Atlanta, Georgia, USA.


Erickson, K.D., Detweiler, C.S., 2006. The Rcs phosphorelay system is specific to enteric pathogens/commensals and activates ydel, a gene important for persistent Salmonella infection of mice. Molecular Microbiology 62, 883-894.


199


haplotype that is also widespread in Southeast Asia. Journal of Clinical Microbiology 48, 2171-2176.


206


Lapidot, A., Yaron, S., 2009. Transfer of Salmonella enterica serovar Typhimurium from contaminated irrigation water to parsley is dependent on curli and cellulose, the biofilm matrix components. Journal of Food Protection 72, 618-623.


Malcova, M., Hradecka, H., Karpiskova, R., Rychlik, I., 2008. Biofilm formation in field strains of Salmonella enterica serovar Typhimurium: identification of a new...
colony morphology type and the role of SGI1 in biofilm formation. Veterinary Microbiology 129, 360-366.


Muhlenberg, W., 1993. [Epidemiologic public health conclusions from observations of illnesses caused by *Salmonella Enteritidis*]. Gesundheitswesen 55, 21-27.


the transition from the intestinal lumen into tissue promotes systemic dissemination of *Salmonella*. PLoS Pathogens 6, e1001060.


Appendix A: List of genes differentially regulated in *S. Typhimurium* planktonic versus biofilms cells grown on glass or cholesterol (>5 fold change).

<table>
<thead>
<tr>
<th>No</th>
<th>Entry</th>
<th>Gene name</th>
<th>Definition</th>
<th>Fold change planktonic vs. biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>STM1730</td>
<td><em>yciE</em></td>
<td>putative cytoplasmic protein</td>
<td>Glass: 51.310 up, Cholesterol: 46.797 up</td>
</tr>
<tr>
<td>2</td>
<td>STM4514.S</td>
<td><em>yjiH</em></td>
<td>inner membrane protein</td>
<td>Glass: 50.495 up, Cholesterol: 42.067 up</td>
</tr>
<tr>
<td>3</td>
<td>STM3242</td>
<td><em>tdcD</em></td>
<td>acetate/propionate kinase</td>
<td>Glass: 43.192 up, Cholesterol: 43.008 up</td>
</tr>
<tr>
<td>4</td>
<td>STM2464</td>
<td><em>eutN</em></td>
<td>ethanolamine utilization protein</td>
<td>Glass: 43.135 up, Cholesterol: 55.011 up</td>
</tr>
<tr>
<td>5</td>
<td>STM0701</td>
<td><em>speF</em></td>
<td>ornithine decarboxylase isozyme</td>
<td>Glass: 40.188 up, Cholesterol: 22.914 up</td>
</tr>
<tr>
<td>6</td>
<td>STM0699</td>
<td></td>
<td>putative cytoplasmic protein</td>
<td>Glass: 39.147 up, Cholesterol: 28.552 up</td>
</tr>
<tr>
<td>7</td>
<td>STM0700</td>
<td><em>potE</em></td>
<td>putrescine/ornithine antiporter</td>
<td>Glass: 34.513 up, Cholesterol: 26.930 up</td>
</tr>
<tr>
<td>8</td>
<td>STM2040</td>
<td><em>pduC</em></td>
<td>propanediol dehydratase large subunit</td>
<td>Glass: 34.473 up, Cholesterol: 45.066 up</td>
</tr>
<tr>
<td>9</td>
<td>STM2463</td>
<td><em>eutE</em></td>
<td>aldehyde oxidoreductase</td>
<td>Glass: 33.353 up, Cholesterol: 32.156 up</td>
</tr>
<tr>
<td>10</td>
<td>STM2460</td>
<td><em>eutH</em></td>
<td>ethanolamine transporter</td>
<td>Glass: 32.960 up, Cholesterol: 36.280 up</td>
</tr>
<tr>
<td>11</td>
<td>STM2045</td>
<td><em>pduJ</em></td>
<td>polyhedral body protein</td>
<td>Glass: 32.798 up, Cholesterol: 42.183 up</td>
</tr>
<tr>
<td>12</td>
<td>STM0597</td>
<td><em>entB</em></td>
<td>2,3-dihydro-2,3-dihydroxybenzoate synthetase</td>
<td>Glass: 32.498 down, Cholesterol: 29.495 down</td>
</tr>
<tr>
<td>13</td>
<td>STM3243</td>
<td><em>tdcC</em></td>
<td>L-threonine/L-serine permease</td>
<td>Glass: 31.793 up, Cholesterol: 29.282 up</td>
</tr>
<tr>
<td>14</td>
<td>STM3241</td>
<td><em>tdcE</em></td>
<td>pyruvate formate-lyase 4/2-ketobutyrate formate-lyase</td>
<td>Glass: 30.739 up, Cholesterol: 40.788 up</td>
</tr>
<tr>
<td>15</td>
<td>STM4513</td>
<td><em>yjiG</em></td>
<td>putative permease</td>
<td>Glass: 30.732 up, Cholesterol: 28.701 up</td>
</tr>
<tr>
<td>16</td>
<td>STM2456</td>
<td><em>eutL</em></td>
<td>carboxysome structural protein</td>
<td>Glass: 30.692 up, Cholesterol: 31.922 up</td>
</tr>
<tr>
<td>17</td>
<td>STM2187</td>
<td><em>yeiA</em></td>
<td>dihydropteridine dehydrogenase</td>
<td>Glass: 29.634 up, Cholesterol: 21.645 up</td>
</tr>
<tr>
<td>18</td>
<td>STM2199</td>
<td><em>cirA</em></td>
<td>colicin I receptor protein</td>
<td>Glass: 28.203 up, Cholesterol: 17.249 down</td>
</tr>
<tr>
<td>19</td>
<td>STM0598</td>
<td><em>entA</em></td>
<td>2,3-dihydroxybenzoate-2,3-dehydrogenase</td>
<td>Glass: 27.804 down, Cholesterol: 22.193 down</td>
</tr>
<tr>
<td>20</td>
<td>STM0595</td>
<td><em>entC</em></td>
<td>isochorismate synthase</td>
<td>Glass: 27.396 down, Cholesterol: 16.554 down</td>
</tr>
<tr>
<td>21</td>
<td>STM2046</td>
<td><em>pduK</em></td>
<td>polyhedral body protein</td>
<td>Glass: 26.834 up, Cholesterol: 36.730 up</td>
</tr>
<tr>
<td>22</td>
<td>STM2457</td>
<td><em>eutC</em></td>
<td>ethanolamine ammonia-lyase small subunit</td>
<td>Glass: 26.715 up, Cholesterol: 27.260 up</td>
</tr>
<tr>
<td>23</td>
<td>STM2458</td>
<td><em>eutD</em></td>
<td>ethanolamine ammonia-lyase heavy chain</td>
<td>Glass: 25.983 up, Cholesterol: 25.829 up</td>
</tr>
<tr>
<td>24</td>
<td>STM1731</td>
<td><em>katN</em></td>
<td>putative catalase</td>
<td>Glass: 25.425 up, Cholesterol: 23.426 up</td>
</tr>
<tr>
<td>25</td>
<td>STM2041</td>
<td><em>pduD</em></td>
<td>propanediol dehydratase medium subunit</td>
<td>Glass: 25.205 up, Cholesterol: 34.322 up</td>
</tr>
<tr>
<td>26</td>
<td>STM0599</td>
<td><em>ybdB</em></td>
<td>hypothetical protein</td>
<td>Glass: 24.845 down, Cholesterol: 21.775 down</td>
</tr>
<tr>
<td>27</td>
<td>STM2844</td>
<td></td>
<td>hypothetical protein</td>
<td>Glass: 23.764 up, Cholesterol: 25.878 up</td>
</tr>
<tr>
<td>28</td>
<td>STM2455</td>
<td><em>eutK</em></td>
<td>carboxysome structural protein</td>
<td>Glass: 22.808 up, Cholesterol: 21.850 up</td>
</tr>
<tr>
<td>No</td>
<td>Entry</td>
<td>Gene name</td>
<td>Definition</td>
<td>Fold change planktonic vs. biofilm</td>
</tr>
<tr>
<td>----</td>
<td>-------------</td>
<td>-----------</td>
<td>----------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>30</td>
<td>STM0585 fepA</td>
<td>outer membrane ferric enterobactin receptor precursor</td>
<td>Glass: 22.494 down, Cholesterol: 15.854 down</td>
<td>Glass: 22.494 down, Cholesterol: 15.854 down</td>
</tr>
<tr>
<td>32</td>
<td>STM3244 tdcB</td>
<td>threonine dehydratase</td>
<td>Glass: 21.399 up, Cholesterol: 25.062 up</td>
<td>Glass: 21.399 up, Cholesterol: 25.062 up</td>
</tr>
<tr>
<td>33</td>
<td>STM2467 eutT</td>
<td>cobalamin adenosyltransferase</td>
<td>Glass: 21.289 up, Cholesterol: 23.250 up</td>
<td>Glass: 21.289 up, Cholesterol: 23.250 up</td>
</tr>
<tr>
<td>34</td>
<td>STM3240 tdhC</td>
<td>L-serine deaminase</td>
<td>Glass: 20.923 up, Cholesterol: 29.010 up</td>
<td>Glass: 20.923 up, Cholesterol: 29.010 up</td>
</tr>
<tr>
<td>36</td>
<td>STM2795 ygaU</td>
<td>LysM domain/BON superfamily protein</td>
<td>Glass: 19.595 up, Cholesterol: 10.459 up</td>
<td>Glass: 19.595 up, Cholesterol: 10.459 up</td>
</tr>
<tr>
<td>38</td>
<td>STM0588 entF</td>
<td>enterobactin synthetase component F</td>
<td>Glass: 17.814 down, Cholesterol: 15.396 down</td>
<td>Glass: 17.814 down, Cholesterol: 15.396 down</td>
</tr>
<tr>
<td>40</td>
<td>STM1765 narK</td>
<td>nitrite extrusion protein</td>
<td>Glass: 16.705 down, Cholesterol: 54.762 down</td>
<td>Glass: 16.705 down, Cholesterol: 54.762 down</td>
</tr>
<tr>
<td>42</td>
<td>STM1653 yciG</td>
<td>membrane transporter of cations</td>
<td>Glass: 16.144 up, Cholesterol: 8.994 up</td>
<td>Glass: 16.144 up, Cholesterol: 8.994 up</td>
</tr>
<tr>
<td>43</td>
<td>STM2459 eutA</td>
<td>ethanolamine utilization protein</td>
<td>Glass: 15.843 up, Cholesterol: 15.078 up</td>
<td>Glass: 15.843 up, Cholesterol: 15.078 up</td>
</tr>
<tr>
<td>44</td>
<td>STM2470 eutS</td>
<td>carboxysome structural protein</td>
<td>Glass: 15.639 up, Cholesterol: 7.131 up</td>
<td>Glass: 15.639 up, Cholesterol: 7.131 up</td>
</tr>
<tr>
<td>45</td>
<td>STM1932 ftnB</td>
<td>ferritin-like protein</td>
<td>Glass: 15.540 up, Cholesterol: 13.194 up</td>
<td>Glass: 15.540 up, Cholesterol: 13.194 up</td>
</tr>
<tr>
<td>46</td>
<td>STM1762 narJ</td>
<td>nitrate reductase 1 delta subunit</td>
<td>Glass: 15.244 down, Cholesterol: 28.123 down</td>
<td>Glass: 15.244 down, Cholesterol: 28.123 down</td>
</tr>
<tr>
<td>47</td>
<td>STM1764 narG</td>
<td>nitrate reductase 1 alpha subunit</td>
<td>Glass: 14.948 up, Cholesterol: 15.095 up</td>
<td>Glass: 14.948 up, Cholesterol: 15.095 up</td>
</tr>
<tr>
<td>50</td>
<td>STM1482 ydgF</td>
<td>multidrug efflux system protein MdtJ</td>
<td>Glass: 14.349 up, Cholesterol: 12.494 up</td>
<td>Glass: 14.349 up, Cholesterol: 12.494 up</td>
</tr>
<tr>
<td>53</td>
<td>STM2038 pduA</td>
<td>polyhedral body protein</td>
<td>Glass: 13.067 up, Cholesterol: 13.891 up</td>
<td>Glass: 13.067 up, Cholesterol: 13.891 up</td>
</tr>
<tr>
<td>54</td>
<td>STM4512 iadA</td>
<td>isoaaspartyl dipeptidase</td>
<td>Glass: 13.060 up, Cholesterol: 12.318 up</td>
<td>Glass: 13.060 up, Cholesterol: 12.318 up</td>
</tr>
<tr>
<td>55</td>
<td>STM2051 pduP</td>
<td>CoA-dependent propionaldehyde dehydrogenase</td>
<td>Glass: 12.154 up, Cholesterol: 19.533 up</td>
<td>Glass: 12.154 up, Cholesterol: 19.533 up</td>
</tr>
<tr>
<td>57</td>
<td>STM4561 osmY</td>
<td>hyperosmotically-inducible periplasmic protein</td>
<td>Glass: 11.724 up, Cholesterol: 7.023 up</td>
<td>Glass: 11.724 up, Cholesterol: 7.023 up</td>
</tr>
<tr>
<td>58</td>
<td>STM2466 eutD</td>
<td>phosphate acetyltransferase</td>
<td>Glass: 11.543 up, Cholesterol: 13.567 up</td>
<td>Glass: 11.543 up, Cholesterol: 13.567 up</td>
</tr>
<tr>
<td>60</td>
<td>STM1121 ymdF</td>
<td>putative cytoplasmic protein</td>
<td>Glass: 11.361 up, Cholesterol: 6.139 up</td>
<td>Glass: 11.361 up, Cholesterol: 6.139 up</td>
</tr>
<tr>
<td>63</td>
<td>STM2184 sanA</td>
<td>vancomycin sensitivity</td>
<td>Glass: 10.957 up, Cholesterol: 9.441 up</td>
<td>Glass: 10.957 up, Cholesterol: 9.441 up</td>
</tr>
<tr>
<td>No</td>
<td>Entry</td>
<td>Gene name</td>
<td>Definition</td>
<td>Fold change planktonic vs. biofilm</td>
</tr>
<tr>
<td>----</td>
<td>--------</td>
<td>-----------</td>
<td>------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>66</td>
<td>STM0586</td>
<td><em>fes</em></td>
<td>enterochelin esterase</td>
<td>10.900 down 5.525 down</td>
</tr>
<tr>
<td>67</td>
<td>STM1729</td>
<td><em>ycIF</em></td>
<td>putative cytoplasmic protein</td>
<td>10.842 up 10.487 up</td>
</tr>
<tr>
<td>68</td>
<td>STM4562</td>
<td>putative inner membrane protein</td>
<td>10.711 up 7.123 up</td>
<td></td>
</tr>
<tr>
<td>69</td>
<td>STM1586</td>
<td>putative periplasmic protein</td>
<td>10.654 down 9.737 down</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>STM4539</td>
<td>putative glucosamine-fructose-6-phosphate aminotransferase</td>
<td>10.539 up 16.030 up</td>
<td></td>
</tr>
<tr>
<td>71</td>
<td>STM0360</td>
<td>cytochrome BD2 subunit I</td>
<td>10.528 up 11.496 up</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>STM1797</td>
<td><em>ymgE</em></td>
<td>transglycosylase-associated protein</td>
<td>10.523 up 6.401 up</td>
</tr>
<tr>
<td>73</td>
<td>STM3577</td>
<td><em>tcp</em></td>
<td>methyl-accepting transmembrane citrate/phenol chemoreceptor</td>
<td>10.521 down 6.747 down</td>
</tr>
<tr>
<td>74</td>
<td>STM2461</td>
<td><em>eutG</em></td>
<td>transporter</td>
<td>10.445 up 11.245 up</td>
</tr>
<tr>
<td>75</td>
<td>STM0587</td>
<td><em>ybdZ</em></td>
<td>putative cytoplasmic protein</td>
<td>10.354 down 7.292 down</td>
</tr>
<tr>
<td>76</td>
<td>STM4055</td>
<td><em>sodA</em></td>
<td>superoxide dismutase</td>
<td>10.231 down 8.619 down</td>
</tr>
<tr>
<td>77</td>
<td>STM1483</td>
<td><em>ydgE</em></td>
<td>multidrug efflux system protein MdtL</td>
<td>10.068 up 13.203 up</td>
</tr>
<tr>
<td>78</td>
<td>STM2047</td>
<td><em>pduL</em></td>
<td>propanediol utilization protein</td>
<td>10.014 up 15.274 up</td>
</tr>
<tr>
<td>79</td>
<td>STM0465</td>
<td><em>ybaY</em></td>
<td>hypothetical protein</td>
<td>9.913 up 6.765 up</td>
</tr>
<tr>
<td>80</td>
<td>STM1761</td>
<td><em>narI</em></td>
<td>nitrate reductase 1 gamma subunit</td>
<td>9.901 down 18.384 down</td>
</tr>
<tr>
<td>81</td>
<td>STM3474</td>
<td><em>nirB</em></td>
<td>nitrite reductase large subunit</td>
<td>9.815 down 16.956 down</td>
</tr>
<tr>
<td>82</td>
<td>STM3475</td>
<td><em>nirD</em></td>
<td>nitrite reductase small subunit</td>
<td>9.813 down 17.585 down</td>
</tr>
<tr>
<td>83</td>
<td>STM1626</td>
<td><em>trg</em></td>
<td>methyl-accepting chemotaxis protein III</td>
<td>9.788 down 5.319 down</td>
</tr>
<tr>
<td>84</td>
<td>STM1515</td>
<td><em>ydel</em></td>
<td>putative periplasmic protein</td>
<td>9.650 up 7.411 up</td>
</tr>
<tr>
<td>85</td>
<td>STM2853</td>
<td><em>hycA</em></td>
<td>formate hydrogenyelase regulatory protein</td>
<td>9.570 up 8.421 up</td>
</tr>
<tr>
<td>86</td>
<td>STM2845</td>
<td><em>hycI</em></td>
<td>hydrogenase 3 large subunit C-terminal protease</td>
<td>9.566 up 11.654 up</td>
</tr>
<tr>
<td>87</td>
<td>STM3476</td>
<td><em>nirC</em></td>
<td>nitrite transport protein</td>
<td>9.538 down 15.313 down</td>
</tr>
<tr>
<td>88</td>
<td>STM0966</td>
<td><em>dmsC</em></td>
<td>anaerobic dimethyl sulfoxide reductase subunit C</td>
<td>9.517 up 5.044 up</td>
</tr>
<tr>
<td>89</td>
<td>STM3143</td>
<td><em>hybG</em></td>
<td>hydrogenase 2 accessory protein</td>
<td>9.454 up 8.579 up</td>
</tr>
<tr>
<td>90</td>
<td>STM1564</td>
<td><em>yddX</em></td>
<td>biofilm-dependent modulation protein</td>
<td>9.367 up 8.647 up</td>
</tr>
<tr>
<td>91</td>
<td>STM0831</td>
<td><em>dps</em></td>
<td>DNA protection during starvation conditions</td>
<td>9.343 up 8.210 up</td>
</tr>
<tr>
<td>92</td>
<td>STM1916</td>
<td><em>cheY</em></td>
<td>chemotaxis regulator</td>
<td>9.332 down 8.281 down</td>
</tr>
<tr>
<td>93</td>
<td>STM0068</td>
<td><em>calF</em></td>
<td>DNA binding transcriptional activator</td>
<td>9.320 up 5.241 up</td>
</tr>
<tr>
<td>94</td>
<td>STM2548</td>
<td><em>asrA</em></td>
<td>anaerobic sulfide reductase</td>
<td>9.320 up 6.713 up</td>
</tr>
<tr>
<td>95</td>
<td>STM1118</td>
<td><em>yccJ</em></td>
<td>putative cytoplasmic protein</td>
<td>9.156 up 4.600 up</td>
</tr>
<tr>
<td>96</td>
<td>STM0359</td>
<td>putative cytoplasmic protein</td>
<td>9.056 up 5.843 up</td>
<td></td>
</tr>
<tr>
<td>97</td>
<td>STM4540.S</td>
<td>glucosamine-fructose-6-phosphate aminotransferase</td>
<td>9.025 up 14.373 up</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>STM2862</td>
<td><em>sitB</em></td>
<td>ATP-binding protein</td>
<td>8.847 down 4.143 down</td>
</tr>
<tr>
<td>99</td>
<td>STM2771</td>
<td><em>fijB</em></td>
<td>flagellar biosynthesis protein</td>
<td>8.795 down 6.428 down</td>
</tr>
<tr>
<td>No</td>
<td>Entry</td>
<td>Gene name</td>
<td>Definition</td>
<td>Fold change planktonic vs. biofilm</td>
</tr>
<tr>
<td>----</td>
<td>---------</td>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glass</td>
</tr>
<tr>
<td>100</td>
<td>STM3611</td>
<td>yhjH</td>
<td>EAL domain-containing protein</td>
<td>8.685 down</td>
</tr>
<tr>
<td>101</td>
<td>STM1184</td>
<td>fglL</td>
<td>flagellar hook-associated protein</td>
<td>8.620 down</td>
</tr>
<tr>
<td>102</td>
<td>STM2063</td>
<td>sitA</td>
<td>periplasmic binding protein</td>
<td>8.485 down</td>
</tr>
<tr>
<td>103</td>
<td>STM2861</td>
<td>sitC</td>
<td>permease</td>
<td>8.306 down</td>
</tr>
<tr>
<td>104</td>
<td>STM1139</td>
<td>csgG</td>
<td>curli operon transcriptional regulator</td>
<td>8.419 up</td>
</tr>
<tr>
<td>105</td>
<td>STM4510</td>
<td>flgM</td>
<td>anti-sigma 28 factor FlgM</td>
<td>8.405 up</td>
</tr>
<tr>
<td>106</td>
<td>STM4440</td>
<td>flaC</td>
<td>flagellar hook-associated protein</td>
<td>8.338 up</td>
</tr>
<tr>
<td>107</td>
<td>STM2863</td>
<td>sitA</td>
<td>thiosulfate reductase electron transport protein</td>
<td>8.278 up</td>
</tr>
<tr>
<td>108</td>
<td>STM2852</td>
<td>hycB</td>
<td>hydrogenase-3 iron-sulfur subunit</td>
<td>8.248 up</td>
</tr>
<tr>
<td>109</td>
<td>STM3325</td>
<td>yrbL</td>
<td>putative cytoplasmic protein</td>
<td>8.245 up</td>
</tr>
<tr>
<td>110</td>
<td>STM1513</td>
<td>flgM</td>
<td>anti-sigma 28 factor FlgM</td>
<td>8.405 up</td>
</tr>
<tr>
<td>111</td>
<td>STM4538</td>
<td>motB</td>
<td>flagellar motor protein</td>
<td>8.111 down</td>
</tr>
<tr>
<td>112</td>
<td>STM0361</td>
<td>flaC</td>
<td>flagellar hook-associated protein</td>
<td>8.044 up</td>
</tr>
<tr>
<td>113</td>
<td>STM1960</td>
<td>motA</td>
<td>flagellar motor protein</td>
<td>8.029 down</td>
</tr>
<tr>
<td>114</td>
<td>STM1922</td>
<td>yhiH</td>
<td>ABC-type multidrug transport system ATPase component</td>
<td>8.015 up</td>
</tr>
<tr>
<td>115</td>
<td>STM1172</td>
<td>flaD</td>
<td>flagellar basal body rod modification protein</td>
<td>7.891 down</td>
</tr>
<tr>
<td>116</td>
<td>STM1563</td>
<td>ansP</td>
<td>L-asparagine transport protein</td>
<td>7.857 down</td>
</tr>
<tr>
<td>117</td>
<td>STM1923</td>
<td>motA</td>
<td>flagellar motor protein</td>
<td>7.803 down</td>
</tr>
<tr>
<td>118</td>
<td>STM3586.S</td>
<td>yhiH</td>
<td>ABC-type multidrug transport system ATPase component</td>
<td>8.015 up</td>
</tr>
<tr>
<td>119</td>
<td>STM3195</td>
<td>ribB</td>
<td>3,4-dihydroxy-2-butaneone 4-phosphate synthase</td>
<td>7.768 up</td>
</tr>
<tr>
<td>120</td>
<td>STM2185</td>
<td>b2145</td>
<td>putative inner membrane protein</td>
<td>7.768 up</td>
</tr>
<tr>
<td>121</td>
<td>STM3145</td>
<td>hybE</td>
<td>hydrogenase-2 specific chaperone</td>
<td>7.769 up</td>
</tr>
<tr>
<td>122</td>
<td>STM3217</td>
<td>aer</td>
<td>aerotaxis sensor receptor</td>
<td>7.634 down</td>
</tr>
<tr>
<td>123</td>
<td>STM0871</td>
<td>ybjM</td>
<td>inner membrane protein</td>
<td>7.632 up</td>
</tr>
<tr>
<td>124</td>
<td>STM2506</td>
<td>inner membrane protein</td>
<td>7.624 up</td>
<td>6.098 up</td>
</tr>
<tr>
<td>125</td>
<td>STM3245</td>
<td>tdcA</td>
<td>transcriptional activator</td>
<td>7.548 down</td>
</tr>
</tbody>
</table>

233
<table>
<thead>
<tr>
<th>No</th>
<th>Entry</th>
<th>Gene name</th>
<th>Definition</th>
<th>Fold change planktonic vs. biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>135</td>
<td>STM3146</td>
<td>hydB</td>
<td>hydrogenase-2 processing element</td>
<td>Glass: 7.422 up, Cholesterol: 6.584 up</td>
</tr>
<tr>
<td>136</td>
<td>STM2846</td>
<td>hycH</td>
<td>hydrogenase 3 large subunit processing protein</td>
<td>Glass: 7.330 up, Cholesterol: 8.338 up</td>
</tr>
<tr>
<td>137</td>
<td>STM1273</td>
<td>ydjN</td>
<td>nitric oxide reductase</td>
<td>Glass: 7.299 up, Cholesterol: 3.913 up</td>
</tr>
<tr>
<td>138</td>
<td>STM1320</td>
<td>asrB</td>
<td>anaerobic sulfite reductase subunit B</td>
<td>Glass: 7.188 down, Cholesterol: 6.724 down</td>
</tr>
<tr>
<td>139</td>
<td>STM2549</td>
<td>rpsV</td>
<td>30S ribosomal subunit protein S22</td>
<td>Glass: 7.156 up, Cholesterol: 5.444 up</td>
</tr>
<tr>
<td>140</td>
<td>STM1565</td>
<td>otsB</td>
<td>trehalose-6-phosphate phosphatase</td>
<td>Glass: 7.144 up, Cholesterol: 5.882 up</td>
</tr>
<tr>
<td>141</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.724 down</td>
</tr>
<tr>
<td>142</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>143</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>144</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>145</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>146</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>147</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>148</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>149</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>150</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>151</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>152</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>153</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>154</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>155</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>156</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>157</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>158</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>159</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>160</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>161</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>162</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>163</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>164</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>165</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>166</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>167</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
<tr>
<td>168</td>
<td>STM1273</td>
<td>ydjN</td>
<td>kinase/transporter-like protein</td>
<td>Glass: 7.188 down, Cholesterol: 6.744 up</td>
</tr>
</tbody>
</table>

234
<table>
<thead>
<tr>
<th>No</th>
<th>Entry</th>
<th>Gene name</th>
<th>Definition</th>
<th>Fold change planktonic vs. biofilm</th>
</tr>
</thead>
<tbody>
<tr>
<td>169</td>
<td>STM0651</td>
<td>2-keto-3-deoxygluconate permease</td>
<td>Glass 6.440 up</td>
<td>Cholesterol 5.337 up</td>
</tr>
<tr>
<td>170</td>
<td>STM2550</td>
<td>asrC</td>
<td>anaerobic sulfide reductase</td>
<td>Glass 6.435 up</td>
</tr>
<tr>
<td>171</td>
<td>STM4537</td>
<td>wrbA</td>
<td>TrpR binding protein WrbA</td>
<td>Glass 6.405 up</td>
</tr>
<tr>
<td>172</td>
<td>STM1119</td>
<td>ygaM</td>
<td>putative inner membrane protein</td>
<td>Glass 6.392 up</td>
</tr>
<tr>
<td>173</td>
<td>STM2802</td>
<td>pseD</td>
<td>DNA replication/recombination/repair protein</td>
<td>Glass 6.382 up</td>
</tr>
<tr>
<td>174</td>
<td>STM3130</td>
<td>STM2854</td>
<td>hypA</td>
<td>hydrogenase nickel incorporation protein</td>
</tr>
<tr>
<td>175</td>
<td>STM1175</td>
<td>flagG</td>
<td>flagellar basal body rod protein</td>
<td>Glass 6.061 down</td>
</tr>
<tr>
<td>176</td>
<td>STM2043</td>
<td>cbiQ</td>
<td>vitamin B12 biosynthetic protein</td>
<td>Glass 6.044 down</td>
</tr>
<tr>
<td>177</td>
<td>STM2411</td>
<td>cysA</td>
<td>sulfate permease A protein</td>
<td>Glass 6.031 up</td>
</tr>
<tr>
<td>178</td>
<td>STM0440</td>
<td>cyoD</td>
<td>cytochrome o ubiquinol oxidase subunit IV</td>
<td>Glass 5.906 down</td>
</tr>
<tr>
<td>179</td>
<td>STM1178</td>
<td>flagF</td>
<td>flagellar basal-body rod protein</td>
<td>Glass 6.016 down</td>
</tr>
<tr>
<td>180</td>
<td>STM1237</td>
<td>yeaJ</td>
<td>methyl-accepting chemotaxis protein</td>
<td>Glass 6.154 up</td>
</tr>
<tr>
<td>181</td>
<td>STM2465</td>
<td>pduG</td>
<td>propanediol dehydratase reactivation protein</td>
<td>Glass 6.110 down</td>
</tr>
<tr>
<td>182</td>
<td>STM2043</td>
<td>sseL</td>
<td>deubiquitinase</td>
<td>Glass 5.968 up</td>
</tr>
<tr>
<td>183</td>
<td>STM1273.1</td>
<td>hypothetical protein</td>
<td>Glass 5.910 up</td>
<td>Cholesterol 4.675 up</td>
</tr>
<tr>
<td>184</td>
<td>STM2287</td>
<td>sseL</td>
<td>deubiquitinase</td>
<td>Glass 6.110 down</td>
</tr>
<tr>
<td>185</td>
<td>STM2023</td>
<td>cbmM</td>
<td>cobalt transport protein CbiM</td>
<td>Glass 5.609 up</td>
</tr>
<tr>
<td>186</td>
<td>STM3516</td>
<td>putative cytoplasmic protein</td>
<td>Glass 5.752 up</td>
<td>Cholesterol 3.966 up</td>
</tr>
<tr>
<td>187</td>
<td>STM1786</td>
<td>hydrogenase-1 small subunit</td>
<td>Glass 5.718 down</td>
<td>Cholesterol 4.763 down</td>
</tr>
<tr>
<td>188</td>
<td>STM2560</td>
<td>ydlL</td>
<td>di-/tripeptide transport protein</td>
<td>Glass 5.662 up</td>
</tr>
<tr>
<td>189</td>
<td>STM4240</td>
<td>yfiJ</td>
<td>stress-response protein</td>
<td>Glass 5.659 up</td>
</tr>
<tr>
<td>190</td>
<td>STM2850</td>
<td>hycD</td>
<td>hydrogenase 3 membrane subunit</td>
<td>Glass 5.604 up</td>
</tr>
<tr>
<td>191</td>
<td>STM1915</td>
<td>cheZ</td>
<td>chemotaxis regulator</td>
<td>Glass 5.600 down</td>
</tr>
<tr>
<td>192</td>
<td>STM3576</td>
<td>zntA</td>
<td>Pb/Cd/Zn/Hg-transporting ATPase</td>
<td>Glass 5.600 down</td>
</tr>
<tr>
<td>193</td>
<td>STM1956</td>
<td>fliA</td>
<td>flagellar biosynthesis sigma factor FliA</td>
<td>Glass 5.594 down</td>
</tr>
<tr>
<td>194</td>
<td>STM2023</td>
<td>cbmM</td>
<td>cobalt transport protein CbiM</td>
<td>Glass 5.609 up</td>
</tr>
<tr>
<td>195</td>
<td>STM3516</td>
<td>putative cytoplasmic protein</td>
<td>Glass 5.752 up</td>
<td>Cholesterol 3.966 up</td>
</tr>
<tr>
<td>196</td>
<td>STM2560</td>
<td>ydlL</td>
<td>di-/tripeptide transport protein</td>
<td>Glass 5.662 up</td>
</tr>
<tr>
<td>197</td>
<td>STM4240</td>
<td>yfiJ</td>
<td>stress-response protein</td>
<td>Glass 5.659 up</td>
</tr>
<tr>
<td>198</td>
<td>STM2023</td>
<td>cbmM</td>
<td>cobalt transport protein CbiM</td>
<td>Glass 5.609 up</td>
</tr>
<tr>
<td>199</td>
<td>STM2560</td>
<td>ydlL</td>
<td>di-/tripeptide transport protein</td>
<td>Glass 5.662 up</td>
</tr>
<tr>
<td>200</td>
<td>STM1915</td>
<td>cheZ</td>
<td>chemotaxis regulator</td>
<td>Glass 5.600 down</td>
</tr>
<tr>
<td>201</td>
<td>STM3576</td>
<td>zntA</td>
<td>Pb/Cd/Zn/Hg-transporting ATPase</td>
<td>Glass 5.600 down</td>
</tr>
<tr>
<td>202</td>
<td>STM1956</td>
<td>fliA</td>
<td>flagellar biosynthesis sigma factor FliA</td>
<td>Glass 5.594 down</td>
</tr>
<tr>
<td>203</td>
<td>STM2796</td>
<td>yqaE</td>
<td>transporter</td>
<td>Glass 5.525 up</td>
</tr>
<tr>
<td>No</td>
<td>Entry</td>
<td>Gene name</td>
<td>Definition</td>
<td>Fold change planktonic vs. biofilm</td>
</tr>
<tr>
<td>----</td>
<td>-----------</td>
<td>-----------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>204</td>
<td>STM1961</td>
<td>fliS</td>
<td>flagellar protein FliS</td>
<td>5.522 down</td>
</tr>
<tr>
<td>205</td>
<td>STM2050</td>
<td>pduO</td>
<td>propanediol utilization protein</td>
<td>5.515 up</td>
</tr>
<tr>
<td>206</td>
<td>STM1171</td>
<td>fliN</td>
<td>FlgK/FlgL export chaperone</td>
<td>5.488 down</td>
</tr>
<tr>
<td>207</td>
<td>STM2558</td>
<td>cadB</td>
<td>lysine/cadaverine antiporter</td>
<td>5.486 up</td>
</tr>
<tr>
<td>208</td>
<td>STM1491</td>
<td></td>
<td>proline/glycine betaine transport systems</td>
<td>5.477 up</td>
</tr>
<tr>
<td>209</td>
<td>STM1928</td>
<td>otsA</td>
<td>trehalose-6-phosphate synthase</td>
<td>5.465 up</td>
</tr>
<tr>
<td>210</td>
<td>STM2164</td>
<td>yehY</td>
<td>ABC-type proline/glycine betaine transport system</td>
<td>5.432 up</td>
</tr>
<tr>
<td>211</td>
<td>STM1177</td>
<td>flgE</td>
<td>flagellar hook protein</td>
<td>5.409 down</td>
</tr>
<tr>
<td>212</td>
<td>STM1265</td>
<td></td>
<td>putative response regulator</td>
<td>5.408 up</td>
</tr>
<tr>
<td>213</td>
<td>STM1318</td>
<td>katE</td>
<td>hydroperoxidase HPII</td>
<td>5.374 up</td>
</tr>
<tr>
<td>214</td>
<td>STM1962</td>
<td>fliT</td>
<td>flagellar biosynthesis protein FliT</td>
<td>5.339 down</td>
</tr>
<tr>
<td>215</td>
<td>STM2065</td>
<td>phsA</td>
<td>thiosulfate reductase precursor</td>
<td>5.309 up</td>
</tr>
<tr>
<td>216</td>
<td>STM2847</td>
<td>hycG</td>
<td>hydrogenase</td>
<td>5.297 up</td>
</tr>
<tr>
<td>217</td>
<td>STM0581</td>
<td></td>
<td>putative regulatory protein</td>
<td>5.294 up</td>
</tr>
<tr>
<td>218</td>
<td>STM2806</td>
<td>nrdl</td>
<td>ribonucleotide reductase stimulatory protein</td>
<td>5.270 down</td>
</tr>
<tr>
<td>219</td>
<td>STM2028</td>
<td>cbiG</td>
<td>cobalamin biosynthesis protein CbiG</td>
<td>5.234 up</td>
</tr>
<tr>
<td>220</td>
<td>STM1833</td>
<td></td>
<td>hypothetical protein</td>
<td>5.227 up</td>
</tr>
<tr>
<td>221</td>
<td>STM2851</td>
<td>hycC</td>
<td>NADH dehydrogenase subunit N</td>
<td>5.201 up</td>
</tr>
<tr>
<td>222</td>
<td>STM0384</td>
<td>psiF</td>
<td>phosphate starvation-inducible protein</td>
<td>5.192 up</td>
</tr>
<tr>
<td>223</td>
<td>STM4464</td>
<td></td>
<td>putative arginine repressor</td>
<td>5.189 up</td>
</tr>
<tr>
<td>224</td>
<td>STM1974</td>
<td>fliK</td>
<td>flagellar hook-length control protein</td>
<td>5.159 down</td>
</tr>
<tr>
<td>225</td>
<td>STM3778</td>
<td></td>
<td>putative DNA-binding protein</td>
<td>5.147 down</td>
</tr>
<tr>
<td>226</td>
<td>STM2049</td>
<td>pduN</td>
<td>polyhedral body protein</td>
<td>5.129 up</td>
</tr>
<tr>
<td>227</td>
<td>STM4535</td>
<td></td>
<td>PTS permease</td>
<td>5.095 up</td>
</tr>
<tr>
<td>228</td>
<td>STM0582</td>
<td>ybdJ</td>
<td>putative inner membrane protein</td>
<td>5.065 up</td>
</tr>
<tr>
<td>229</td>
<td>STM2454</td>
<td>eutR</td>
<td>ethanolamine operon regulator</td>
<td>5.045 up</td>
</tr>
<tr>
<td>230</td>
<td>STM2278</td>
<td>nrdB</td>
<td>ribonucleotide-diphosphate reductase beta subunit</td>
<td>5.028 down</td>
</tr>
<tr>
<td>231</td>
<td>STM1996</td>
<td>cspB</td>
<td>cold-shock protein</td>
<td>4.921 up</td>
</tr>
<tr>
<td>232</td>
<td>STM2027</td>
<td>cbiH</td>
<td>precorrin-3B C17-methyltransferase</td>
<td>4.912 up</td>
</tr>
<tr>
<td>233</td>
<td>STM2259</td>
<td>napA</td>
<td>periplasmic nitrate reductase</td>
<td>4.801 down</td>
</tr>
<tr>
<td>234</td>
<td>STM2057</td>
<td>pduW</td>
<td>acetate/propionate kinase</td>
<td>4.724 up</td>
</tr>
<tr>
<td>235</td>
<td>STM0489</td>
<td>hemH</td>
<td>ferrochelatase</td>
<td>4.685 up</td>
</tr>
<tr>
<td>236</td>
<td>STM3909</td>
<td>ilvC</td>
<td>ketol-acid reductoisomerase</td>
<td>4.679 down</td>
</tr>
<tr>
<td>237</td>
<td>STM2026</td>
<td>cbiJ</td>
<td>precorrin-6x reductase</td>
<td>4.676 up</td>
</tr>
<tr>
<td>238</td>
<td>STM2260</td>
<td>napD</td>
<td>periplasmic nitrate reductase</td>
<td>4.636 down</td>
</tr>
<tr>
<td>239</td>
<td>STM2032</td>
<td>cbiD</td>
<td>cobalt-precorrin-6A synthase</td>
<td>4.442 up</td>
</tr>
<tr>
<td>No</td>
<td>Entry</td>
<td>Gene name</td>
<td>Definition</td>
<td>Fold change planktonic vs. biofilm</td>
</tr>
<tr>
<td>----</td>
<td>---------</td>
<td>-----------</td>
<td>------------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Glass down</td>
</tr>
<tr>
<td>240</td>
<td>STM2261</td>
<td>napF</td>
<td>electron transfer protein</td>
<td>4.384</td>
</tr>
<tr>
<td>241</td>
<td>STM0067</td>
<td>carB</td>
<td>carbamoyl-phosphate synthase large subunit</td>
<td>4.359</td>
</tr>
<tr>
<td>242</td>
<td>STM0442</td>
<td>cyoB</td>
<td>cytochrome o ubiquinol oxidase subunit I</td>
<td>4.346</td>
</tr>
<tr>
<td>243</td>
<td>STM2022</td>
<td>cbiN</td>
<td>cobalt transport protein CbiN</td>
<td>4.305</td>
</tr>
<tr>
<td>244</td>
<td>STM3477</td>
<td>cysG</td>
<td>siroheme synthase</td>
<td>4.154</td>
</tr>
<tr>
<td>245</td>
<td>STM0439</td>
<td>cyoE</td>
<td>protoheme IX farnesytransferase</td>
<td>4.145</td>
</tr>
<tr>
<td>246</td>
<td>STM4466</td>
<td></td>
<td>carbamate kinase</td>
<td>4.109</td>
</tr>
<tr>
<td>247</td>
<td>STM3898</td>
<td>yifE</td>
<td>putative transcriptional regulator</td>
<td>4.057</td>
</tr>
<tr>
<td>248</td>
<td>STM2962</td>
<td>gudT</td>
<td>D-glucarate permease</td>
<td>3.972</td>
</tr>
<tr>
<td>249</td>
<td>STM0362</td>
<td></td>
<td>putative cytoplasmic protein</td>
<td>3.906</td>
</tr>
<tr>
<td>250</td>
<td>STM0708</td>
<td>ybfA</td>
<td>putative periplasmic protein</td>
<td>3.797</td>
</tr>
<tr>
<td>251</td>
<td>STM2044</td>
<td>pduH</td>
<td>propanediol dehydratase reactivation protein</td>
<td>3.737</td>
</tr>
<tr>
<td>252</td>
<td>STM0490</td>
<td>aes</td>
<td>acetyl esterase</td>
<td>3.668</td>
</tr>
<tr>
<td>253</td>
<td>STM0441</td>
<td>cyoC</td>
<td>cytochrome o ubiquinol oxidase subunit III</td>
<td>3.328</td>
</tr>
<tr>
<td>254</td>
<td>STM2408</td>
<td>mntH</td>
<td>manganese transport protein MntH</td>
<td>2.993</td>
</tr>
<tr>
<td>255</td>
<td>STM1787</td>
<td></td>
<td>hydrogenase-1 large subunit</td>
<td>2.918</td>
</tr>
<tr>
<td>256</td>
<td>STM1793</td>
<td></td>
<td>putative cytochrome oxidase subunit II</td>
<td>2.774</td>
</tr>
</tbody>
</table>
Appendix B: Biofilms from Type 1 fimbriae and ycfR mutants at 72 hours post-inoculation.

Representative confocal images of biofilms on cholesterol coated-surfaces produced by the wild type, mutants and complemented strains after 72h of flow in the presence of bile. Biofilms were stained with live/dead stain (Invitrogen), fixed with 4% paraformaldehyde and observed by confocal microscopy. All strains harbor the empty vector (pWSK29 or pWSK129) or the respective complementation vector (pGGE1 and pGGE2 for fimAICDHF and ycfR, respectively). All images have a magnification of 40x. All mutants showed more biomass but similar thickness as the wild-type. ∆ycfR also showed increased cell damage/death (increased red staining). The complemented strains demonstrated a reduction of biofilm thickness and biomass.