NOVEL GENETIC REGULATORS AND THE INTESTINAL CHEMICAL SIGNAL PROPIONATE CONTROL *SALMONELLA* INVASION GENE EXPRESSION

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Chien-Che Hung

January 2012

NOVEL GENETIC REGULATORS AND THE INTESTINAL CHEMICAL SIGNAL PROPIONATE CONTROL *SALMONELLA* INVASION GENE EXPRESSION

Chien-Che Hung, Ph.D.

Cornell University 2012

Salmonella is an important enterobacterial pathogen causing diseases ranging from mild enteritis to severe systemic infection. To cause disease, it needs to invade the intestinal epithelium using genes encoded within the *Salmonella* pathogenicity Island 1 (SPI1), which comprise a type III section system (TTSS) to translocate effector proteins into host cells. In this thesis, we investigated the effects of three novel genetic regulators FliT, Pat, and CobB, and the intestinal chemical signal propionate on SPI1. Further, we used molecular and genetic approaches to study the associated mechanisms by which these internal genetic elements and the external signal propionate control *Salmonella* invasion.

SPI1 gene expression has been shown to be controlled by complex interrelated regulatory networks and involves numerous regulators inside and outside SPI1. Using a random transposon mutagenesis strategy, we discovered that a novel genetic regulator FliT, encoded within the *fliDST* operon, can negatively control SPI1 gene expression. Since FliT is known to be a negative regulator of the flagellar regulon, we used genetic approaches to investigate the roles of additional flagellar regulators controlled by FliT in the repression of invasion. We found that deletion of *flhDC* or the *flhDC*-regulated gene, *fliZ,* completely abrogated the negative effect of FliT, suggesting that FliT controls *Salmonella* invasion through this *flhDC–fliZ* route.

Our previous studies have shown that propionate, an intestinal short chain fatty acid found predominantly in the large intestine, represses SPI1 gene expression. To further understand the mechanism by which propionate represses *Salmonella* invasion, we examined

the importance of intestinal pH in this repressive effect, and investigated the metabolic and genetic pathways required for this fatty acids to control SPI1 gene expression. We found that the repressive effect was observed at mildly acidic, physiologically relevant pH, but not under alkaline conditions, suggesting that propionate must enter the bacterial cytoplasm to have its effects. Additionally, we found that metabolism of propionate is essential for its repressive effect, and the high-energy metabolic intermediate propionyl-CoA is apparently required. Using genetic approaches, we also found that the repressive effect of propionate is through the central SPI1 regulator *hilD*. However, propionate control of HilD is through neither transcription nor translation, suggesting a post-translational modification of HilD may be required for regulation.

Previous studies have shown that Pat and CobB act as post-translational regulators and control the function of their targets through N-lysine acyl modification. Interestingly, the known targets of Pat and CobB in *Salmonella* are PrpE and Acs that play roles in fatty acid (propionate and acetate) metabolic pathways. As the intestinal short chain fatty acids are known to affect SPI1, we examined whether Pat and CobB affect *Salmonella* invasion. We found that Pat and CobB were able to repress and induce SPI1 expression, respectively, when over-expressed. However, using genetic approaches, we demonstrated that their effects on invasion were not through their known targets PrpE and Acs. Instead, HilD and Lon are required for their control on SPI1. These results suggest that Pat and CobB may work through the modification of HilD and its subsequent degradation by Lon protease, and consequently may affect SPI1 gene expression.

BIOGRAPHICAL SKETCH

Chien-Che Hung was born in 1976 in Kaohsiung, Taiwan. He attended National Chung-Hsing University, Taichung, Taiwan where he received his Doctor of Veterinary Medicine degree in 1999 and a Master degree in Veterinary Pathology in 2001. He studied at the College of Veterinary Medicine at North Carolina State University as a Ph.D. student in the field of Comparative Biomedical Science in 2005. He joined the lab of Dr. Craig Altier in the winter of 2005 and transferred to the College of Veterinary Medicine at Cornell University with his advisor in the summer of 2006.

DEDICATION

我謹以此論文獻給我親愛的父母洪漢濱先生與許淑瑛女士，我的女友蕭君倪，我的妹妹洪琳雅。沒有你們的陪伴與支持，此論文將無法順利完成。同時我也要以此論文告慰在天上的爺爺奶奶洪秋木先生與洪戴春花女士還有我的外公許振芳先生。謝謝妳們給我的一切，讓我得以出國留學並順利的完成學業。

ACKNOWLEDGMENTS

I would like to thank my advisor Dr. Craig Altier for his patience, encouragement, and immense knowledge. Without his guidance I would not have become a qualified bacteriologist and an independent scientist. I would also like to thank Mitsu Suyemoto for her expertise with experiment, her encouragement and above all her friendship, my labmates Cherilyn Garner, Yanyan Huang, Kellie Cicconi for their wise counsel and friendship, Leanne Haines for assisting with experimental execution, and Julie Siler for providing critical review of manuscripts and helping with grammar correction of my thesis. I would also like to thank Dr. Jonathan Frye, who provided *Salmonella* LT2 microarray and expert mentoring in experimental design, execution, and data analysis, Dr. Sara Lawhon, Dr. Cherilyn Garner and Zach Dwyer for contributing several valuable results in the propionate study. I would like to thank Dr. Brian Ahmer who shared the invasion gene-lux fusions, and Dr. James Slauch who provided *hilD*-transcriptional and -translational fusions.

Finally, I would like to thank the members of my committee Dr. Marci Scidmore, Dr. Ailong Ke, and Dr. John Parker for their insight and advice, and for sharing their experience.

This work was partially supported by Cornell funding to Dr. Craig Altier and the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, award number 2005-35201-16270.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Importance of salmonellosis in public health

Salmonellosis is one of the most significant causes of gastroenteritis in the United States, causing 1.4 million infections each year (13). Based on reports from the Centers for Disease Control and Prevention (CDC) and the World Health Organization (79), about 15,000 *Salmonella* infected patients require hospitalization with more than 500 deaths reported every year (11, 13, 79). Most patients develop symptoms such as fever, vomiting, diarrhea, abdominal cramps, and nausea within 1 to 3 days after infection and recover completely within 4 to 7 days without treatment. Some patients develop Reiter's syndrome, including symptoms such as arthritis, redness of the eyes, and painful urination. Infections caused by *Salmonella* mainly result from consumption of *Salmonella*-contaminated foods including meats, water, eggs, and vegetables. To date, >2500 different serotypes have been identified based on different bacterial surface antigens (O antigen and H antigen). In the United States, *Salmonella enterica* serovar Typhimurium (19%) and *Salmonella enterica* serovar Enteritidis (14%) are the two most common serotypes isolated form human patients (11). As such, salmonellosis is a significant issue in public health, particularly in cases where *Salmonella enterica* serovar Typhimurium is the causative agent in *Salmonella* outbreaks.

***Salmonella* invasion of intestinal epithelial cells**

In the infection process, *Salmonella* needs to first penetrate the intestinal epithelium. Most of the genes required for this invasion process are located on a 40 kb gene cluster at centisome 63 termed *Salmonella* Pathogenicity Island I (SPI1) (8, 32, 37, 44, 55, 61). The genes on SPI1 encode proteins required for the synthesis of a type III secretion apparatus, the needle complex, which spans the bacterial inner and outer membranes and translocates secreted effector proteins into the host cell cytoplasm. Once these proteins are delivered into a targeted epithelial cell, they induce cytoskeleton rearrangement and membrane ruffling, leading to internalization of *Salmonella* organisms into the host cell (16, 31, 38, 48, 61, 82).

Invasion gene regulatory cascade within SPI1

SPI1 genes are known to be controlled by several transcriptional regulator proteins encoded within SPI1 (Fig. 1.1). In the SPI1 gene regulatory cascade, HilA, an OmpR family protein induces the *prg/org* and *inv/spa* operons that encode proteins of the type III secretion apparatus and a second transcriptional regulator, InvF (18, 19, 21, 46). Also, HilA activates the *sic/sip* operon which encodes effector proteins via the read-through transcription of the *inv/spa* operons (18, 21). InvF induces the expression of downstream SPI1 effector proteins encoded by the *sipBCDA* operon and other effectors outside SPI1, such as SopB, SopE and SigD (18, 20, 21).

The expression of *hilA* is controlled by three transcriptional regulators, HilD, HilC, and RtsA (24, 66, 72). *hilD* and *hilC* both are within SPI1, whereas *rtsA* is located at centisome 93.3 (24). These regulators have been shown to bind to the promoter of *hilA* and directly induce *hilA* expression (24, 66, 72). Furthermore, HilD, HilC, and RtsA are able to regulate their own gene expression and activate expression of *hilD*, *hilC* and *rtsA* independently of each other to constitute a regulatory circuit for controlling *hilA* expression (23). Among these regulators, it has been suggested that HilD is at the top of the hierarchy and controls *hilC* and *rtsA* expression (25). In addition, a *hilD* mutant is unable to invade epithelial cells and significantly reduces *hilA* expression, indicating that *hilD* is important for *Salmonella* invasion (71). However, the mutation of *hilC* merely causes a slight decrease in invasion and modestly decreases SPI1 gene expression, but over-expression of *hilC* increases invasion gene expression (22). Thus, the importance of *hilC* in *Salmonella* invasion is still unclear. In addition, HilD and HilC have been shown to induce the expression of the *sipBCDA* operon by directly controlling *invF,* where HilD and HilC bind to a second promoter of *invF* not used by HilA (1).

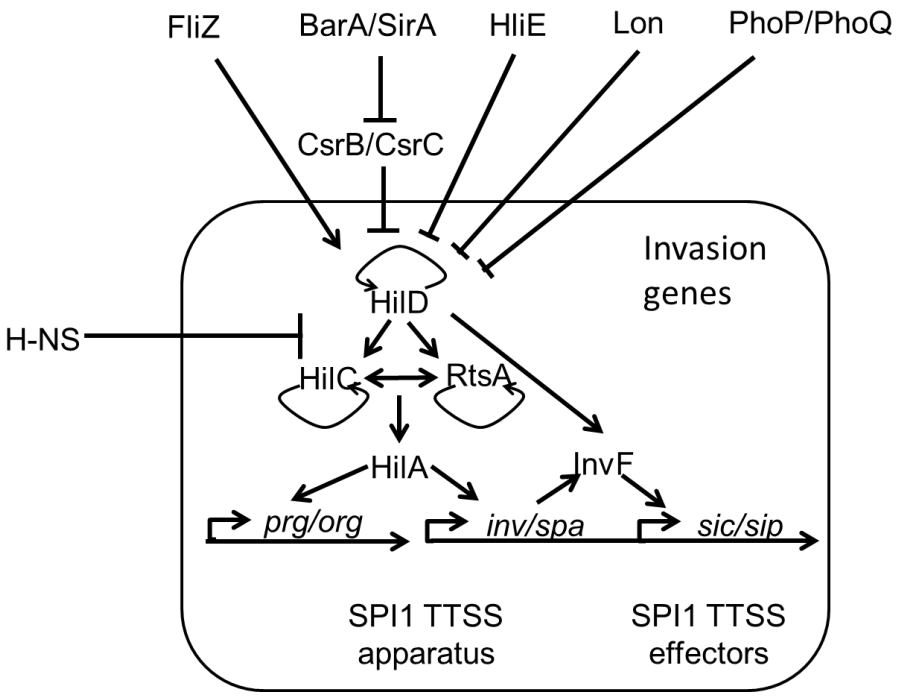

Figure 1.1. *Salmonella* invasion gene regulatory cascade.

SPI1 refers to the Salmonella Pathogenicity Island 1. TTSS refers to the Type Three Secretion System.

Invasion regulators outside SPI1

Several genetic regulators outside SPI1 have also been shown to regulate SPI1 gene expression (Fig. 1.1). The BarA/SirA two-component system (TCS) has been shown to positively control invasion gene expression through the control of the Csr (carbon storage regulator) system. The Csr system is constituted by CsrA, an RNA-binding protein, that binds to the *hilD* mRNA and prevents the translation of this message, and two small RNAs, CsrB and CsrC, that interacted with CsrA and titrate it from the *hilD* message. CsrB and CsrC are two targets of SirA. Thus, BarA/SirA positively regulates SPI1 gene expression by inducing CsrB and CsrC, both of which inhibit the activity of CsrA, increasing the level of HilD, and subsequently induce SPI1 gene expression (2, 3, 29, 53, 59). FliZ, a class 2 flagellar gene, can

positively regulate SPI1 gene expression. Previous studies have shown that FliZ positively regulates invasion genes thorough post-transcriptional control of HilD (15, 45). Additionally, PhoP/PhoQ, HilE, and Lon have been shown to negatively control SPI1 (6, 7, 26, 74, 77, 78). PhoP/PhoQ is a two-component system that primarily regulates *Salmonella* Pathogenicity Island 2, which is essential for intracellular survival (28). Activation of PhoP/PhoQ has been shown to negatively control SPI1 though HilA (6, 74). HilE is suggested to interact with HilD and prevent it from inducing the expression of downstream invasion genes (7, 26). Lon is an ATP–dependent protease and is suggested to play roles in the degradation of HilD and HilC (77, 78). In addition to the above regulators, the global regulator H-NS, a histone-like protein, has been shown to negatively control SPI1. H-NS has been suggested to bind to multiple sites on the AT-rich SPI1 DNA, bending the DNA and silencing the transcription of SPI1 genes (27, 62, 63).

Regulation of flagellar gene expression

The flagellar regulon of *Salmonella* contains more than 60 genes, which build a flagellar type III secretion system allowing this bacterium to construct flagella on its surface ((30) and reviewed in reference (14)). In this regulon, the *flhDC* operon is characterized as the sole class1 flagellar gene encoding proteins of the flagellar master regulator $FlhD_4C_2$ (50, 81). This regulator is at the top of the hierarchy of the flagellar regulon and functions as a transcriptional activator, promoting transcription from class 2 flagellar genes (56, 57). Most class 2 genes encode proteins required for the structure and assembly of the hook-basal body (HBB) of the flagellar type III secretion apparatus. In addition, several transcriptional regulators such as FliA, FliZ, FlgM, and FliT are involved in this group (36, 49, 64). FliA is a flagellar-specific transcription factor, σ^{28}, which activates transcription of the class 3 genes that encode proteins needed for the flagellar filaments and for bacterial swimming and chemotaxis (30, 64). FliZ is a minor activator of the class 2 flagellar genes, and it has been suggested to activate flagellar genes though the post-translational control of $FlhD_4C_2$ (49, 70). FlgM is an anti-FliA factor,

which associates with FliA and prevents FliA from activating class 3 genes before the HBB is fully constructed on the bacterial surface (47, 65). When the HBB is made, FlgM is exported from the bacterial cytoplasm to the culture media though the HBB, relieving its inhibitory effect on FliA, and consequently allowing FliA to activate the transcription of class 3 genes (40, 47, 65). FliT is an important negative regulator in the flagellar regulon. Previous studies have shown that FliT represses flagellar gene expression by post-translationally controlling the activity of $FlhD_4C_2$, in which FliT interacts with $FlhD_4C_2$ and prevents this flagellar master regulator from binding to the promoter of class 2 genes (42, 49, 80). Among the above regulators, $FlhD_4C_2$ and FliZ have been shown to positively regulate *Salmonella* invasion (15, 58). FliZ has been shown to affects invasion through the post-transcriptional control of SPI1 regulator HilD (15, 45).

Environmental conditions affecting invasion gene expression

In addition to interior genetic regulators, numerous *in vitro* growth conditions that mimic the animal intestinal environment can induce invasion gene expression, including high osmolality, low oxygen and near the neutral pH (6, 73). Importantly, our work and previous studies have shown that the short chain fatty acids (SCFAs) normally present in the intestinal tract affect *Salmonella* invasion; specifically, formate and acetate induce invasion gene expression, while propionate and butyrate repress it (10, 33, 41, 54). It is known that weak acids can diffuse into the cell and concentrate inside the cytoplasm when the external pH is lower than the internal pH. Thus, for formate and acetate, both weak acids, to affect invasion gene expression, pH values (pH 6.7) slightly below the neutral pH are required. Additionally, it has been shown that the effects of these SCFAs on invasion gene expression were abrogated at pH 8.0 (41, 54). Thus, these results suggest that these fatty acids need to diffuse into *Salmonella* and accumulate inside the cytoplasm to exhibit their effects. The mechanism by which acetate induces invasion gene expression is through SirA, the response regulator of the BarA/SirA TCS (3, 43), but not though its corresponding sensor kinase BarA (54). In addition,

the production of acetyl-phosphate in the acetate metabolic pathway is required for acetate to induce SPI1 gene expression. Therefore, it has been suggested that acetate induces invasion by working through the production of acetyl-phosphate and subsequently activates the positive invasion regulator SirA via phosphorylation (54). The mechanism by which formate induces SPI1 gene expression is still uncharacterized. However, it has been shown that neither BarA nor SirA is required for formate control on invasion (41).

In various mammalian species, formate, acetate, and propionate are found in the distal small intestine (ileum), while acetate, propionate and butyrate predominate in the large intestine (cecum and colon) (4, 5, 9, 17, 34, 51, 52, 60). Previous studies have shown that the distal intestine is the site where salmonellosis normally occurs (12, 44). This phenomenon shows a correlation between the location where formate and acetate predominately exist and the known positive effect of these two fatty acids on invasion. In contrast to the finding in the small intestine, conditions in the large intestine apparently inhibit *Salmonella* invasion (54). In our previous work, using a combination of acetate, propionate and butyrate to mimic the conditions of large intestine, we demonstrated that this mixture of SCFAs represses invasion gene expression, and the repressing effects of the latter two outweigh the inducing effect of the former (54). Additionally, *in vivo* studies showed that streptomycin-treated mice had significantly reduced SCFAs in the large intestine and show a correlation with the increased susceptibility to *Salmonella* infection (34). Thus, these results suggest that SCFAs are important environmental cues to control *Salmonella* invasion in the host intestine.

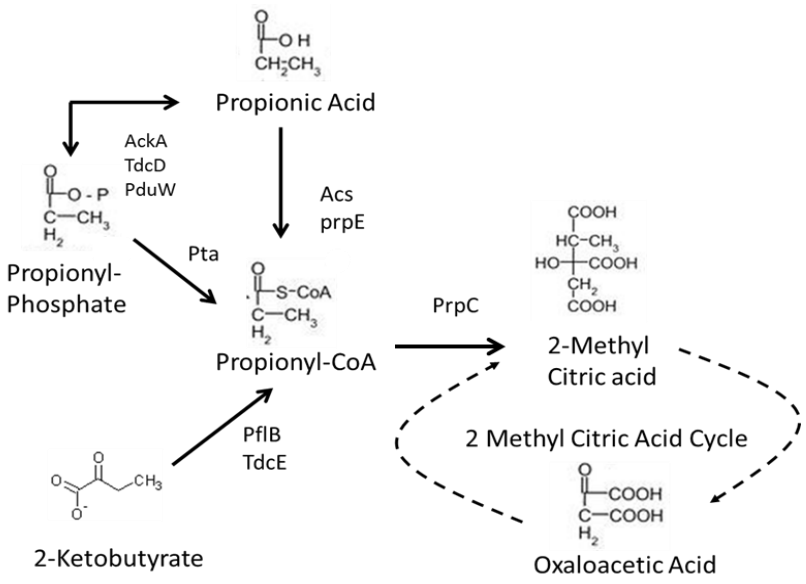

Figure 1.2. Propionate metabolic pathways.

Metabolic pathways of propionate

The metabolic fate of propionate in *Salmonella* involves complex and redundant pathways (Fig. 1.2). When propionate is supplied exogenously, it is converted into the high-energy metabolic intermediate propionyl-CoA through either of two known routes, the propionate kinase/ phosphotransacetylase (39) pathway or the propionyl-CoA synthetase pathway (39, 69). In the former pathway, propionate is first converted into propionyl-phosphate by propionate kinases encoded by *ackA, tdcD,* and *pdeW*, and this intermediate is metabolized into propionyl-CoA through phosphotransacetylase, encoded by *pta*. The propionyl-CoA synthetase pathway metabolizes propionate directly to propionyl-CoA by using PrpE and Acs (39, 69). In addition to the above two routes, propionyl-CoA can also be produced from endogenously synthesized 2-ketobutyrate via TdcE and PflB (39). Once propionyl-CoA is produced, it is metabolized to 2-methyl citric acid by PrpC, and this intermediate further feeds into the 2-methyl citric acid cycle (67-69).

Pat and CobB regulate the activity of PrpE and Acs via N-lysine acyl-modification.

Post-translational modification of proteins is an important means to manipulate the function of regulatory proteins and the activity of enzymes. Interestingly, enzymes needed in fatty acid metabolism, PrpE and Acs, are known to be modified by Pat and CobB through N-lysine acyl-modification (35, 75, 76). PrpE functions as a propionyl-CoA synthase to catalyze propionate to propionyl-CoA (67, 76). In addition, Acs can act as an acetyl-CoA synthase to convert acetate to acetyl-CoA (74). Previous *in vitro* studies have shown that Pat can use propionyl-CoA or acetyl-CoA as its substrate and transfer the acyl group to the specific lysine residue of PrpE, which inactivates its enzyme activity (35). Similar to PrpE, the enzyme activity of Acs is known to be inhibited by Pat through N-lysine acetylation (75). In contrast to Pat, CobB, a Sir2 family protein, acts as a depropionylase or a deacetylase. CobB reversely removes the acyl group from the propionylated or acetylated lysine of PrpE and Acs and restores their functions (35, 75, 76). Thus, these results suggest that Pat and CobB can modulate the level of propionyl-CoA and Acetyl-CoA inside the bacterial cytoplasm by controlling the activity of PrpE and Acs by N-acyl modification of these two enzymes.

Summary of results

In this work, we investigated the effects of three novel genetic elements FliT, Pat and CobB, and the intestinal chemical signal propionate on *Salmonella* invasion gene (SPI1 gene) expression. Further, we used molecular and genetic approaches to study the associated mechanisms by which these genetic regulators and propionate control *Salmonella* invasion.

In chapter 2, we used a random transposon mutagenesis stratagem to seek a new genetic regulator that can induce invasion gene expression under conditions designed to repress SPI1. We found that the disruption of *fliT* can induce SPI1 expression under our designed SPI1 repressing conditions, indicating that this flagellar gene negatively controls invasion. As FliT is known to be a negative regulator of the flagellar regulon, we used genetic

approaches to investigate the roles of additional flagellar regulators controlled by FliT in the repression of invasion. We found that two of the flagellar regulators $FlhD_4C_2$ and FliZ are required for this repressive effect, suggesting that FliT controls SPI1 through this *flhDC-fliZ* pathway.

Our previous work has demonstrated that the intestinal short chain fatty acid propionate can repress SPI1 gene expression (54). Thus, in chapter 3, we further studied the mechanism by which propionate negatively controls SPI1. Using microarray approaches, we found that this fatty acid predominately repressed SPI1 genes, but this repressive effect was not seen with the fatty acid acetate, indicating this repressive effect on invasion is specific to propionate. We also found the propionate-induced repression was observed at mildly acidic, physiologically relevant pH, but not under alkaline conditions, suggesting that propionate must enter the bacterial cytoplasm to have its effects. Additionally, using genetic approaches, we found the metabolism of propionate was essential for its repressive effect on invasion. Also, the high energy intermediate propionyl-CoA was apparently important for this effect. Further, we investigated the roles of the SPI1 regulators HilD, SirA, FliZ, HilA, HilE and Lon in the propionate control of invasion. Genetic studies indicated that among these regulators *hilD* was required for propionate to control invasion gene expression. However, regulation via *hilD* was not achieved through the control of either transcription or translation of this gene, indicating a post-translational mechanism of control.

Previous studies have shown that Pat and CobB act as post-translational regulators and control the function of their targets through N-lysine acyl-modification (35, 75, 76). Interestingly, the known targets of Pat and CobB in *Salmonella* are PrpE and Acs that play roles in fatty acid (propionate and acetate) metabolic pathways (35, 67, 75, 76). Since the intestinal short chain fatty acids are known to affect SPI1 gene expression, in chapter 4 we investigated the effect of Pat and CobB on SPI1 gene expression. We showed that over-expression of Pat induced SPI 1 gene expression, while over-expression of CobB repressed it. We demonstrated that the effects

of Pat and CobB on SPI1 were not through their known targets PrpE and Acs. Instead, HilD and Lon are required for their effects, while other SPI1 regulators SirA, FliZ, HilA, are dispensable. These results suggest that Pat and CobB may work through the modification of HilD and its subsequent degradation by Lon protease, consequently affecting SPI1 gene expression**.**

REFERENCES

1. **Akbar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee.** 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in Salmonella typhimurium. Molecular Microbiology **47:**715-28.
2. **Altier, C., M. Suyemoto, and S. D. Lawhon.** 2000. Regulation of Salmonella enterica serovar typhimurium invasion genes by csrA. Infection and Immunity **68:**6790-7.
3. **Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer.** 2000. Characterization of two novel regulatory genes affecting Salmonella invasion gene expression. Molecular Microbiology **35:**635-46.
4. **Argenzio, R. A., and M. Southworth.** 1975. Sites of organic acid production and absorption in gastrointestinal tract of the pig. Am J Physiol **228:**454-60.
5. **Argenzio, R. A., M. Southworth, and C. E. Stevens.** 1974. Sites of organic acid production and absorption in the equine gastrointestinal tract. Am J Physiol **226:**1043-50.
6. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Molecular Microbiology **22:**703-14.
7. **Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones.** 2003. HilE interacts with HilD and negatively regulates hilA transcription and expression of the Salmonella enterica serovar Typhimurium invasive phenotype. Infection and Immunity **71:**1295-305.

8. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells. Journal of Bacteriology **175:**4475-84.

9. **Bohnhoff, M., C. P. Miller, and W. R. Martin.** 1964. Resistance of the Mouse's Intestinal Tract to Experimental Salmonella Infection. I. Factors Which Interfere with the Initiation of Infection by Oral Inoculation. J Exp Med **120:**805-16.

10. **Boyen, F., F. Haesebrouck, A. Vanparys, J. Volf, M. Mahu, F. Van Immerseel, I. Rychlik, J. Dewulf, R. Ducatelle, and F. Pasmans.** 2008. Coated fatty acids alter virulence properties of Salmonella Typhimurium and decrease intestinal colonization of pigs. Vet Microbiol **132:**319-27.

11. **Braden, C. R.** 2006. Salmonella enterica serotype Enteritidis and eggs: a national epidemic in the United States. Clin Infect Dis **43:**512-7.

12. **Carter, P. B., and F. M. Collins.** 1974. The route of enteric infection in normal mice. J Exp Med **139:**1189-203.

13. **CDC.** 2008. Diseases Listing: Salmonellosis, http://www.cdc.gov/nczved/dfbmd/disease_listing/salmonellosis_gi.html.

14. **Chilcott, G. S., and K. T. Hughes.** 2000. Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and Escherichia coli. Microbiol Mol Biol Rev **64:**694-708.

15. **Chubiz, J. E., Y. A. Golubeva, D. Lin, L. D. Miller, and J. M. Slauch.** 2010. FliZ regulates expression of the Salmonella pathogenicity island 1 invasion locus by controlling HilD protein activity in Salmonella enterica serovar typhimurium. J Bacteriol **192:**6261-70.

16. **Collazo, C. M., and J. E. Galan.** 1997. The invasion-associated type-III protein secretion system in Salmonella--a review. Gene **192:**51-9.

17. **Cummings, J. H.** 1981. Short chain fatty acids in the human colon. Gut **22:**763-79.

18. **Darwin, K. H., and V. L. Miller.** 1999. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in Salmonella typhimurium. Journal of Bacteriology **181:**4949-54.

19. **Darwin, K. H., and V. L. Miller.** 1999. Molecular basis of the interaction of Salmonella with the intestinal mucosa. Clin Microbiol Rev **12:**405-28.

20. **Darwin, K. H., and V. L. Miller.** 2001. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in Salmonella typhimurium. EMBO J **20:**1850-62.

21. **Eichelberg, K., and J. E. Galan.** 1999. Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA. Infection and Immunity **67:**4099-105.

22. **Eichelberg, K., W. D. Hardt, and J. E. Galan.** 1999. Characterization of SprA, an AraC-like transcriptional regulator encoded within the Salmonella typhimurium pathogenicity island 1. Molecular Microbiology **33:**139-52.

23. **Ellermeier, C. D., J. R. Ellermeier, and J. M. Slauch.** 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. Mol Microbiol **57:**691-705.

24. **Ellermeier, C. D., and J. M. Slauch.** 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in Salmonella enterica serovar Typhimurium. Journal of Bacteriology **185:**5096-108.

25. **Ellermeier, J. R., and J. M. Slauch.** 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. Curr Opin Microbiol **10:**24-9.

26. **Fahlen, T. F., N. Mathur, and B. D. Jones.** 2000. Identification and characterization of mutants with increased expression of hilA, the invasion gene transcriptional activator of Salmonella typhimurium. FEMS Immunol Med Microbiol **28:**25-35.

27. **Fang, F. C., and S. Rimsky.** 2008. New insights into transcriptional regulation by H-NS. Curr Opin Microbiol **11:**113-20.

28. **Fass, E., and E. A. Groisman.** 2009. Control of Salmonella pathogenicity island-2 gene expression. Curr Opin Microbiol **12:**199-204.

29. **Fortune, D. R., M. Suyemoto, and C. Altier.** 2006. Identification of CsrC and characterization of its role in epithelial cell invasion in Salmonella enterica serovar Typhimurium. Infect Immun **74:**331-9.

30. **Frye, J., J. E. Karlinsey, H. R. Felise, B. Marzolf, N. Dowidar, M. McClelland, and K. T. Hughes.** 2006. Identification of new flagellar genes of Salmonella enterica serovar Typhimurium. J Bacteriol **188:**2233-43.

31. **Fu, Y., and J. E. Galan.** 1998. The Salmonella typhimurium tyrosine phosphatase SptP is translocated into host cells and disrupts the actin cytoskeleton. Molecular Microbiology **27:**359-68.

32. **Galan, J. E., and R. Curtiss, 3rd.** 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc Natl Acad Sci U S A **86:**6383-7.

33. **Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, I. Hautefort, A. Thompson, J. C. Hinton, and F. Van Immerseel.** 2006. Butyrate specifically down-regulates salmonella pathogenicity island 1 gene expression. Appl Environ Microbiol **72:**946-9.

34. **Garner, C. D., D. A. Antonopoulos, B. Wagner, G. E. Duhamel, I. Keresztes, D. A. Ross, V. B. Young, and C. Altier.** 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a Salmonella enterica serovar typhimurium murine model of infection. Infect Immun **77:**2691-702.

35. **Garrity, J., J. G. Gardner, W. Hawse, C. Wolberger, and J. C. Escalante-Semerena.** 2007. N-lysine propionylation controls the activity of propionyl-CoA synthetase. J Biol Chem **282:**30239-45.

36. **Gillen, K. L., and K. T. Hughes.** 1991. Molecular characterization of flgM, a gene encoding a negative regulator of flagellin synthesis in Salmonella typhimurium. J Bacteriol **173:**6453-9.

37. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. Embo J **12:**3779-87.

38. **Hardt, W. D., H. Urlaub, and J. E. Galan.** 1998. A substrate of the centisome 63 type III protein secretion system of Salmonella typhimurium is encoded by a cryptic bacteriophage. Proc Natl Acad Sci U S A **95:**2574-9.

39. **Hesslinger, C., S. A. Fairhurst, and G. Sawers.** 1998. Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in Escherichia coli that degrades L-threonine to propionate. Mol Microbiol **27:**477-92.

40. **Hirano, T., T. Minamino, K. Namba, and R. M. Macnab.** 2003. Substrate specificity classes and the recognition signal for Salmonella type III flagellar export. J Bacteriol **185:**2485-92.

41. **Huang, Y., M. Suyemoto, C. D. Garner, K. M. Cicconi, and C. Altier.** 2008. Formate acts as a diffusible signal to induce Salmonella invasion. J Bacteriol **190:**4233-41.

42. **Imada, K., T. Minamino, M. Kinoshita, Y. Furukawa, and K. Namba.** 2010. Structural insight into the regulatory mechanisms of interactions of the flagellar type III chaperone FliT with its binding partners. Proc Natl Acad Sci U S A **107:**8812-7.

43. **Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller.** 1996. Transcriptional activation of Salmonella typhimurium invasion genes by a member of the phosphorylated response-regulator superfamily. Molecular Microbiology **22:**715-27.

44. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a Salmonella typhimurium oxygen-regulated gene required for bacterial internalization. Infection and Immunity **62:**3745-52.

45. **Kage, H., A. Takaya, M. Ohya, and T. Yamamoto.** 2008. Coordinated regulation of expression of Salmonella pathogenicity island 1 and flagellar type III secretion systems by ATP-dependent ClpXP protease. J Bacteriol **190:**2470-8.

46. **Kaniga, K., J. C. Bossio, and J. E. Galan.** 1994. The Salmonella typhimurium invasion genes invF and invG encode homologues of the AraC and PulD family of proteins. Molecular Microbiology **13:**555-68.

47. **Karlinsey, J. E., S. Tanaka, V. Bettenworth, S. Yamaguchi, W. Boos, S. I. Aizawa, and K. T. Hughes.** 2000. Completion of the hook-basal body complex of the Salmonella typhimurium flagellum is coupled to FlgM secretion and fliC transcription. Mol Microbiol **37:**1220-31.

48. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science **280:**602-5.

49. **Kutsukake, K., T. Ikebe, and S. Yamamoto.** 1999. Two novel regulatory genes, fliT and fliZ, in the flagellar regulon of Salmonella. Genes Genet Syst **74:**287-92.

50. **Kutsukake, K., Y. Ohya, and T. Iino.** 1990. Transcriptional analysis of the flagellar regulon of Salmonella typhimurium. J Bacteriol **172:**741-7.

51. **Laerke, H. N., and B. B. Jensen.** 1999. D-tagatose has low small intestinal digestibility but high large intestinal fermentability in pigs. J Nutr **129:**1002-9.

52. **Laerke, H. N., B. B. Jensen, and S. Hojsgaard.** 2000. In vitro fermentation pattern of D-tagatose is affected by adaptation of the microbiota from the gastrointestinal tract of pigs. J Nutr **130:**1772-9.

53. **Lawhon, S. D., J. G. Frye, M. Suyemoto, S. Porwollik, M. McClelland, and C. Altier.** 2003. Global regulation by CsrA in Salmonella typhimurium. Molecular Microbiology **48:**1633-45.

54. **Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier.** 2002. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Molecular Microbiology **46:**1451-64.

55. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a Salmonella typhimurium invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci U S A **89:**1847-51.

56. **Liu, X., and P. Matsumura.** 1996. Differential regulation of multiple overlapping promoters in flagellar class II operons in Escherichia coli. Mol Microbiol **21:**613-20.

57. **Liu, X., and P. Matsumura.** 1994. The FlhD/FlhC complex, a transcriptional activator of the Escherichia coli flagellar class II operons. J Bacteriol **176:**7345-51.

58. **Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee.** 2000. Multiple factors independently regulate hilA and invasion gene expression in Salmonella enterica serovar typhimurium. Journal of Bacteriology **182:**1872-82.

59. **Martinez, L. C., H. Yakhnin, M. I. Camacho, D. Georgellis, P. Babitzke, J. L. Puente, and V. H. Bustamante.** 2011. Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the Salmonella SPI-1 and SPI-2 virulence regulons through HilD. Mol Microbiol **80:**1637-56.

60. **Meynell, G. G.** 1963. Antibacterial mechanisms of the mouse gut. II. The role of Eh and volatile fatty acids in the normal gut. Br J Exp Pathol **44:**209-19.

61. **Mills, D. M., V. Bajaj, and C. A. Lee.** 1995. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol Microbiol **15:**749-59.

62. **Navarre, D. A., and T. J. Wolpert.** 1995. Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. Plant Cell **7:**463-471.

63. **Navarre, W. W., M. McClelland, S. J. Libby, and F. C. Fang.** 2007. Silencing of xenogeneic DNA by H-NS-facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. Genes Dev **21:**1456-71.

64. **Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Iino.** 1990. Gene fliA encodes an alternative sigma factor specific for flagellar operons in Salmonella typhimurium. Mol Gen Genet **221:**139-47.

65. **Ohnishi, K., K. Kutsukake, H. Suzuki, and T. Lino.** 1992. A novel transcriptional regulation mechanism in the flagellar regulon of Salmonella typhimurium: an antisigma factor inhibits the activity of the flagellum-specific sigma factor, sigma F. Mol Microbiol **6:**3149-57.

66. **Olekhnovich, I. N., and R. J. Kadner.** 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of Salmonella enterica serovar Typhimurium. Journal of Bacteriology **184:**4148-60.

67. **Palacios, S., and J. C. Escalante-Semerena.** 2004. 2-Methylcitrate-dependent activation of the propionate catabolic operon (prpBCDE) of Salmonella enterica by the PrpR protein. Microbiology **150:**3877-87.

68. **Palacios, S., and J. C. Escalante-Semerena.** 2000. prpR, ntrA, and ihf functions are required for expression of the prpBCDE operon, encoding enzymes that catabolize propionate in Salmonella enterica serovar typhimurium LT2. J Bacteriol **182:**905-10.

69. **Palacios, S., V. J. Starai, and J. C. Escalante-Semerena.** 2003. Propionyl coenzyme A is a common intermediate in the 1,2-propanediol and propionate catabolic pathways needed for expression of the prpBCDE operon during growth of Salmonella enterica on 1,2-propanediol. J Bacteriol **185:**2802-10.

70. **Saini, S., J. D. Brown, P. D. Aldridge, and C. V. Rao.** 2008. FliZ Is a posttranslational activator of FlhD4C2-dependent flagellar gene expression. J Bacteriol **190:**4979-88.

71. **Schechter, L. M., S. M. Damrauer, and C. A. Lee.** 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the hilA promoter. Molecular Microbiology **32:**629-42.

72. **Schechter, L. M., and C. A. Lee.** 2001. AraC/XylS family members, HilC and HilD, directly bind and derepress the Salmonella typhimurium hilA promoter. Molecular Microbiology **40:**1289-99.

73. **Schiemann, D. A., and S. R. Shope.** 1991. Anaerobic growth of Salmonella typhimurium results in increased uptake by Henle 407 epithelial and mouse peritoneal cells in vitro and repression of a major outer membrane protein. Infect Immun **59:**437-40.

74. **Soncini, F. C., E. Garcia Vescovi, F. Solomon, and E. A. Groisman.** 1996. Molecular basis of the magnesium deprivation response in Salmonella typhimurium: identification of PhoP-regulated genes. Journal of Bacteriology **178:**5092-9.

75. **Starai, V. J., I. Celic, R. N. Cole, J. D. Boeke, and J. C. Escalante-Semerena.** 2002. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. Science **298:**2390-2.

76. **Starai, V. J., and J. C. Escalante-Semerena.** 2004. Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in Salmonella enterica. J Mol Biol **340:**1005-12.

77. **Takaya, A., Y. Kubota, E. Isogai, and T. Yamamoto.** 2005. Degradation of the HilC and HilD regulator proteins by ATP-dependent Lon protease leads to downregulation of Salmonella pathogenicity island 1 gene expression. Mol Microbiol **55:**839-52.

78. **Takaya, A., T. Tomoyasu, A. Tokumitsu, M. Morioka, and T. Yamamoto.** 2002. The ATP-dependent lon protease of Salmonella enterica serovar Typhimurium regulates

invasion and expression of genes carried on Salmonella pathogenicity island 1. Journal of Bacteriology **184:**224-32.

79. **WHO.** 2008. Salmonella, http://www.who.int/mediacentre/factsheets/fs139/en/.

80. **Yamamoto, S., and K. Kutsukake.** 2006. FliT acts as an anti-FlhD2C2 factor in the transcriptional control of the flagellar regulon in Salmonella enterica serovar typhimurium. J Bacteriol **188:**6703-8.

81. **Yanagihara, S., S. Iyoda, K. Ohnishi, T. Iino, and K. Kutsukake.** 1999. Structure and transcriptional control of the flagellar master operon of Salmonella typhimurium. Genes Genet Syst **74:**105-11.

82. **Zhou, D., M. S. Mooseker, and J. E. Galan.** 1999. Role of the S. typhimurium actin-binding protein SipA in bacterial internalization. Science **283:**2092-5.

CHAPTER 2

The flagellar regulator FilT represses *Salmonella* Pathogenicity Island 1 through *flhDC* and *fliZ* *

*Hung, C., Haines, L., and Altier, C. The flagellar regulator FilT represses *Salmonella* Pathogenicity Island 1 through *flhDC* and *fliZ*. Submitted to PLoS ONE.

ABSTRACT

Salmonella pathogenicity island 1 (SPI1), comprising a type III section system (TTSS) that translocates effector proteins into host cells, is essential for the enteric pathogen *Salmonella* to penetrate the intestinal epithelium and subsequently to cause disease. Using random transposon mutagenesis, we found that a *Tn*10 disruption in the flagellar *fliDST* operon induced SPI1 expression when the strain was grown under conditions designed to repress SPI1, by mimicking the environment of the large intestine through the use of the intestinal fatty acid butyrate. Our genetic studies showed that only *fliT* within this operon was required for this effect, and that exogenous over-expression of *fliT* alone significantly reduced the expression of SPI1 genes, including the invasion regulator *hilA,* and the *sipBCDA* operon, encoding TSSIII effector proteins. *fliT* has been known to inhibit the flagellar machinery through repression of the flagellar master regulator *flhDC*. We found that the repressive effect of *fliT* on invasion was completely abolished in the absence of *flhDC* or *fliZ,* the latter previously shown to induce SPI1, indicating that this regulatory pathway is required for invasion control by *fliT.* Although this *flhDC-fliZ* pathway was necessary for *fliT* to negatively control invasion, *fliZ* was not essential for the repressive effect of *fliT* on motility, placing *fliT* high in the regulatory cascade for both invasion and motility.

INTRODUCTION

Salmonella is an important bacterial pathogen that is a leading source of food-borne illness, causing diseases ranging from transient enteritis to life-threatening septicemia. To infect its animal hosts, *Salmonella* first must penetrate the intestinal epithelium, a process termed invasion. Most of the genes required for invasion lie within a 40 kb gene cluster at centisome 63 termed *Salmonella* Pathogenicity Island 1 (SPI1), which is used by *Salmonella* to construct a type III secretion apparatus, the needle complex, to deliver secreted effector proteins into the host cell cytoplasm (8, 26, 29, 37, 41, 45). Once these proteins are translocated into a targeted epithelial cell, they induce cytoskeleton rearrangement and membrane ruffling, resulting in internalization of *Salmonella* by the host cell (14, 25, 30, 57).

SPI1 genes are known to be controlled by several transcriptional regulators encoded within and outside SPI1 through a complex network. Four transcriptional regulators, *hilD*, *hilC*, *hilA* and *invF* are present within SPI1 (15, 17, 41, 47, 51). Among these, *hilD* is at the top of the regulatory cascade and controls *hilC* as well as a regulator located outside SPI1, *rtsA* (19, 20) . HilD, HilC, and RtsA are able to regulate their own gene expression and can activate expression of *hilD*, *hilC* and *rtsA* independent of each other to constitute a regulatory circuit for the control of the SPI1 central regulator *hilA* (18). HilA controls the *sic/sip* operon, encoding effector proteins, and the *prg/org* and *inv/spa* operons that encode proteins composing the type III secretion apparatus (15, 17). HilA also induces the expression of the transcriptional regulator *invF*, encoding a member of the AraC family that activates the expression of genes encoding effector proteins within and outside SPI1 (15, 17). In addition, *invF* has been shown to be directly regulated by *hilD* and *hilC* through a *hilA*-independent pathway (1). Several genetic regulators outside SPI1 have also been shown to transcriptionally or post-transcriptionally control invasion gene expression. Regulators affecting SPI1 at the level of transcription include the two-component regulators *phoP/phoQ*, *envZ/ ompR*, *phoB/phoR* and *barA/sirA* (5, 8, 43). In addition, the DNA binding proteins H-NS and Hha have been demonstrated to bind to multiple

A-T rich sequences in SPI1, occupying the binding sites of positive regulators, and consequently preventing transcription (23, 46, 48). Among the post-transcriptional regulators of SPI1, the Csr system, PNPase, Lon protease and HilE have been shown to control invasion genes by affecting protein production or by manipulating the level or activity of the controller, HilD (4, 7, 10, 13, 53).

In addition to the mechanisms of control described above, two regulators of the flagellar regulon, *flhDC* and *fliZ*, have been described as inducers of SPI1 (12, 43). In the *Salmonella* flagellar regulatory cascade, the $FlhD_4C_2$ complex, encoded by *flhDC*, functions as a master regulator that binds to the class 2 flagellar promoters and to its own promoter to induce downstream flagellar gene expression (11, 42). However, the function of $FlhD_4C_2$ is antagonized by another flagellar protein, FliT, which associates with $FlhD_4C_2$ and neutralizes its activity (2, 56). *fliZ* has been characterized as a class 2 flagellar gene (33). Previous studies have shown that mutation of *fliZ* significantly reduces *hilA* expression and *Salmonella* intestinal colonization in mice. In addition, overproduction of FliZ increases *hilA* expression only when *hilD* is present, indicating that *fliZ* controls invasion gene expression through *hilD* (12). Although FliZ has been demonstrated to negatively control *hilD*, the mechanism by which this is accomplished remains uncertain (12, 35).

Expression of invasion genes can also be induced using various laboratory conditions that mimic the host intestinal environment, such as low oxygen, high osmolarity, and a near neutral pH (6, 21, 52). In addition, short-chain fatty acids (SCFA), produced by the intestinal microbiota through fermentative metabolic pathways, have been shown to play important roles in controlling *Salmonella* invasion (28, 39). Among these, butyrate, which exists in high concentration in the large intestine where salmonellosis rarely occurs, represses SPI1 gene expression (27, 39).

To identify additional genetic elements involved in *Salmonella* invasion control, here we applied a transposon mutagenesis approach and identified a mutation in the flagellar gene *fliT*

that affects the expression of SPI1 genes. As *fliT* was known to be a negative regulator of the flagellar regulon, we used genetic approaches to study the role of *fliT* and associated regulatory elements in the repression of invasion. Here, we demonstrate that *fliT* controls *Salmonella* invasion through *flhDC* and the *flhDC*-regulated gene, *fliZ*.

MATERIALS AND METHODS

Construction of mutant strains. *Salmonella enterica* serovar Typhimurium strain ATCC 14028S and isogenic mutants were used throughout this study, and are shown in Table 2.1. Gene deletions were made using the previously reported one-step inactivation method (16). In brief, PCR reactions were performed to amplify the fragments containing the FRT sequences flanking the antibiotic resistance markers from plasmids pKD3 or pKD4 using primers carrying 40 bases of homologous sequence flanking the coding region of the target gene. The resulting PCR product was purified and transformed into a *Salmonella* strain carrying the plasmid pKD46, which expresses the Red λ recombinase, allowing allelic exchange. The resulting deletion mutants were cultured at 42°C to remove the temperature-sensitive pKD46 plasmid, and the loss of the target gene was determined by PCR. The chromosomal *sipC::gfp* translational fusion was created using the above one-step gene exchange method. A promoterless *gfp* linked to a chloramphenicol resistance marker was amplified from the plasmid pZEP07 (31) with primers

TGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTCACCTGTAGGCTGGAGCTGCTTCG

and

TTAAATCACACCCATGATGGCGTATAGATGACCTTTCAGACATATGAATATCCTCCTTAG,

which encode DNA homologous to the regions immediately adjacent to the *sipC* open reading frame. The resulting PCR product was purified and treated with DpnI to remove the pZEP07 template and transformed into the *Salmonella* strain carrying pKD46 with selection on 25 μg/ml chloramphenicol to allow recombination of *gfp*, creating a translational fusion to *sipC* with an

adjacent chloramphenicol cassette. To create the *fliT* expression plasmid (pFliT), a PCR product was produced that included a synthetic ribosome binding site, based upon that of *lacZ*, and the *fliT* ORF with an extended 175 bp 3' of the *fliT* sequence to include the predicted transcriptional termination site. This product was amplified using primers CCCATCGATCAATTTCACACAGGAAACAGCTATGACCTCAACCGTGGAGTTTATCAAC and TCCCCCGGGGATATCATTCAGCCCATCAGCACG. The PCR product was then cloned into the unique ClaI and SmaI sites of pACYC177 to place *fliT* under the control of the kanamycin resistance gene (*npt*) promoter on this vector.

Table 2.1. Strains and plasmids used in this study

Strain or Plasmid	Genotype	Source or reference
Strains		
Salmonella enterica serovar *Typhimurium* 14028S	wild type	American Type Culture Collection
CA412	*sipC::lacZY*	[36]
CA2312	Δ*ackA sipBCDA*::MudJ	This study
CA2311	Δ*ackA sipC::gfp*	This study
CA1274	Δ*ackA sipC::lacZY*	This study
CA2064	Δ*ackA fliD::Tn*10 *sipC::lacZY*	This study
CA2123	Δ*ackA fliDST::kan sipC::lacZY*	This study
CA2124	Δ*ackA fliST::kan sipC::lacZY*	This study
CA2125	Δ*ackA fliS::kan sipC::lacZY*	This study
CA2126	Δ*ackA fliT::kan sipC::lacZY*	This study
CA2047	*fliD::Tn*10 *sipC::lacZY*	This study
CA2060	*flhDC::cam*	This study
CA1854	*fliZ::kan*	This study
CA2121	*flhDC::cam sipC::lacZY*	This study
CA2122	*fliZ::cam sipC::lacZY*	This study
Plasmids		
pNK2883	Plasmid carrying IPTG-inducible *Tn*10 transposon	[56]
pZEP07	Plasmid carrying *gfp*	[55]
pACYC177	Cloning vector	[58]
pFliT (pCA173)	*fliT* ORF on pACYC177	This study

***Tn*10 random transposon mutagenesis screening.** To create a random transposon *Tn*10 library, a wild type strain carrying the IPTG-inducible *Tn*10 plasmid pNK2883 was used (36). The strain was grown overnight in LB broth with 100 µg/ml of ampicillin at 37ºC with shaking, and sub-cultured in the same medium to mid-log phase. To induce transposon insertion, IPTG was added at a final concentration of 0.1 mM to the mid-log culture, which was grown for another 16 hr. The resulting random *Tn*10 insertion library was moved into the *ackA* mutant strain carrying the chromosomal *sipC*::*gfp* translational fusion by P22 phage transduction. Transductants were plated on LB agar with 25 µg/ml tetracycline, 100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 6.7 and 10 mM butyric acid and incubated at 37ºC overnight. The green florescence of individual colonies was determined using the OV100 Observation Intravital System (Olympus Corp., Tokyo, Japan). This work was assisted by Leanne Haines.

Determining the DNA sequence flanking the *Tn*10 element. The sequences flanking the *Tn*10 insertions were identified using a method previously reported (40). In brief, *Tn*10 insertion strains and a control strain (the isogenic strain without a *Tn*10 insertion) were grown overnight in LB broth with aeration. The overnight culture was diluted 100-fold with nuclease-free water, and bacteria were frozen and thawed three times to expose the genomic DNA. Five µl of the sample was used as a template to perform an initial PCR using primer AATTGCTGCTTATAACAGGCACTG in combination with arbitrary primers GGCCAGCGAGCTAACGAGACNNNNGTTGC, GGC CAGCGAGCTAACGAGACNNNNGATAT, and GGCCAGCGAGCTAACGAGACNNNNAGTAC with a cycle of 3 min at 95ºC followed by 30 cycles of 30 sec denaturation at 95ºC, 30 sec annealing at 38ºC, 90 second extension at 72ºC and an additional cycle of 3 min final extension at 72ºC. Five µl of this PCR reaction was next used as template to perform a second PCR using the primer set GGCCAGCGAGCTAACGAGAC and ACCTTTGGTCACCAAAGCTTT, beginning with a cycle of 3 min denaturation at 95 ºC followed by 30 cycles of 15 sec denaturation at 95ºC, 30 sec

annealing at 56ºC, 90 sec extension at 72ºC and a final cycle of 3 min extension at 72ºC. PCR products were separated by electrophoresis on a 2% agarose gel. The DNA fragments produced from the *Tn*10 insertion mutants but not from the control strain were harvested from the gel. Purified DNA fragments were sequenced using the primer ACCTTTGGTCACCAAAGCTTT.

β-galactosidase assays. Triplicate cultures of tested bacterial strains were grown standing overnight at 37°C in LB broth buffered to pH 6.7 with 100 mM MOPS and with 10 mM butyric acid if needed. β-galactosidase activity was measured as described previously (44) .

Secreted protein isolation and analysis. Strains were grown in LB with100 mM MOPS pH 6.7 and 100 µg/ml ampicillin at 37ºC with shaking at 60 rpm for 16 hr. Proteins secreted into the culture supernatant were prepared and analyzed as previously described (5).

Bacterial swimming activity. Strains were grown overnight in LB with 100 µg/ml ampicillin at 37º C with shaking at 200 rpm. Ten µl of overnight culture of each strain was dotted onto the LB swimming agar plates (containing 0.35% agar) with100 µg/ml ampicillin, and incubated at 37º C for 6-7 hr in a humidified incubator.

Statistical analysis. Results from β-galactosidase assays were analyzed using a one-way analysis of variance to determine if the mean of at least one strain or condition differed from any of the others. The Tukey-Kramer HSD multiple comparison test was then used to determine which means were statistically different. A p-value <0.05 was considered significant. Statistical analysis was performed using Jmp 9.0 software (SAS, Cary, NC).

RESULTS

Identifying *fliT, a* novel negative regulator of *Salmonella* invasion, using random transposon mutagenesis screening. Regulation of *Salmonella* Pathogenicity Island 1(SPI1) gene expression is controlled by various regulatory elements inside and outside the island, and is also affected by environmental cues (3). To identify novel regulators that negatively control *Salmonella* invasion, we used random *Tn*10 transposon mutagenesis in a strain carrying a *gfp* reporter fusion to the SPI1 gene *sipC*, with the strain grown in the presence of butyrate, a short chain fatty acid found in abundance within the mammalian intestine. As butyrate has been shown to repress *Salmonella* invasion (27, 39), the bacterial colonies carrying the *sipC::gfp* reporter showed little fluorescence on LB agar containing 10 mM butyric acid. We surmised that transposon insertions in negative regulators of invasion would increase *sipC::gfp* expression, producing fluorescent colonies. The strain used for this screen also carried a deletion of *ackA*, encoding acetyl kinase, as our studies showed that the *ackA* mutation partially restored *sipC* expression in media containing butyric acid (Fig. 2.1 and data not shown). This strain thus allowed the screen to be performed without the repeated isolation of *ackA* mutants, and thus provided the possibility of identifying novel regulators of invasion. In total, we screened approximately 40,000 colonies, representing an 8-fold screening of the genome, with 31 fluorescent colonies being found. We next sought to determine the transposon insertion sites in candidate mutants. Previously, it has been reported that *Tn*10 insertions near the promoter region of the SPI1 regulator *hilD* could cause increased expression of the downstream regulator *hilA*, which is essential to induce *sipC* (22). To rule out these and other potential mutations within SPI1, we examined the linkage of *Tn*10 insertions to *sipC* using P22 bacteriophage-mediated transductional mapping. The results showed that 22 candidates possessed a *Tn*10 insertion linked to *sipC*; these mutants were not further characterized.

For the remaining nine candidate colonies, the *Tn*10 insertions were moved by transduction into an *ackA* mutant carrying a MudJ insertion encoding a *lacZY* fusion to the

sipBCDA operon to quantify the increase in invasion gene expression using β-galactosidase assays. Based upon the increased level of *sipBCDA::lacZY* expression, candidates were categorized into two classes; those that had an increase in expression only when butyrate was present (six mutants), and those with increased expression under both repressing and inducing conditions (three mutants). As individual mutants in each group possessed a similar effect on *sipBCDA* expression, their phenotypes suggested that they might carry *Tn*10 disruptions in the same gene or operon. To identify the sites of transposon insertion, we amplified the region flanking the *Tn*10 for one candidate from each of the two groups by arbitrary PCR (40). We found that the mutant affected only under repressing conditions, in the presence of butyrate, carried a *Tn*10 insertion in *fliD*, the first gene of the *fliDST* operon. A representative of the second class, showing increased *sipC* expression under both repressing and inducing conditions, carried a *Tn*10 insertion in *pnp*. Further, we determined the genetic linkage of *Tn*10 in all of the remaining candidates of both groups to *fliD* and *pnp* by transductional mapping, finding that all insertions within a group were 100% linked to these respective genes. These results, taken together, demonstrate that all of the mutations residing outside SPI1 that induced the expression of *sipC* under our tested conditions resulted from disruptions in or near either *fliD* or *pnp*

FliT is a negative regulator of SPI1 expression. *pnp*, encoding a 3- to 5-phosphorolytic exonuclease, a subunit of RNA degradosome, has been shown to affect SPI1 genes expression by interfering with RNA half-life (13). However, genes in the *fliDST* operon have not been reported to control invasion by *Salmonella*. For this reason, we focused our study on the role of the *fliDST* operon in the control of SPI1. To quantify the effects of the *Tn*10 disruption of *fliD* on invasion, we compared *sipC::lacZY* expression in various mutants grown with or without butyric acid by β–galactosidase assays. In the wild type, *sipC* expression decreased 3.5-fold

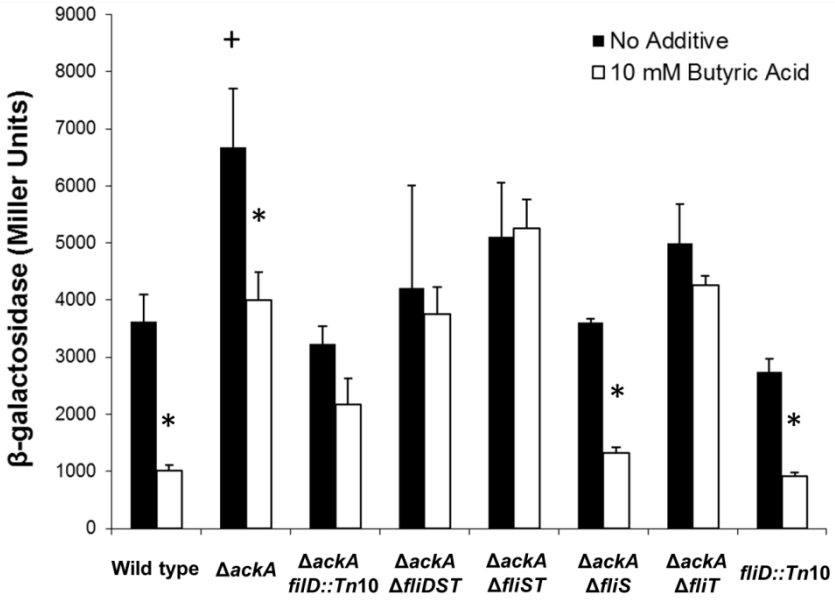

Figure 2.1. Mutation of *fliT* restores *sipC* invasion gene expression in the *ackA* mutant under SPI1-repressing conditions. Wild type and mutants strains carrying the *sipC*::*lacZY* fusion were grown in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration with no additive (black bars) or with 10 mM butyric acid (white bars). *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to butyrate as compared to the same strain with no additive at $p < 0.01$. A plus (+) indicates a statistically significant difference due to deletion of a gene as compared to the wild type when grown under the same conditions at $p < 0.05$.

when the strain was grown in media containing 10 mM butyric acid compared to media with no additive (with all media stably buffered to pH 6.7), and an *ackA* mutant, as expected, demonstrated a lesser, 1.5-fold repression with butyric acid present (Fig. 2.1). Importantly, *sipC* expression was unaffected by butyric acid in the *ackA, fliD::Tn*10 double mutant (Fig. 2.1). As *fliD* is the first gene in the *fliDST* operon, the increase of *sipC* expression caused by the disruption of *fliD* may have resulted from polar effects on any of the downstream genes in the operon. Thus, we next determined which genes played important roles in control of *sipC* expression by testing the effects of mutations of operon genes, singly and in combination. The results showed that *ackA* mutant strains with an additional deletion of *fliDST*, *fliST*, or *fliT* restored *sipC* expression in the presence of butyric acid (Fig. 2.1). There remained, however, a significant decrease in *sipC* expression by butyrate in strains with disruptions of *fliD* (data not

shown) or *fliS* (Fig. 2.1), the first two genes of the *fliDST* operon. From these results, we concluded that the last gene of the *fliDST* operon, *fliT*, is required for the negative control of *Salmonella* invasion. The increased *sipC* expression caused by deletion of *fliT* was, however, seen only in the *ackA* mutant and with the repression of SPI1 genes provided by butyric acid. To confirm the negative effect of *fliT* on invasion, we cloned the *fliT* ORF onto a low-copy number plasmid, on which *fliT* was constitutively expressed under the control of an exogenous promoter. Again using a *sipC::lacZY* fusion, we found a significant 3.1-fold decrease in *sipC* expression in the wild type strain with the *fliT* plasmid compared to the isogenic strain carrying the control plasmid, pACYC177 (Fig. 2.2). We next verified the repressive effect of *fliT* on additional secreted effectors of SPI1 by characterizing the proteins secreted by this strain into the culture media. SPI1 invasion proteins encoded by the *sipBCDA* operon have been shown to

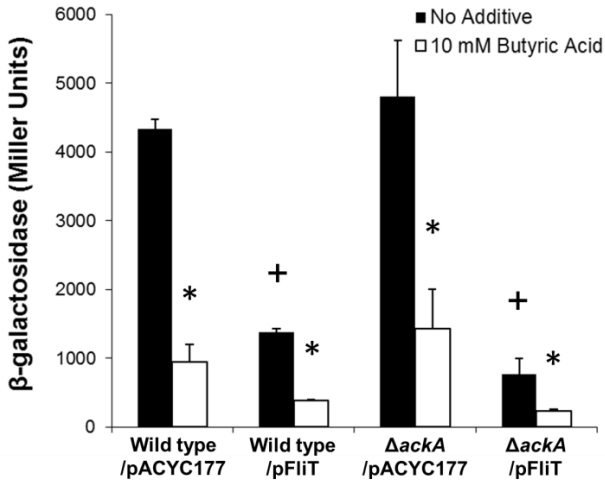

Figure 2.2. Over-expression of *fliT* negatively controls *sipC* invasion gene expression. The expression of *sipC::lacZY* was measured in strains carrying a low copy-number plasmid expressing *fliT* (pFliT) or its vector control (pACYC177). Bacterial strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration with no additive (black bars) or with 10 mM butyric acid (white bars). *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to butyrate as compared to the same strain with no additive at $p < 0.01$. A plus (+) indicates a statistically significant difference due to pFliT as compared to the isogenic strain carrying the control plasmid pACYC177 at $p < 0.05$.

be secreted into the culture media when *Salmonella* is grown in laboratory culture medium (32). We extracted the secreted proteins from overnight bacterial culture and examined the SPI1 effector protein profile using SDS-PAGE with Coomassie blue staining. Four bands had molecular weights equivalent to the invasion proteins SipA (89 kDa), SipB (67 kDa), SipC (43 kDa) and SipD/invJ (38 kDa) (protein sequences of these bands were determined by mass spectrometry, with the band for SipD overlapping that of another invasion protein, InvJ, due to their similar molecular weights). These bands were significantly diminished in the wild type strain carrying the *fliT* plasmid compared to the strain with the control plasmid (Fig. 2.4, lanes 1 and 2). Based on the results of β-galactosidase and secreted protein profile assays, we thus demonstrated a negative effect by *fliT* on SPI1 gene expression. However, a *fliT* mutant carrying a *sipC*::*lacZY* fusion in an otherwise wild type background demonstrated no significant change in *sipC* expression (data not shown). These results, in combination with those shown in Fig. 2.1, confirm that *fliT* acts as a repressor of SPI1 gene expression and *Salmonella* invasion.

The fact that the loss of *fliT* in the *ackA* deletion mutant could relieve the butyrate–induced repressive effect on the SPI1 gene *sipC* (Fig. 2.1) led us to speculate that butyrate might function through the induction of *fliT* itself. To test this hypothesis, we used a *fliT-lacZ* transcriptional fusion in wild type and *ackA* mutant strains, and examined whether *fliT* expression was increased by butyrate. We found that there was no significant difference in *fliT* expression in either strain background with the addition of butyric acid (data not shown), indicating that *fliT* expression is not affected by butyrate at the transcriptional level. To further investigate whether the negative effects of *fliT* and butyrate on invasion were independent, we determined whether the addition of butyrate promoted the repressive effect on SPI1 when *fliT* was overproduced. We over-expressed *fliT* in the wild type and the *ackA* mutant strains carrying the *fliT* plasmid and compared *sipC* expression with or without the addition of butyric acid. As expected, we found that there was a significant further reduction of *sipC* expression by butyrate in these strains, 3.6-fold for the wild type and 3.3-fold for the *ackA* mutant (Fig. 2.2).

These results therefore demonstrate that *fliT* is not involved in the negative control of butyrate on invasion.

FliT negatively controls invasion genes through the flagellar regulators *flhDC* and *fliZ.*

Having shown that over-expression of *fliT* from an exogenous promoter repressed invasion gene expression, we further asked how this member of the flagellar regulon negatively controls *Salmonella* invasion. FliT has been shown to function as a chaperone to facilitate export of the flagella capping protein, FliD, in the assembly of flagella (24, 34). More importantly, FliT has also been demonstrated to negatively control flagellar gene expression by binding to the class 1 flagellar regulator, the $FlhD_4C_2$ complex, and preventing this transcriptional activator from binding to class 2 flagellar promoters, consequently reducing downstream flagellar gene expression (38, 54, 56). Since FliT can function as a negative regulator of the flagellar regulon, it is possible that the repressive effect of FliT on invasion may result from its negative effects on other flagellar genes that can positively control invasion gene expression. In *Salmonella*, two flagella genes, *flhDC* and the *flhDC*-controlled downstream regulator *fliZ*, have been shown to positively regulate SPI1 (12, 43). In addition, FliZ has been shown to regulate *Salmonella* invasion through the control of the SPI1 regulator, HilD (12, 35). To test whether *fliT* affects invasion through the negative control of this *flhDC-fliZ* pathway, we first examined *sipC* expression in the wild type, the *flhDC* mutant, and the *fliZ* mutant, each carrying the *fliT* plasmid or the control plasmid pACYC177. Using the *sipC*::*lacZY* fusion, there was a significant reduction in *sipC* expression, 4.6-fold for the *flhDC* mutant and 6.8-fold for the *fliZ* mutant, (Fig. 2.3A). These results demonstrate that these two flagella regulators are important to induce *Salmonella* invasion gene expression, and are consistent with results published by other groups (12, 43). Additionally, we found that over-expression of *fliT* did not further reduce *sipC*

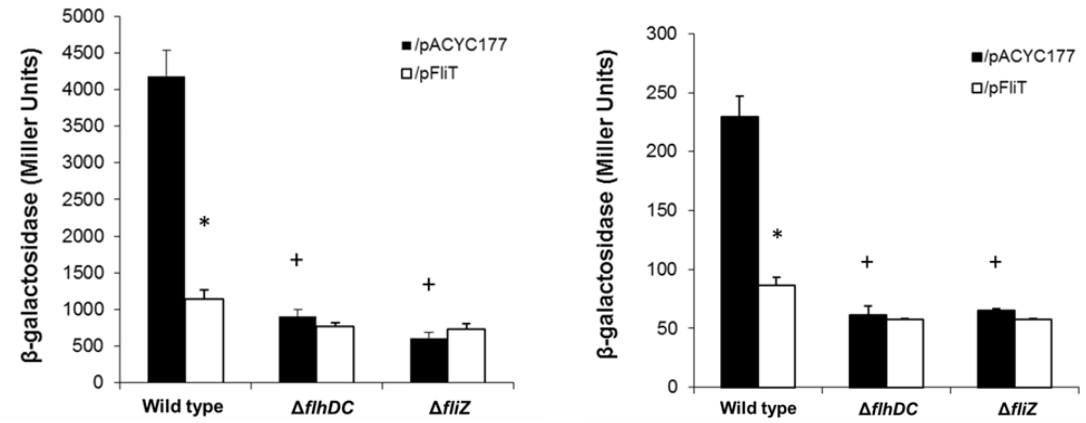

Figure 2.3. *fliT* affects SPI1 gene expression through the *flhDC-fliZ* pathway. The *fliT* expression plasmid pFliT (white bars) and the control plasmid pACYC177 (black bars) were tested in the wild type, the *flhDC* mutant and the *fliZ* mutant carrying A) the *sipC::lacZY* fusion, and B) the *hilA::lacZY* fusion. Strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration and *lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to butyrate as compared to the same strain with no additive at $p < 0.01$. A plus (+) indicates a statistically significant difference due to deletion of a gene as compared to the wild type at $p < 0.05$.

expression in the *flhDC* or the *fliZ* mutant (Fig. 2.3A). These results suggest that *fliT* negatively controls *sipC* through this recognized pathway of regulation. To confirm the negative effect of *fliT* on invasion through *flhDC* and *fliZ*, we further examined the secreted invasion protein profiles using culture conditions identical to those employed for the β-galactosidase assays. The result showed that the secreted invasion effector proteins SipA, SipB, SipC and SipD were significantly diminished in the *flhDC* and the *fliZ* mutants compared to those in the wild type (Fig. 2.4, lanes 1, 3 and 5). Additionally, there was no further reduction in these proteins in the *flhDC* or *fliZ* mutant carrying the *fliT* plasmid (Fig. 2.4, lanes 4 and 6). As we had shown that downstream SPI1 effector proteins were affected by over-expression of *fliT*, in parallel we also determined whether their upstream regulator, *hilA,* was affected. As for the previous β-galactosidase results using *sipC*, *hilA* expression was significantly reduced in the *flhDC* and *fliZ* mutants carrying the control plasmid compared to the wild type with the same plasmid. A 2.8-

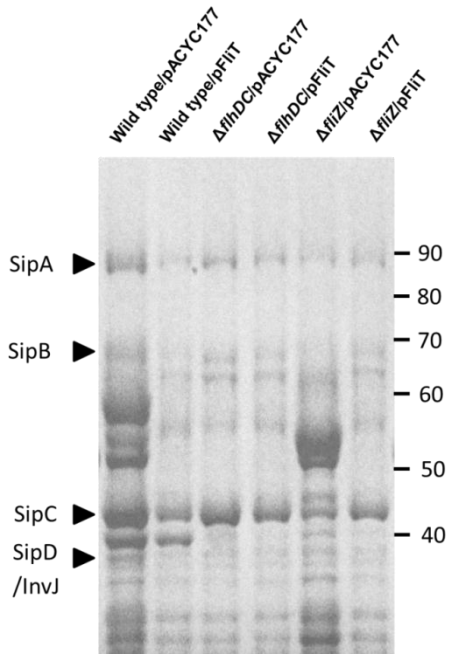

Figure 2.4. Over-expression of *fliT* decreases SPI1 effector protein production. The wild type, the *flhDC* mutant and the *fliZ* mutant carrying the control plasmid pACYC177 (lanes 1, 3 and 5) or the *fliT* expression plasmid pFliT (lanes 2, 4 and 6) were grown in LB broth buffered to pH 6.7 with 100 mM MOPS with low aeration (60 rpm) overnight. Proteins secreted into the culture media were purified as described and separated using 10% SDS-PAGE. The locations of four SPI1 effector proteins, SipA (89 kDa), SipB (67 kDa), SipC (42 kDa) and SipD/InvJ (38 kDa), are shown on the left. Molecular weights (kDa) are shown on the right.

fold decreased in *hilA* expression was also observed due to the expression of *fliT*, and there was no additional decrease in *hilA* expression in the *flhDC* and *fliZ* mutants (Fig. 2.3B). Based upon these results, we conclude that *fliT* negatively affects *Salmonella* invasion gene expression through *flhDC* and *fliZ*.

The *flhDC-fliZ* pathway is specific for the repressive effects of *fliT* on invasion, but not for its effects on flagellar regulation. Our results demonstrate that *fliT* acts as a negative regulator of invasion, and previous studies have shown that *fliT* affects flagellar control in *Salmonella* (38, 54, 56). Additionally, our data suggest that the repressive effect of *fliT* on

invasion is accomplished through the *flhDC-fliZ* pathway. Since *flhDC* and *fliZ* have been implicated as regulators in the flagellar regulon (49), we further asked whether this *flhDC-fliZ* route is used by *fliT* in its control of flagella. To test this, we used the wild type, the *flhDC* mutant, and the *fliZ* mutant carrying either the control plasmid or the *fliT* plasmid, and examined their swimming ability on 0.35 % LB agar plates (Fig. 2.5). We found that in the wild type strain over-expression of *fliT* completely eliminated *Salmonella* motility. The same phenotype was also observed in the *flhDC* mutant carrying the control plasmid, pACYC177, or the *fliT* plasmid. As previously described, *fliT* is able to negatively control flagellar gene expression by post-translational regulation of $FlhD_4C_2$ activity. Our results thus suggest that *fliT* controls *Salmonella* motility through *flhDC*, and are consistent with other studies. However, unlike *flhDC*, the *fliZ* mutant showed only a slight reduction in swimming ability, and over-expression of *fliT* in this strain fully inhibited its motility, suggesting that the repression of motility by *fliT* was not mediated through *fliZ*. Therefore, our results, taken together, suggested that the *flhDC-fliZ* pathway is specific for repression of *Salmonella* invasion by *fliT*, but this pathway is not required for *fliT* repression of the flagellar regulon.

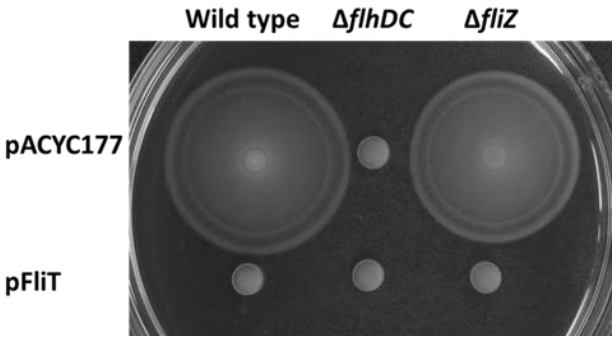

Figure 2.5. *fliT* affects *Salmonella* motility through *flhDC,* but not through *fliZ.* The wild type, the *flhDC* mutant and the *fliZ* mutant carrying the control plasmid pACYC177 or the *fliT* expression plasmid pFliT were grown in LB broth with aeration overnight. Cultures were dotted onto LB swimming agar plates (0.35% agar) and incubated at 37°C for 7 hours in a humidified incubator.

DISCUSSION

For serovars of *Salmonella*, the genes of SPI1 are key elements that dictate the ability of the pathogen to penetrate the intestinal epithelium and cause further systemic infection. The control of SPI1 gene expression has been shown to be evoked by complex interrelated regulatory networks. In this work, using a random transposon mutagenesis strategy, we discovered that the flagellar regulator *fliT*, encoded within the *fliDST* operon, can negatively control SPI1 gene expression (Fig. 2.1). In addition, we showed that *fliT* over-expression reduced the expression of the invasion gene regulator *hilA* and downstream effector proteins through the negative control of the *flhDC-fliZ* pathway (Figs. 2.2, 2.3 and 2.4). *fliZ* has been shown to positively control invasion genes by regulating the SPI1 regulator *hilD*, which exists high in the regulatory hierarchy of this pathogenicity island (12, 20). It has been suggested that FliZ post-transcriptionally controls HilD through an unidentified mechanism, rather than affecting *hilD* expression at the level of transcription (12). Kage and coworkers showed that HilD protein level, when expressed from a constitutive promoter, was significantly decreased by the deletion of *fliZ.* However, the half-life of HilD was not changed when *fliZ* was missing. Their studies thus suggest that *fliZ* controls HilD at the translational level (35). In contrast, Chubiz and colleagues showed that the HilD protein, when constitutively expressed from a single-copy chromosomal tetracycline-inducible promoter, was only slightly reduced in the *fliZ* null strain compared to that in the wild type. Additionally, they measured the stability of HilD in the *fliZ* mutant and in the wild type and showed that the stability of HilD was not significantly altered when *fliZ* was missing. Therefore, they suggested that the mechanism by which *fliZ* regulates HilD is by post-translationally affecting HilD activity (12). As we have shown that *fliT* negatively controls invasion through *fliZ*, we suggest that FliT negatively controls *Salmonella* invasion by changing the amount or activity of HilD, and subsequently affects expression of downstream invasion genes (Fig. 2.6).

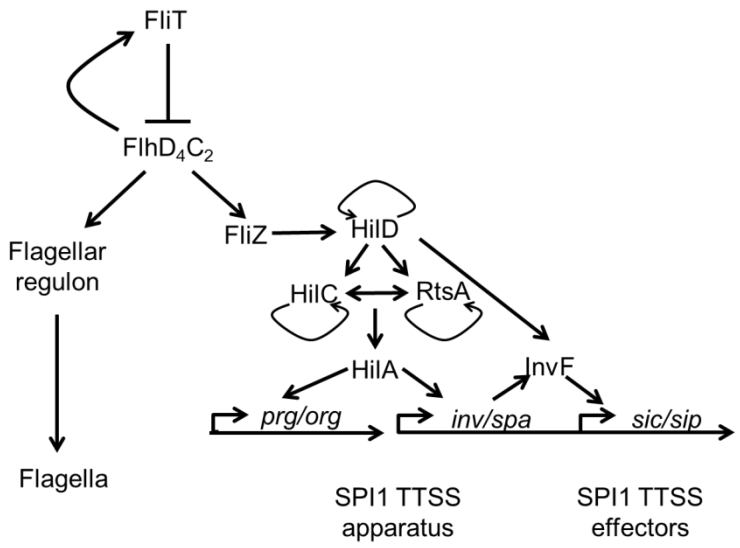

Figure 2.6. A model for *fliT* regulation of SPI1 and flagellar genes.

FliT has been shown to possess two functions in *Salmonella*, acting both as a regulator and a chaperone (9, 24, 38). In its chaperone function, FliT directly interacts with several flagellar proteins, including FliD, FliI and FliJ, preventing their pre-maturation and aggregation within the bacterial cytoplasm and thus facilitating flagellar assembly (24, 34). As a regulator of flagellar expression, FliT binds to the class 1 flagellar regulator FlhC as part of the $FlhD_4C_2$ complex and inhibits $FlhD_4C_2$ from binding to its target promoters, consequently repressing downstream flagellar gene expression (54). In our work, we showed that *fliT* was the only gene of the *fliDST* operon able to restore invasion gene expression under our test conditions. We would expect that if FliT had affected invasion through its role as a chaperone, by interacting with FliD, the deletion of *fliD* would similarly restore *sipC* expression, as did the *fliT* mutant. However, restoration of *sipC* expression was not observed in the *fliD* mutant (data not shown), suggesting that *fliT* does not affect SPI1 gene expression through its function as a chaperone. Instead, our results indicate that *fliT* acts on *Salmonella* invasion in its role as a regulator, as we

have demonstrated that the *flhDC-fliZ* pathway with which FliT is known to interact is required for its negative control of invasion.

In agreement with previous studies (2, 56), our work showed that *flhDC* is also required for FliT to control *Salmonella* motility (Fig. 2.5). However, we found that *fliZ* was required for the effects of *fliT* only on invasion, and not on motility, as only a slight reduction in swimming was observed in the *fliZ* mutant (Fig. 2.5). These results, taken together, thus demonstrate that FliT is able to coordinately regulate invasion and flagellar gene expression though the single flagellar master regulator *flhDC*, but that the control of these two regulons diverges at subsequent steps in their regulatory cascades.

In *Salmonella*, flagella and invasion have been shown to be coordinately regulated by regulators in addition to FliT through the *flhDC-fliZ* pathway. ClpXP is an ATP-dependent protease and has been demonstrated to repress both flagella and SPI1 gene expression (35). ClpXP negatively controls the flagellar regulon by facilitating the degradation of the master flagellar regulators FlhD and FlhC and subsequently repressing downstream flagella genes (54). Kage and coworkers have demonstrated that the repressive effect of ClpXP on the *flhDC-fliZ* cascade is required for this protease to negatively control *Salmonella* invasion (35). TviA is another regulator that negatively co-regulates *Salmonella* invasion and motility though this pathway. TviA is a regulator within *Salmonella* Pathogenicity Island 7(SPI7) unique to *Salmonella* serovar Typhi that does not exist in *S.* Typhimurium. This regulator has been shown to respond to stimulation by low osmolarity and also negatively controls both flagella and invasion gene expression. TviA affects flagella by repressing the transcription of *flhDC*. This inhibitory effect on *flhDC* has been suggested to consequently cause the reduction of invasion gene expression through the *flhDC–fliZ* pathway (55). The above two regulators and FliT have thus been demonstrated to either transcriptionally or post-translationally affect the flagellar master regulator *flhDC*, while previous studies and the results we present here demonstrate that the *flhDC-fliZ* pathway is essential for these regulators to control invasion. Based on this

evidence, we suggest that the *flhDC–fliZ* pathway is an important common route used by *Salmonella* to allow the flagellar regulon to coordinately control invasion gene expression.

As previously described, FliT has been shown to negatively control $FlhD_4C_2$ activity by its interaction with FlhC and subsequently to inhibit the binding of $FlhD_4C_2$ to target DNA (56). Interestingly, Aldridge and coworkers showed that FliT was able to interact with $FlhD_4C_2$ that has not bound to its target DNA. leading to the dissociation of the $FlhD_4C_2$ complex *in vitro* (2). However, when $FlhD_4C_2$ was pre-associated with its target DNA, this protein-DNA complex was insensitive to FliT (2). Their studies suggest a means by which *Salmonella* can efficiently control the flagellar regulon in response to rapidly changing environments. When FliT is produced, it binds existing $FlhD_4C_2$, dissociating the $FlhD_4C_2$ complex and resulting in a quick down-regulation of flagella gene expression. When the level of FliT is low, however, the $FlhD_4C_2$ complex associates with its target DNA and thus efficiently activates the flagellar regulon. A recent study has also shown that flagella and invasion are coordinately regulated in response to growth phase. Both are highly expressed in the early stages of growth in laboratory medium (50). However, when these two regulons were both repressed in late stationary phase, and alternatively fimbrial genes were highly expressed (50). This phenomenon may be relevant to the control of *Salmonella* gene expression within the intestine of an animal host. Infecting bacteria first utilize flagella and invasion genes to reach and invade the intestinal epithelium. For those unable not penetrate the epithelium, expression of fimbrial genes would allow bacteria to better colonize within the intestine (50). Thus, although the environmental and genetic cues of the intestinal tract that elicit control of flagella and invasion, including that mediated by FliT, are not well known, the coordinated regulation of these two important functions is clearly essential to productive infection and disease.

ACKNOWLEDGEMENTS

We thank Cherilyn Garner for the construction of the *sipC*::*gfp* fusion and Julie Siler for the critical review of this manuscript. This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, award number 2005-35201-16270.

REFERENCES

1. Akbar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee. 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in Salmonella typhimurium. Molecular Microbiology 47:715-28.
2. Aldridge, C., K. Pooncharoen, S. Saini, T. Ewen, A. Soloyva, C. V. Rao, K. Imada, T. Minamino, and P. D. Aldridge. 2010. The interaction dynamics of a negative feedback loop regulates flagellar number in Salmonella enterica serovar Typhimurium. Mol Microbiol 78:1416-30.
3. Altier, C. 2005. Genetic and environmental control of salmonella invasion. J Microbiol 43 Spec No:85-92.
4. Altier, C., M. Suyemoto, and S. D. Lawhon. 2000. Regulation of Salmonella enterica serovar typhimurium invasion genes by csrA. Infection and Immunity 68:6790-7.
5. Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer. 2000. Characterization of two novel regulatory genes affecting Salmonella invasion gene expression. Molecular Microbiology 35:635-46.
6. Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee. 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Molecular Microbiology 22:703-14.

7. Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones. 2003. HilE interacts with HilD and negatively regulates hilA transcription and expression of the Salmonella enterica serovar Typhimurium invasive phenotype. Infection and Immunity 71:1295-305.

8. Behlau, I., and S. I. Miller. 1993. A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells. Journal of Bacteriology 175:4475-84.

9. Bennett, J. C., J. Thomas, G. M. Fraser, and C. Hughes. 2001. Substrate complexes and domain organization of the Salmonella flagellar export chaperones FlgN and FliT. Mol Microbiol 39:781-91.

10. Boddicker, J. D., and B. D. Jones. 2004. Lon protease activity causes down-regulation of Salmonella pathogenicity island 1 invasion gene expression after infection of epithelial cells. Infection and Immunity 72:2002-13.

11. Chilcott, G. S., and K. T. Hughes. 2000. Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and Escherichia coli. Microbiol Mol Biol Rev 64:694-708.

12. Chubiz, J. E., Y. A. Golubeva, D. Lin, L. D. Miller, and J. M. Slauch. 2010. FliZ regulates expression of the Salmonella pathogenicity island 1 invasion locus by controlling HilD protein activity in Salmonella enterica serovar typhimurium. J Bacteriol 192:6261-70.

13. Clements, M. O., S. Eriksson, A. Thompson, S. Lucchini, J. C. Hinton, S. Normark, and M. Rhen. 2002. Polynucleotide phosphorylase is a global regulator of virulence and persistency in Salmonella enterica. Proc Natl Acad Sci U S A 99:8784-9.

14. Collazo, C. M., and J. E. Galan. 1997. The invasion-associated type III system of Salmonella typhimurium directs the translocation of Sip proteins into the host cell. Molecular Microbiology 24:747-56.

15. Darwin, K. H., and V. L. Miller. 1999. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in Salmonella typhimurium. Journal of Bacteriology 181:4949-54.

16. Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-5.

17. Eichelberg, K., and J. E. Galan. 1999. Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA. Infection and Immunity 67:4099-105.

18. Ellermeier, C. D., J. R. Ellermeier, and J. M. Slauch. 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. Mol Microbiol 57:691-705.

19. Ellermeier, C. D., and J. M. Slauch. 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in Salmonella enterica serovar Typhimurium. Journal of Bacteriology 185:5096-108.

20. Ellermeier, J. R., and J. M. Slauch. 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. Curr Opin Microbiol 10:24-9.

21. Ernst, R. K., D. M. Dombroski, and J. M. Merrick. 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by Salmonella typhimurium. Infect Immun 58:2014-6.

22. Fahlen, T. F., N. Mathur, and B. D. Jones. 2000. Identification and characterization of mutants with increased expression of hilA, the invasion gene transcriptional activator of Salmonella typhimurium. FEMS Immunol Med Microbiol 28:25-35.

23. Fahlen, T. F., R. L. Wilson, J. D. Boddicker, and B. D. Jones. 2001. Hha is a negative modulator of transcription of hilA, the Salmonella enterica serovar Typhimurium invasion gene transcriptional activator. Journal of Bacteriology 183:6620-9.

24. Fraser, G. M., J. C. Bennett, and C. Hughes. 1999. Substrate-specific binding of hook-associated proteins by FlgN and FliT, putative chaperones for flagellum assembly. Mol Microbiol 32:569-80.

25. Fu, Y., and J. E. Galan. 1998. Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of Salmonella typhimurium. Journal of Bacteriology 180:3393-9.

26. Galan, J. E., and R. Curtiss, 3rd. 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc Natl Acad Sci U S A 86:6383-7.

27. Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, I. Hautefort, A. Thompson, J. C. Hinton, and F. Van Immerseel. 2006. Butyrate specifically down-regulates salmonella pathogenicity island 1 gene expression. Appl Environ Microbiol 72:946-9.

28. Garner, C. D., D. A. Antonopoulos, B. Wagner, G. E. Duhamel, I. Keresztes, D. A. Ross, V. B. Young, and C. Altier. 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a Salmonella enterica serovar typhimurium murine model of infection. Infect Immun 77:2691-702.

29. Groisman, E. A., and H. Ochman. 1993. Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. Embo J 12:3779-87.

30. Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galan. 1998. S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell 93:815-26.

31. Hautefort, I., M. J. Proenca, and J. C. Hinton. 2003. Single-copy green fluorescent protein gene fusions allow accurate measurement of Salmonella gene expression in vitro and during infection of mammalian cells. Appl Environ Microbiol 69:7480-91.

32. Hueck, C. J., M. J. Hantman, V. Bajaj, C. Johnston, C. A. Lee, and S. I. Miller. 1995. Salmonella typhimurium secreted invasion determinants are homologous to Shigella Ipa proteins. Molecular Microbiology 18:479-90.

33. Ikebe, T., S. Iyoda, and K. Kutsukake. 1999. Promoter analysis of the class 2 flagellar operons of Salmonella. Genes Genet Syst 74:179-83.

34. Imada, K., T. Minamino, M. Kinoshita, Y. Furukawa, and K. Namba. 2010. Structural insight into the regulatory mechanisms of interactions of the flagellar type III chaperone FliT with its binding partners. Proc Natl Acad Sci U S A 107:8812-7.

35. Kage, H., A. Takaya, M. Ohya, and T. Yamamoto. 2008. Coordinated regulation of expression of Salmonella pathogenicity island 1 and flagellar type III secretion systems by ATP-dependent ClpXP protease. J Bacteriol 190:2470-8.

36. Kleckner, N., J. Bender, and S. Gottesman. 1991. Uses of transposons with emphasis on Tn10. Methods Enzymol 204:139-80.

37. Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa. 1998. Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science 280:602-5.

38. Kutsukake, K., T. Ikebe, and S. Yamamoto. 1999. Two novel regulatory genes, fliT and fliZ, in the flagellar regulon of Salmonella. Genes Genet Syst 74:287-92.

39. Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier. 2002. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Molecular Microbiology 46:1451-64.

40. Lee, C., C. Wozniak, J. E. Karlinsey, and K. T. Hughes. 2007. Genomic screening for regulatory genes using the T-POP transposon. Methods Enzymol 421:159-67.

41. Lee, C. A., B. D. Jones, and S. Falkow. 1992. Identification of a Salmonella typhimurium invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci U S A 89:1847-51.

42. Liu, X., N. Fujita, A. Ishihama, and P. Matsumura. 1995. The C-terminal region of the alpha subunit of Escherichia coli RNA polymerase is required for transcriptional activation of the flagellar level II operons by the FlhD/FlhC complex. J Bacteriol 177:5186-8.

43. Lucas, R. L., C. P. Lostroh, C. C. DiRusso, M. P. Spector, B. L. Wanner, and C. A. Lee. 2000. Multiple factors independently regulate hilA and invasion gene expression in Salmonella enterica serovar typhimurium. Journal of Bacteriology 182:1872-82.

44. Miller, J. H. 1992. A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

45. Mills, D. M., V. Bajaj, and C. A. Lee. 1995. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol Microbiol 15:749-59.

46. Navarre, W. W., S. Porwollik, Y. Wang, M. McClelland, H. Rosen, S. J. Libby, and F. C. Fang. 2006. Selective silencing of foreign DNA with low GC content by the H-NS protein in Salmonella. Science 313:236-8.

47. Olekhnovich, I. N., and R. J. Kadner. 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of Salmonella enterica serovar Typhimurium. Journal of Bacteriology 184:4148-60.

48. Olekhnovich, I. N., and R. J. Kadner. 2007. Role of nucleoid-associated proteins Hha and H-NS in expression of Salmonella enterica activators HilD, HilC, and RtsA required for cell invasion. J Bacteriol 189:6882-90.

49. Saini, S., J. D. Brown, P. D. Aldridge, and C. V. Rao. 2008. FliZ Is a posttranslational activator of FlhD4C2-dependent flagellar gene expression. J Bacteriol 190:4979-88.

50. Saini, S., J. M. Slauch, P. D. Aldridge, and C. V. Rao. 2010. Role of cross talk in regulating the dynamic expression of the flagellar Salmonella pathogenicity island 1 and type 1 fimbrial genes. J Bacteriol 192:5767-77.

51. Schechter, L. M., S. M. Damrauer, and C. A. Lee. 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the hilA promoter. Molecular Microbiology 32:629-42.

52. Schiemann, D. A., and S. R. Shope. 1991. Anaerobic growth of Salmonella typhimurium results in increased uptake by Henle 407 epithelial and mouse peritoneal cells in vitro and repression of a major outer membrane protein. Infect Immun 59:437-40.

53. Takaya, A., T. Tomoyasu, A. Tokumitsu, M. Morioka, and T. Yamamoto. 2002. The ATP-dependent lon protease of Salmonella enterica serovar Typhimurium regulates invasion and expression of genes carried on Salmonella pathogenicity island 1. Journal of Bacteriology 184:224-32.

54. Tomoyasu, T., A. Takaya, E. Isogai, and T. Yamamoto. 2003. Turnover of FlhD and FlhC, master regulator proteins for Salmonella flagellum biogenesis, by the ATP-dependent ClpXP protease. Mol Microbiol 48:443-52.

55. Winter, S. E., M. G. Winter, P. Thiennimitr, V. A. Gerriets, S. P. Nuccio, H. Russmann, and A. J. Baumler. 2009. The TviA auxiliary protein renders the Salmonella enterica serotype Typhi RcsB regulon responsive to changes in osmolarity. Mol Microbiol 74:175-93.

56. Yamamoto, S., and K. Kutsukake. 2006. FliT acts as an anti-FlhD2C2 factor in the transcriptional control of the flagellar regulon in Salmonella enterica serovar typhimurium. J Bacteriol 188:6703-8.

57. Zhou, D., M. S. Mooseker, and J. E. Galan. 1999. Role of the S. typhimurium actin-binding protein SipA in bacterial internalization. Science 283:2092-5.

CHAPTER 3

The intestinal fatty acid propionate inhibits *Salmonella* invasion through the post-translational control of HilD*

*Hung, C., Garner, C.D., Dwyer, Z. W., Lawhon, S. D., Frye J. G., Ahmer B. M. M., Slauch, J. M., and Altier C. The intestinal fatty acid propionate inhibits *Salmonella* invasion through the post-translational control of HilD. Manuscript is in preparation.

ABSTRACT

For *Salmonella* to cause disease, it must first invade the intestinal epithelium using genes encoded within *Salmonella* Pathogenicity Island 1 (SPI1). Previous work has shown that propionate, a short chain fatty acids abundant in the intestine of animal hosts, negatively regulates SPI1 *in vitro*. Here we investigated how propionate represses invasion. Repression was observed at mildly acidic, physiologically relevant pH, but not under alkaline conditions, suggesting that propionate must enter the bacterial cytoplasm to exert its effects. Transcriptome analyses revealed that propionate predominantly affected SPI1, and that the closely related fatty acid acetate did not share this effect, indicating a specific mechanism for the control of virulence. Using concentrations comparable to those present in the normal mouse cecum, propionate reduced expression of the central SPI1 regulators *hilD*, *hilA*, and *invF*, consequently decreasing the expression and secretion of effector proteins and reducing bacterial penetration of cultured epithelial cells. This control centered on *hilD*, as the loss of only this gene among those of the regulatory cascade prevented repression by propionate. Genetic studies indicated that regulation through *hilD*, however, was not achieved through the control of either transcription or translation, suggesting a post-translational mechanism. Further, repression by propionate on SPI1 was significantly lessened in a mutant unable to produce the metabolic intermediate propionyl-CoA by any known route, an effect specific to this fatty acid, while further metabolism of propionyl-CoA appeared not to be required. These results thus suggest a mechanism by which HilD is post-transcriptionally modified using the high energy intermediate propionyl-CoA.

INTRODUCTION

The complex chemical environment of the animal intestinal tract is created in large part by the vast number of bacteria that populate that organ. This environment is nearly devoid of oxygen, particularly in the large intestine, allowing the growth of bacterial species capable of the anaerobic metabolism of nutrients. In the absence of oxygen and other terminal electron acceptors, this is accomplished through fermentation, where energy is derived through substrate-level phosphorylation reactions. To balance oxidation and reduction during fermentation, however, bacteria must synthesize reducing potential. They do so by producing metabolic intermediates during the fermentation process itself that can be reduced and then excreted into the surrounding medium. Thus, the consequence of fermentation is the production and excretion by bacteria of organic molecules in large concentrations. Predominant among these in the animal intestine are the short chain fatty acids acetate, butyrate and propionate, which can be present in high concentrations (5, 6, 26, 77). These fatty acids have been suggested to have numerous potential effects on the host. Butyrate is the best studied among them, shown to be the primary nutrient source for the colonic epithelium (4, 93). In addition, fatty acids are known to affect smooth muscle contraction, and thus intestinal motility, and are proposed to possess immune modulating functions (20, 64, 75, 83, 85, 97, 104), as well as anti-carcinogenic effects (15, 47, 51, 61, 86, 114). Fatty acids in high concentrations also inhibit the growth of some bacterial species, and so have been proposed as possible preventatives or therapies for enteric bacterial infections (72, 79, 98, 99). It has thus been proposed that manipulation of the microbiota might cause global changes in fatty acid concentrations that could be used to affect the health of the host or prevent infections.

Salmonella is a ubiquitous pathogen that has evolved to survive and proliferate within this intestinal environment, infecting a wide variety of animal species and causing enteric disease. Serovars of *Salmonella enterica* are important sources of bacterial food-borne disease, causing illnesses ranging from self-limiting enteritis to life-threatening septicemia, with

virulence beginning with the invasion of the epithelium that lines the intestine. Invasion of the intestinal tract is mediated by a type three secretion system (TTSS) that is encoded within *Salmonella* Pathogenicity Island 1 (SPI1) (14, 41, 46, 59, 71, 82, 94) . The TTSS forms a "needle complex" (65) that is used to inject effector proteins into host cells, causing rearrangement of the host cell cytoskeleton and leading to engulfment of the bacteria (reviewed by 40). Invasion, and hence the genes encoded in SPI1, are thus required for both the intestinal and septicemic forms of disease (12, 41, 59, 111).

Salmonella invasion is mediated by a number of environmental cues known or likely to be present within the intestine. Activators of SPI1 include low oxygen, log-phase growth, high osmolarity, changes in DNA supercoiling, and pH (8, 38, 42, 70, 96). Importantly, short chain fatty acids have been demonstrated to affect invasion in complex ways. Acetate and the weak acid formate induce the expression of SPI1 genes (56, 69). The action of acetate requires SirA, a recognized activator of invasion and the response regulator of the BarA/SirA two-component regulator (3, 58), but not its sensor kinase partner BarA (69). In addition, induction of invasion by acetate requires the *ackA-pta* operon, encoding acetate kinase and phosphotransacetylase, that converts acetate to acetyl-phosphate and on to acetyl-CoA. These findings thus suggest that acetate works through the production of acetyl-phosphate and the subsequent activation of SirA by phosphorylation. Formate induces invasion by a mechanism that is uncharacterized, but one that requires neither BarA nor SirA (56). For both of these acids, the effects on invasion are observed only at a pH of the growth medium below neutrality and are abrogated at basic pH (56, 69). Fatty acids, being weak acids, concentrate within the bacterial cytoplasm when the cytoplasmic pH exceeds that of the external medium. These findings thus suggest that acetate and formate must concentrate within *Salmonella* to have their effects, rather than acting as extracellular signals. Indeed, the requirement for the cytoplasmic protein SirA in the induction of invasion by acetate, but not BarA with its periplasmic sensing domain, supports such a model.

In contrast to the inducing effects of acetate and formate on invasion, two other predominant intestinal short chain fatty acids, butyrate and propionate, have been shown to have the opposite, repressive effect on the genes and functions of SPI1 (19, 43, 69). It has also been demonstrated that the concentrations and composition of fatty acids vary within regions of the intestine. Thus, the ability of specific classes of these metabolites to have opposing effects on *Salmonella* invasion suggests that the pathogen uses fatty acids as signals for the identification of specific areas within the intestinal tract most productive for invasion. Studies in various mammalian species have shown that acetate, propionate, and butyrate are found in the large intestine (the cecum and the colon), while acetate, propionate, and formate predominate in the small intestine (5, 18, 25, 44, 66, 67, 80). In addition, total fatty acid concentrations can be much higher in the large intestine than in the small intestine, reaching more than 100 mM in some animal species (5, 6, 26, 77). The pH throughout the intestine varies between 6.0 and 7.0 (6, 18, 44), conducive to the cytoplasmic effects of fatty acids on *Salmonella*. Thus, the conditions in the distal small intestine, the area previously described as the location for invasion (21, 60), reflect those known to induce invasion genes (56, 69). Conversely, the high propionate and butyrate concentrations of the cecum and colon may be more likely to repress invasion (69). In fact, *in vivo* studies in mice have shown a correlation between a decrease in large intestinal short chain fatty acid concentrations and an increased susceptibility to *Salmonella* infection (44, 110). Studies using a combination of acetate, propionate, and butyrate designed to mimic the conditions of the mammalian large intestine have also demonstrated that the repressive effects of the latter two outweigh induction by the former (69), indicating that these fatty acids likely affect *Salmonella* virulence in the animal host.

The metabolic fate of fatty acids in *Salmonella* often involves complex and redundant pathways but has been well defined for only some members of this group. The routes of acetate metabolism have been well characterized, with its conversion to acetyl-CoA by either of two routes (113). Similarly, multiple means have been described for the oxidation of formate by

formate dehydrogenases (27, 63). Among the fatty acids repressive for invasion, little is known about butyrate metabolism in *Salmonella*, as no catabolic pathway has yet been identified in this or closely related organisms. In contrast, several mechanisms for the metabolism of propionate have been shown to exist. Propionate, when supplied exogenously, can be converted to the high-energy metabolic intermediate propionyl-CoA by either of two routes, one of which leads through propionyl-phosphate, and both of which are catalyzed by redundant enzymes (50, 89). Propionyl-CoA can also be produced endogenously (50), and eventually is metabolized through the TCA cycle by way of the 2-methylcitrate pathway (87-89).

In addition to the environmental regulators of invasion, an array of genetic factors has been shown to mediate invasion gene expression. Invasion genes are controlled by a complex regulatory network of transcriptional and post-transcriptional regulators both within and outside SPI1. Transcriptional regulators within the island include HilA, HilC, HilD, and InvF. HilA, a member of the OmpR/ToxR family and characterized as a central regulator, is a transcriptional activator of the *inv/spa*, *prg/org*, and *sic/sip* operons that encode the secretion apparatus and secreted effector proteins required for cytoskeletal rearrangement (7, 28, 74). InvF, encoded within the *inv* operon, is itself a transcriptional regulator of the AraC family (62) that controls secreted effectors both within and outside SPI1 (28, 34). InvF can additionally be regulated by mechanisms that are independent of HilA (2, 3, 91). HilA is itself controlled by HilC and HilD from within the island and RtsA and HilE outside SPI1 (37), and can negatively regulate itself (30).

To better understand the mechanism by which intestinal fatty acids repress *Salmonella* invasion, in this work we examined the effects of propionate on SPI1 gene expression and invasion, and investigated the metabolic and genetic pathways required for these effects. We found that at physiologically relevant concentrations, propionate repressed invasion and that pH was important for the repressive effect. Additionally, as the pathways for propionate metabolism have previously been characterized in *Salmonella*, we used genetic approaches to further study

the metabolic routes and products required for the repressive effect of this fatty acid. We show here that metabolism of propionic acid is necessary for its repressive effect and that in particular, the high-energy metabolic intermediate propionyl-CoA is likely required. We further demonstrate that the repressive effect of propionate functions through the central SPI1 regulator *hilD*, but that this control is evoked through neither transcription nor translation, implicating a post-translational modification of HilD as required for regulation.

MATERIALS AND METHODS

Bacterial Strains. Bacterial strains and plasmids used in this study are shown in Table 3.1. All strains are isogenic to *Salmonella enterica* serovar Typhimurium strain ATCC 14028s. All gene deletions were created using the previously described one-step inactivation method (29). Briefly, PCR products were generated from the chloramphenicol or kanamycin resistance genes of pKD3 and pKD4, respectively, using primers carrying at their 5' ends 40 bp of homology to the regions flanking the start and stop codons of the gene to be deleted. A *Salmonella* strain carrying pKD46, containing the λ Red recombinase for allelic exchange, was transformed with the resultant PCR products. All deletion mutants were checked for the loss of genes by PCR. P22 transduction was used to transfer marked deletions and to create multiple mutations in strains. To create unmarked deletions, the FLP recombinase was used to remove resistance markers (29). The *hilD'-'lacZ* chromosomal translational fusion was constructed using FRT-mediated integration as previously described (36), and carried 296 bp of the *hilD* ORF. Plasmid fusions of *hilD* and *sopB* to *luxCDABE* were constructed as previously described (105). The *hilD* complementation plasmid was created by amplifying the *hilD* open reading frame (ORF) including sufficient upstream sequence to include the ribosome binding site. The PCR product was then cloned into the kanamycin resistance gene of the cloning vector pACYC177 (22) using the unique *Sma*I and *Cla*Isites, placing *hilD* under the control of the promoter for this gene.

Table 3.1. Strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or reference
Strains		
ATCC 14028s	Wild type	American Type Culture Collection
CA412	*sip*C::*lac*ZY	(8)
CA414	*hilA::lacZY*	(57)
CA1694	*invF::lacZY*	(8)
CA921	*sip*C::*lac*ZY Δ*sirA*	This study
CA2122	*sip*C::*lac*ZY Δ*fliZ*	This study
CA1983	*sip*C::*lac*ZY Δ*hilD*	This study
CA504	*sip*C::*lac*ZY Δ*hilA*	This study
CA749	*sip*C::*lac*ZY Δ*hilE*	This study
CA2294	*sip*C::*lac*ZY Δ*lon*	This study
CA1959	Δ*hilC*	This study
CA1601	Δ*hilD*	(95)
CA2304	Δ*hilC* Δ*hilD*	This study
CA1005	Δ*csrB* Δ*csrC*	(39)
CA1512	*sipC::lacZY* Δ*prpC*	This study
CA1796	*sipC::lacZY* Δ*acs*, *prpE*, *pta*	This study
CA1821	*sipC::lacZY* Δ*acs*, *prp*E, *pta*, *pflB*, *tdcE*	This study
Plasmids		
	hilD::luxCDABE	(105)
	sopB::luxCDABE	(105)
pACYC177	Cloning vector	(22)
pCA174	*hilD* ORF on pCAYC177	This study

β-galactosidase assays. For assays using media of an acidic pH, cultures were grown overnight standing at 37°C in LB with100 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 6.7 containing either no additive, 10 mM propionic acid, or 10 mM butyric acid. For experiments where a pH of 8.0 was used, 100 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) pH 8.0 was used in place of the MOPS. All cultures were grown in triplicate and β-galactosidase activity was measured as previously described (81).

HEp-2 invasion assays. Cultures were grown overnight standing at 37°C in LB with100 mM MOPS pH 6.7 with no additive or 10 mM propionic acid. The invasion assay was performed as previously described (3), except that upon infection plates were centrifuged at 100 x g. Quadruplicates were tested for each strain under each condition.

Reverse transcription real-time PCR. The wild type *Salmonella* strain was grown overnight with aeration in N-minimal media with 0.2% glucose to repress SPI1 gene expression. Aliquots were then sub-cultured into LB broth with100 mM MOPS, pH 6.7 with either no additive or with 10 mM propionic acid. Cultures were grown at 37 ºC with slow shaking (60 rpm) for 4.5 hr to reach an optical density at 600 nm (OD_{600}) of 0.37-0.45. Three independent cultures were used for each condition. Total RNA was extracted and cDNA synthesis was performed as previously described (56). cDNA samples were diluted 2000-fold for detection of 16S rRNA as the control, and 20-fold for detection of all other gene products. cDNA was used as template for real-time PCR using B-R Syber green reagent (Quanta Biosciences) with cycling once at 95 ºC for 3 min followed by 40 cycles at 95 ºC for 20 sec and 58 ºC for 1 min. Individual samples were each tested in triplicate. Primers for *hil*D and *hil*C expression were used as previously described (56). Other primers used were GGGAGTATATTACGGCATCAG and TTCATGAGTCTCTTCCATAGTG for *rts*A; GTGCCAGCMGCCGCGGTAA and GACTACCAGGGTATCTAAT for 16S rRNA. The relative expression of invasion genes was normalized to that of 16S rRNA using iQ5 software (Bio-Rad).

Secreted protein isolation and analysis. The wild type *Salmonella* strain was grown in LB 100 mM MOPS pH 6.7 with no additive, 10 mM propionic acid or 10 mM butyric acid at 37 ºC with shaking at 60 rpm for 16 hr. Proteins secreted into the culture supernatant were prepared and analyzed as previously described (3).

Microarrays. Microarray analysis was performed as previously described (68). Bacteria were grown prior to RNA extraction in either 15 mM propionic acid or acetic acid in buffered LB medium.

Luciferase assays. Strains were grown overnight in LB 100 mM MOPS pH 6.7 and then diluted 100-fold in the same medium with appropriate additives. Samples of 150 μl were inoculated into 96-well plates, and luminescence and OD_{600} were read every 20 minutes for 15

hours using a Synergy 2 luminescence microplate reader (BioTek, Winooski, VT). Samples were tested in replicates of six or more.

Statistical Analysis. Results from β-galactosidase assays, invasion assays, and reverse transcription real-time PCR were analyzed using a one-way analysis of variance to determine if the mean of at least one strain or condition differed from any of the others. The Tukey-Kramer HSD multiple comparison test was then used to determine which means were statistically different. A p-value <0.05 was considered significant. Statistical analysis was performed using Jmp 8.0 and 9.0 software (SAS, Cary, NC).

RESULTS

Fatty acids are the predominant metabolic product of the anaerobic intestinal microbiota and so largely define the environment of the mammalian intestinal tract. Previous work in our laboratory and those of others has shown that propionate, a major constituent of the large intestinal environment, reduces *Salmonella* SPI1 invasion gene expression *in vitro* when supplied alone or in combination with other fatty acids (31, 44, 69). To identify the propionate regulon more completely, we preformed microarray analyses, comparing gene expression of *Salmonella* serovar Typhimurium grown in buffered LB broth to that grown in buffered LB with the addition of propionate. We found that the expression of a majority of SPI1 genes was repressed by growth in the medium containing propionate. Of the 35 genes of SPI1, 22 were reduced in their expression by at least 2-fold with the addition of this fatty acid (Table 3.2). Among these were the central transcriptional regulators of SPI1 *hilD* and *hilA*, although the expression of *hilC*, an additional transcriptional regulator involved in the complex regulation of invasion, was not repressed. Propionate, being a weak acid, can enter the bacterial cytoplasm and can, at least in high concentration, reduce transmembrane potential and cytoplasmic pH (92, 100). To determine whether the observed changes in gene expression might be due to this generic mechanism, we also employed microarrays to examine gene expression when bacteria were grown in medium containing acetate, a short chain fatty acid with a pKa similar to that of propionate. In contrast to the effects of propionate, no SPI1 gene demonstrated reduced expression in the presence of acetate (Table 3.2). In fact, 16 of 35 SPI1 genes were induced at least 2-fold by acetate, supporting our previous finding that this fatty acid can induce SPI1 gene expression (69), and demonstrating that repression by propionate is not accomplished simply by the accumulation of weak acid within the bacterial cytoplasm. Additionally, we found that the most severe repressive effects of propionate were manifested within SPI1. Of the genes demonstrating repression by propionate but not by acetate, 20 of the 30 with the greatest

repression are either encoded within SPI1 or are themselves controlled by SPI1 regulators (Table 3.3).

Table 3.2. Effects of the intestinal fatty acids propionate and acetate on expression of SPI1 genes

Gene number	Gene symbol	Median Expression with Propionate*	Median Expression with Acetate*
STM2865	*avrA*	1.0	1.7
STM2866	*sprB*	1.0	2.4
STM2867	*hilC*	1.8	2.6
STM2868	*orgC*	0.6	1.1
STM2869	*orgA*	0.6	2.1
STM2871	*prgK*	0.3	1.8
STM2872	*prgJ*	0.4	1.8
STM2873	*prgI*	0.3	1.8
STM2874	*prgH*	0.3	1.9
STM2875	*hilD*	0.4	1.4
STM2876	*hilA*	0.5	1.8
STM2877	*iagB*	0.8	1.4
STM2878	*sptP*	0.4	1.1
STM2879	*sicP*	0.4	1.3
STM2880	-	0.4	1.3
STM2881	*iacP*	0.5	1.2
STM2882	*sipA*	0.3	2.0
STM2883	*sipD*	0.2	2.2
STM2884	*sipC*	0.3	2.0
STM2885	*sipB*	0.3	2.1
STM2886	*sicA*	0.3	1.8
STM2887	*spaS*	1.3	3.6
STM2888	*spaR*	0.7	2.0
STM2889	*spaQ*	0.7	1.2
STM2890	*spaP*	0.5	2.6
STM2891	*spaO*	0.7	3.1
STM2892	*invJ*	0.5	1.7
STM2893	*invI*	0.5	2.3
STM2894	*invC*	0.6	3.1
STM2895	*invB*	0.5	2.4
STM2896	*invA*	0.6	2.2
STM2897	*invE*	0.4	1.9
STM2898	*invG*	0.3	1.5
STM2899	*invF*	5.6	3.7
STM2900	*invH*	0.3	1.5

*Proportion of expression for each gene is given in comparison to that produced during growth in medium without fatty acid added.
This work was done by Sara Lawhon and Jonathan Frye.

Table 3.3. *Salmonella* genes most severely repressed by propionate but not repressed by acetate.

Gene number[a]	Gene symbol	Median Expression with Propionate[b]
STM1091	***sopB***	**0.2**
STM4315	***rtsA***	**0.2**
STM2883	***sipD***	**0.2**
STM2898	***invG***	**0.3**
STM2900	***invH***	**0.3**
STM2886	***sicA***	**0.3**
STM2884	***sipC***	**0.3**
STM2874	***prgH***	**0.3**
STM2885	***sipB***	**0.3**
STM2882	***sipA***	**0.3**
STM1090	***pipC***	**0.3**
STM3138	*mcpA*	0.3
STM2873	***prgI***	**0.3**
STM2871	***prgK***	**0.3**
STM4464	-	**0.4**
STM4465	-	**0.4**
STM2872	***prgJ***	**0.4**
STM3216	*mcpC*	0.4
STM1690	*pspA*	0.4
STM2153	*yehE*	0.4
STM2315	*yfbK*	0.4
STM2897	***invE***	**0.4**
STM0271	-	0.4
STM4265	*soxS*	0.4
STM1513	-	0.4
STM2879	***sicP***	**0.4**
STM1774	***sirC***	**0.4**
STM2880	-	**0.4**
STM1239	-	0.4
STM2875	***hilD***	**0.4**

[a]Genes shown in bold are located within SPI1 or are controlled by SPI1 regulators.
[b]Proportion of expression for each gene is given in comparison to that produced during growth in medium without fatty acid added.
This work was done by Sara Lawhon and Jonathan Frye.

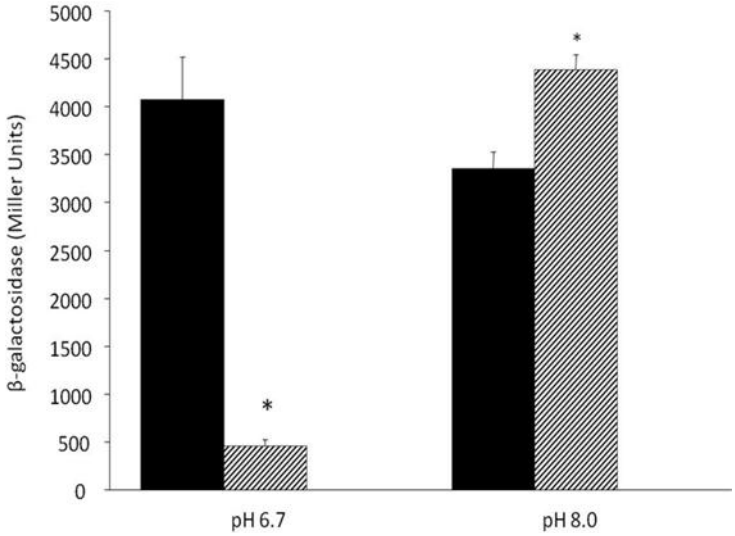

Figure 3.1. Propionate represses *sip*C invasion gene expression. Cultures of the strain with the *sip*C::*lac*ZY reporter fusion were grown overnight standing in LB with either 100 mM MOPS pH 6.7 or 100 mM HEPES pH 8.0, containing no additive (black bars) or10 mM propionic acid (striped bars). β-galactosidase assays were used to assess *sip*C expression. Error bars indicate standard deviation. An asterisk (*) indicates a statistically significant difference from wild type with no additive at $p < 0.05$.
This work was done by Cherilyn Garner.

To confirm the microarray findings, we tested the effects of propionate on key SPI1 regulators and effectors by independent methods. Using media buffered to pH 6.7 and 10 mM propionic acid, conditions designed to mimic those of the murine large intestine (44), we employed *lacZY* reporter fusions to the SPI1 invasion genes *sipC*, encoding a secreted effector protein, and *hilA* and *invF*, both transcriptional regulators. We found there to be a significant decrease in gene expression for each of these fusions, more than 8-fold for *sipC* and 5-fold each for *invF* and *hilA*, in the presence of propionic acid (Figs. 3.1 and 3.2). Additionally, previous work had shown that pH is important for the effects of fatty acids on invasion gene expression, suggesting that accumulation of the fatty acid in the bacterial cytoplasm is necessary for the observed effects (56, 69). In contrast to the repression observed when the medium was maintained at pH 6.7, we found that at pH 8.0 propionate failed to repress *sipC*, *invF*, or *hilA*. Instead, there was a slight increase in gene expression in the presence of propionate (Fig. 3.1 and not shown). Thus, these combined results suggest that, although

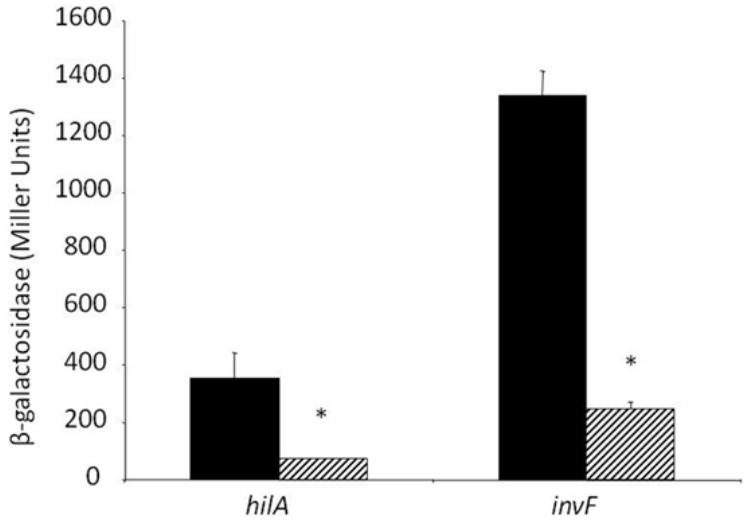

Figure 3.2. Propionate represses regulators of *Salmonella* SPI1. Cultures of wild type with the *hil*A::*lac*ZY or *inv*F::*lac*ZY reporter fusion were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing no additive (black bars) or 10 mM propionic acid (striped bars). β-galactosidase assays were used to assess *hil*A *and inv*F expression. Error bars indicate standard deviation. An asterisk (*) indicates a statistically significant difference from no additive at $p < 0.05$.
This work was done by Gerilyn Garner.

propionate does not exert its repressive effects by acidification of the bacterial cytoplasm, it must still enter the bacterium to repress the genes of SPI1.

To confirm the effects of propionate on phenotypes associated with invasion, we next examined whether this fatty acid had any effect on SPI1 secreted effector proteins or on invasion of epithelial cells *in vitro*. When grown in laboratory media, *Salmonella* secretes into the culture media the invasion proteins SipA, B, C, and D (57). Using wild type bacteria grown overnight in media buffered to pH 6.7 with no additive, proteins extracted from the culture supernatant, separated by SDS-PAGE and stained with Coomassie blue demonstrated the presence of four bands of apparent molecular weights equivalent to these proteins (Fig. 3.3A). These four bands were greatly diminished, however, in extracts from the same strain grown in the presence of propionic acid, suggesting that the repression of invasion genes is also manifested as a reduction of the proteins they encode. Subsequent analysis by mass spectroscopy showed these four proteins to indeed be SipA, B, C, and D (not shown). We similarly tested the ability of this fatty acid to affect the penetration of epithelial cells, a function

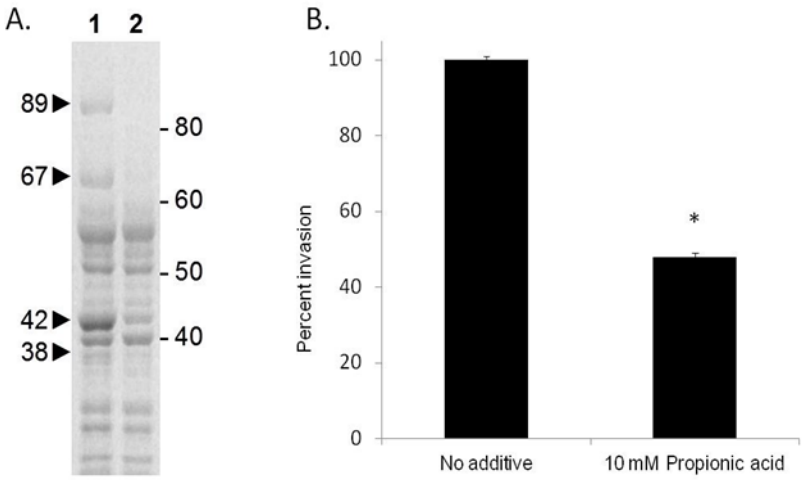

Figure 3.3. Propionate diminishes production of SPI1 secreted effector proteins and decreases *Salmonella* invasion of HEp-2 cells. A) Wild type *Salmonella* was grown in LB with 100 mM MOPS pH 6.7 and no additive (lane 1) or 10 mM propionic acid (lane 2). Secreted proteins were then isolated and analyzed by SDS PAGE. Proteins with apparent molecular weights of 89, 67, 42, and 38 kDa that were reduced in cultures grown with fatty acids are designated with arrowheads. Molecular weights are shown on the right. B) The wild type strain was grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive or10 mM propionic acid. Invasion of HEp-2 cells was assessed using a gentamicin protection assay. Invasion is shown relative to wild type invasion in no additive which was set to 100%. Error bars indicate standard deviation. An asterisk (*) indicates a statistically significant difference from wild type with no additive at $p < 0.05$.
The work of Figure 3.3B was done by Cherilyn Garner.

requiring SPI1. We found using a gentamicin-protection assay that overnight growth in propionic acid prior to infection significantly decreased invasion of cultured HEp-2 cells, by 2-fold (Fig. 3.3B). Therefore, these results, consistent with those of other studies for epithelial cell invasion (19, 32, 107), confirm that propionate not only affects expression of invasion genes, but also affects the production of secreted effector proteins and the invasion of epithelial cells.

As pH and thus the ability of propionate to enter the bacterial cytoplasm was important for its repressive effects on SPI1 invasion genes, we next determined whether this fatty acid acted directly or whether it must first have been converted to a metabolic product to have its effect. The pathways for propionate metabolism have been well characterized in *Salmonella* and *E. coli* (50, 52-55, 87-89). Therefore, we next tested the genes of these pathways to determine whether they were important for the repressive effect of propionate. There are two

characterized pathways for the initial steps of propionate metabolism, through propionate kinase and phosphotransacetylase or independently through propionyl-CoA synthetase, that both lead to the intermediate propionyl-CoA, which then feeds into the 2-methyl citric acid cycle (89). The production of TCA cycle intermediates from 2-methylcitrate is then catalyzed by 2-methylcitrate synthase, encoded by *prpC*. In the propionate kinase/phosphotransacetylase pathway, propionyl phosphate is produced from propionate and then metabolized to propionyl-CoA by a phosphotransacetylase encoded by *pta* (89). The propionyl-CoA synthetase pathway metabolizes propionate directly to propionyl-CoA, using two additional genes, *acs* and *prp*E, which encode acetyl-CoA and propionyl-CoA synthetases (54). To determine whether metabolism of propionate was important for its repressive effect on invasion, we first examined the importance of the 2-methylcitrate pathway. Using the *sipC::lacZY* reporter, we tested a non-

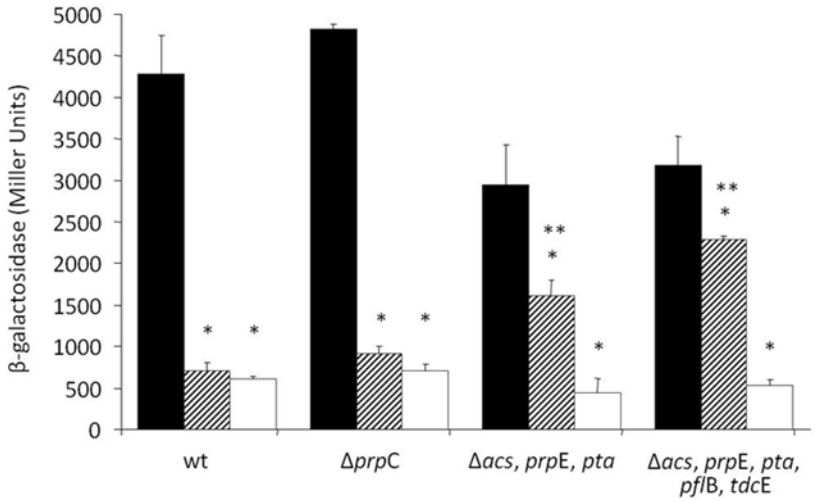

Figure 3.4. Production of the intermediate propionyl-CoA is important for the repressive effect of propionate. Wild type and mutant strains with the *sip*C::*lac*ZY reporter fusion were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive (black bars), 10 mM propionic acid (striped bars), or 10 mM butyric acid (white bars). β-galactosidase assays were used to assess *sip*C expression. Error bars indicate standard deviation. An asterisk (*) indicates a statistically significant difference from each respective strain with propionate or butyrate compared to no additive at $p < 0.01$. Two asterisks (**) indicates a statistically significant difference of a mutant strain grown in propionate compared to wild type with propionate or mutant strain grown in butyrate compared to wild type with butyrate at $p < 0.05$.
This work was done by Cherilyn Garner

polar null mutant of *prpC* and found that the mutation had no effect on the repression of *sipC* in the presence of propionate, suggesting that metabolic intermediates downstream from propionyl-CoA were unnecessary for this effect (Fig. 3.4). We next tested a null mutant of *prp*E, *acs*, and *pta* in the *sipC*::*lac*ZY fusion strain, eliminating all of the known routes of metabolism from propionate to propionyl-CoA. Using this strain, we found a significant, 2-fold increase in invasion gene expression in the presence of propionic acid when compared to that of the wild type grown under the same conditions, but the combined mutations failed to restore invasion gene expression to the level seen in the mutant without propionic acid added. However, there are previous reports that *tdc*E and *pfl* can produce propionyl-CoA from endogenous sources through 2-ketobutyrate (50). Thus, we created a mutant that deleted all known pathways for the production of propionyl-CoA from endogenous and exogenous sources (a *prpE, acs, pta, pflB, tdcE* mutant). We found that invasion gene expression was increased 3-fold from the wild type level in the presence of propionic acid in this mutant strain. To examine the specificity of these genetic pathways for the effects of propionate, we additionally tested a second short chain fatty acid, butyrate, which is chemically similar to propionate and has also been shown to repress SPI1 genes (31, 32, 43, 69, 109). For both the *prpE*, *acs*, *pta* mutant and the *prpE*, *acs*, *pta*, *pflB*, *tdcE* mutant, the increase in invasion gene expression seen in the presence of propionic acid was specific for that fatty acid, as butyric acid continued to fully repress *sipC* expression in these mutants, identical to its effect on the wild type strain (Fig. 3.4). Therefore, these results implicate the production of propionyl-CoA as necessary for the negative effect of propionate on invasion and demonstrate its effect to be independent of generic means of gene regulation that might be induced by fatty acids.

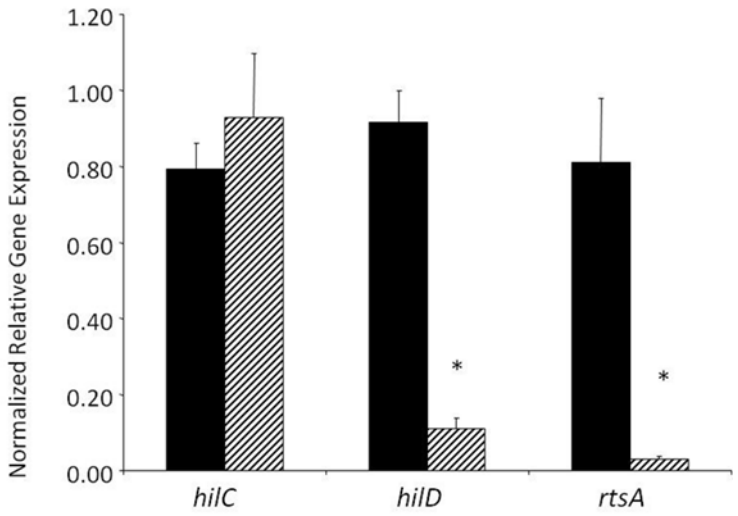

Figure 3.5. Propionate represses the central SPI1 regulator *hilD*. The wild type strain was grown in LB with100 mM MOPS, pH 6.7 with either no additive (black bars) or with 10 mM propionic acid (striped bars) to an optical density at 600 nm of 0.37-0.45. Total RNA was extracted and cDNA synthesis was performed. cDNA was then used as template for real-time PCR to measure relative gene expression of *hilC*, *hilD*, and *rtsA*. An asterisk (*) indicates a statistically significant difference from no additive at $p < 0.05$.

As we had shown that two important regulators of SPI1, *invF* and *hilA*, were repressed in the presence of fatty acids, we next determined whether other regulators of invasion were affected similarly. To address this, we examined three SPI1 regulators that occur further upstream in the invasion regulatory cascade: *hilD* and *hilC*, both encoded within SPI1, and *rts*A, encoded outside the island. As all of these are known to regulate their own expression (35), we used quantitative reverse transcription real-time PCR to determine relative gene expression without manipulation of the genes themselves. We found that messages of both *hilD* and *rtsA* were significantly decreased in the presence of propionic acid, while consistent with our microarray data, that of *hilC* was not changed (Fig. 3.5). These results thus show that propionate acts to decrease the expression of many regulators of invasion and acts either at the level of *hilD* or higher in the SPI1 regulatory cascade.

HilD occupies a position at the apex of the regulatory cascade within SPI1, but can itself be controlled, both positively and negatively, by several genetic elements outside the island

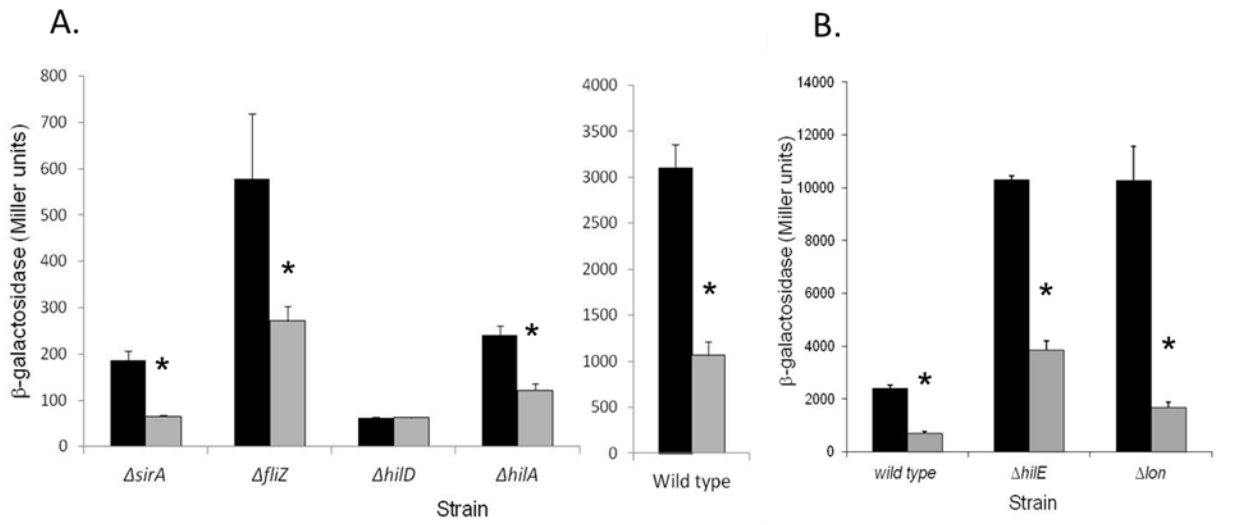

Figure 3.6. The loss of *hilD* alone prevents the repressive effects of propionate on *sipC* expression. Wild type and mutant strains with the *sip*C::*lac*ZY reporter fusion were grown overnight standing in LB with 100 mM MOPS pH 6.7 containing either no additive (black bars) or 10 mM propionic acid (grey bars). β-galactosidase assays were used to assess *sip*C expression. Error bars indicate standard deviation. The wild type in panel A is shown using a separate axis to better discern the differences among mutant strains. An asterisk (*) indicates a statistically significant difference for a given strain with the addition of propionate as compared to no additive at $p < 0.05$.

(Fig. 3.10). To determine the importance of HilD specifically for the repressive effects of propionate, we next examined the effects of mutations in genes known to encode regulators of *hilD*. Using the *sipC::lacZY* fusion to assess repression within SPI1, we first tested the positive regulators *sirA* and *fliZ*. SirA encodes a response regulator in *Salmonella* that induces expression of the regulatory RNAs CsrB and CsrC, which titrate the protein CsrA that can bind to *hilD* message and prevent translation (2, 39, 78). FliZ has been shown to also affect HilD by controlling its protein activity (24). Although expression of *sipC* was reduced, as expected, in mutants of either *sirA* or *fliZ*, culture of these strains in the presence of propionic acid continued to repress *sipC* to a degree proportionate to that of the wild type grown under the same conditions (Fig. 3.6A), indicating that propionate represses by a means independent of these two regulators. In contrast, the mutant of *hilD* itself became completely refractory to reduced *sipC* expression when propionic acid was present. We tested next two negative regulators of *hilD*: HilE is a well characterized repressor of *hilD*, while Lon protease also represses through its

effects on either HilD itself or FliZ (13, 16, 17, 24). Again using the *sipC::lacZY* fusion, we found in both cases that gene expression was increased when strains were grown without additive, compared to the wild type, but that propionic acid continued to significantly reduce expression (Fig.3.6B), thus indicating that neither of these repressors of *hilD* was important for HilD-mediated repression by propionate. Immediately downstream from HilD in the regulatory cascade lies *HilA*, itself a transcriptional activator (Fig. 3.10). Work presented here shows *hilA* to be one of the SPI1 genes repressed by propionate (Table 3.1 and Fig. 3.2). To define the role of *HilA* in SPI1 repression, we further tested the effects of a *hilA* null mutant on *sipC* expression. We found that propionic acid continued to repress *sipC* in this mutant to a degree similar to that in the wild type (Fig. 3.6A). Thus, although propionate represses *hilA* through its control of *hilD*, *hilA* does not play a significant role in the control of downstream SPI1 genes produced by this fatty acid. To verify the importance of HilD in repression of invasion by propionate, we additionally tested its effect on *sopB*, encoding a type III secreted effector located outside SPI1 but controlled by SPI1 regulators (1, 33, 90). Using a plasmid-borne *luxCDABE* fusion to *sopB* and measuring light production over a time-course experiment, we observed that *sopB* exhibited the typical rise and fall in gene expression associated with growth phase that has been reported for genes controlled via SPI1 when grown in culture (38, 70, 76). The addition of propionic acid, however, significantly repressed this gene, greatly reducing its cumulative expression (Fig. 3.7A). Additionally, the loss of *hilD* severely reduced *sopB* expression and made *sopB* refractory to the effect of propionic acid (Fig. 3.7B). By contrast, *sopB* expression in a mutant of *hilC*, shown above to be unaffected by propionate (Fig. 3.7C) was indistinguishable from that of the wild type with or without the addition of propionic acid, and a *hilC*, *hilD* double mutant reproduced the phenotype of the *hilD* mutant alone (Fig. 3.7D). Thus, these results show that *hilD* is required for the propionate-mediated repression of *Salmonella* genes essential for tissue invasion.

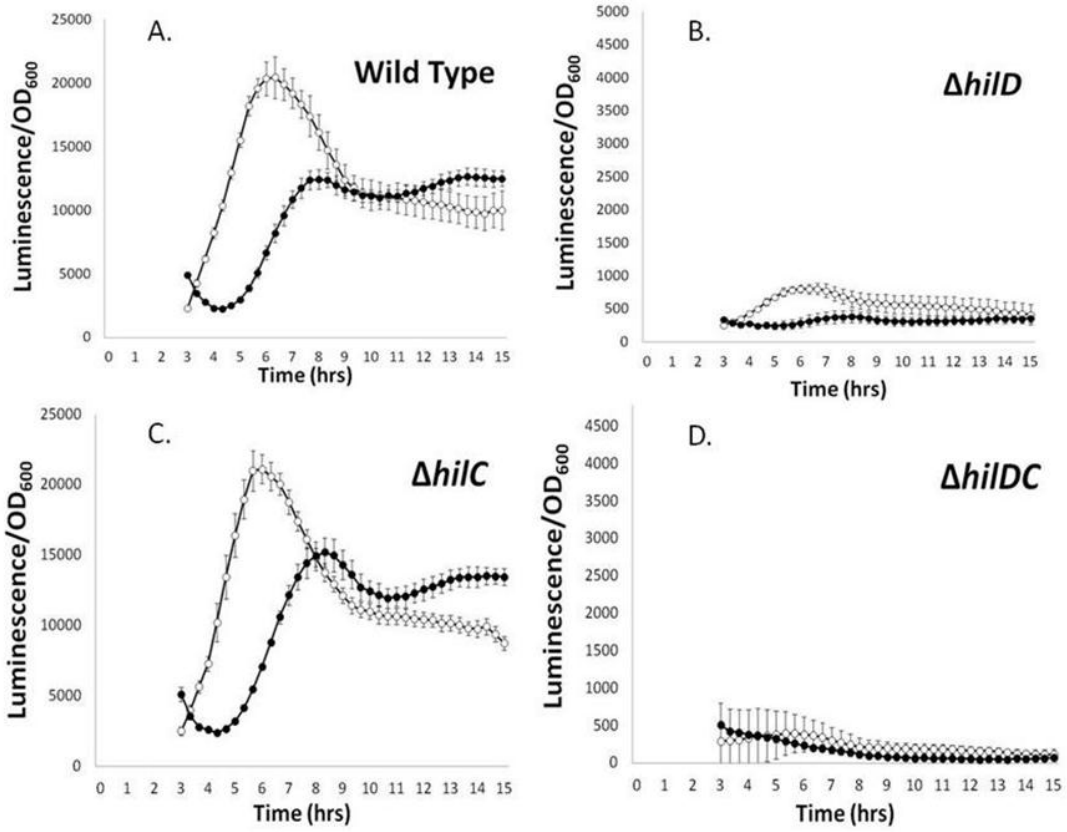

Figure 3.7. *hilD*, but not *hilC*, is required for the repressive effect of propionate. Strains of the genotype shown and with the *sopB::luxCDABE* reporter fusion plasmid were grown in 96-well plates in LB with 100 mM MOPS pH 6.7 containing either no additive (open symbols) or 10 mM propionic acid (closed symbols). Light production was used to assess *sopB* expression, and optical density at 600 nm (OD_{600}) was used to measure growth. The measure of luminescence/OD_{600} was used to normalize for variation in growth rate. Error bars indicate standard deviation.

Although our results indicate that propionate functions to repress *hilD* by a means independent of the tested regulators, the control of this gene remains complex. HilD has been shown to regulate its own expression (35), and thus mutants unable to produce the protein exhibit reduced expression of the gene. To determine specifically how propionate might affect *hilD*, we next examined the effects it had on transcription in the presence and absence of functional HilD. For this, we employed a transcriptional *hilD::luxCDABE* fusion carried on a

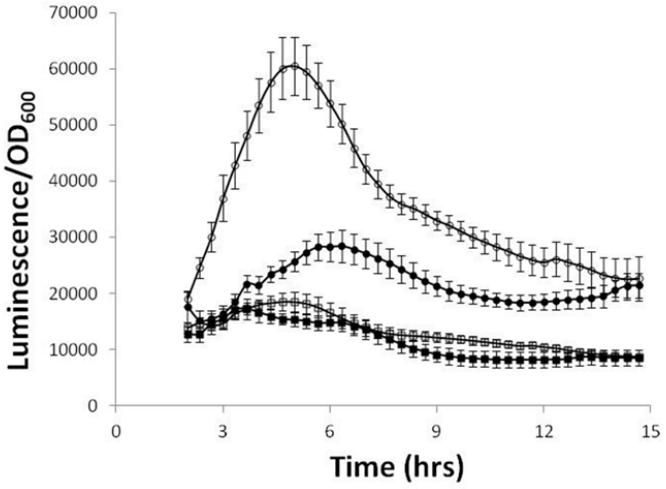

Figure 3.8. Functional HilD is required for the repressive effect of propionate on *hilD* expression. Wild type (circles) and the *hilD* mutant (squares) with the *hilD::luxCDABE* reporter fusion plasmid were grown in 96-well plates in LB with 100 mM MOPS pH 6.7 containing either no additive (open symbols) or 10 mM propionic acid (closed symbols). Light production was used to assess *hilD* expression, and optical density at 600 nm (OD_{600}) was used to measure growth. The measure of luminescence/OD_{600} was used to normalize for variation in growth rate. Error bars indicate standard deviation.
This work was done by Zach Dwyer.

plasmid in both wild type and Δ*hilD* strains. The fusion construct included the entire upstream region (to position -283 from the transcriptional start site) known to be required for maximal expression (84), and so would be predicted to respond to the genetic and environmental regulators of *hilD*. As expected, we found that in the wild type strain propionic acid greatly affected *hilD* expression, reducing peak expression by 2-fold and reducing the total expression over the course of the experiment by 3.3-fold (Fig. 3.8). The loss of the chromosomal copy of *hilD*, however, both further reduced *hilD::luxCDABE* expression and completely eliminated the effect of propionic acid, demonstrating the necessity of intact HilD for these effects.

For genes that do not undergo auto-regulation, such control of a transcriptional fusion in a wild type strain would clearly indicate regulation at the level of transcription. The requirement here, however, that HilD be present allows the possibility that propionate functions by controlling the chromosomal copy of *hilD*, which then affects the *hilD::luxCDABE* fusion, and that such

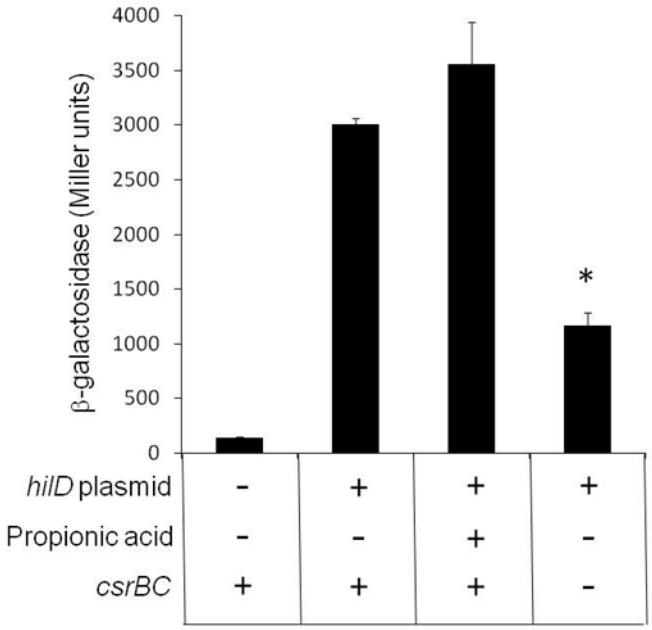

Figure 3.9. Propionate does not affect the translation of *hilD*. Strains carrying a single-copy translational fusion of *hilD* to *lacZY* and of the genotype shown were grown overnight in LB with100 mM MOPS, pH 6.7 and were assessed for b-galactosidase production. The *hilD* plasmid encoded the gene on pACYC177 under the control of the *npt* promoter. Propionic acid was supplied at 10 mM, and the + symbol for *csrBC* indicates the presence of these two genes. An asterisk (*) indicates a statistically significant difference of the *csrBC* mutant from the *csrBC*$^+$ strain at $p < 0.05$.

control could be gained at any one of a number of levels. To examine the specific mechanism by which *hilD* was controlled, we next tested a chromosomally encoded, single-copy translational fusion of *hilD* to *lacZ*. Because *hilD* is disrupted in this strain, fusion expression is low (Fig. 3.9). The addition of *hilD* expressed from a constitutive promoter on a low copy-number plasmid (the *npt* promoter of pACYC177) provided greatly increased gene expression. In this strain, we found that propionic acid had no effect on *hilD* expression demonstrating that, with the over-expression of *hilD*, propionate exhibited no control over the transcription or translation of *hilD*. To ensure that the experimental conditions used were adequate to detect such control, we also tested *hilD* expression, with the *hilD* plasmid present, in a double mutant of CsrB and CsrC. These mutations allow increased activity of the regulatory protein CsrA, which has been shown to bind to *hilD* message the repress translation. As seen in Fig. 3.9, *hilD*

expression was reduced by 3-fold in this mutant, demonstrating that translational control could be detected and therefore that, with the exogenous production of HilD, propionate fails to affect *hilD* transcription or translation, and thus that the repression of invasion by propionate is exerted through the post-translational control of HilD.

DISCUSSION AND FUTURE DIRECTIONS

Propionate is a fatty acid produced as a metabolic byproduct of bacterial fermentation and is thus found in high concentration within the intestinal lumen of humans and other animals. It has been previously demonstrated that this short chain fatty acid can repress the genes of SPI1 (31, 32, 69, 108, 109). The work presented here demonstrates that this repression occurs as a result of the post-translational control of the central SPI1 regulator HilD, and requires the high energy metabolic product of propionate metabolic, propionyl-CoA. Our studies show that propionate functions as a cytoplasmic signal to repress invasion. Although this and other fatty acids have been proposed to act as extracellular signals of the BarA/SirA two-component regulator of invasion (23), we found this regulatory pathway to be dispensable to the effects of propionate (Fig. 3.6). The importance of a pH below neutrality provided further support for a cytoplasmic effect (Fig. 3.1), as the pH differential between the bacterial cytoplasm and the external milieu dictates the internal concentration of this fatty acid. The requirement for propionyl-CoA production (Fig. 3.4) and the specific effect on *hilD* (Fig. 3.6) additionally indicate

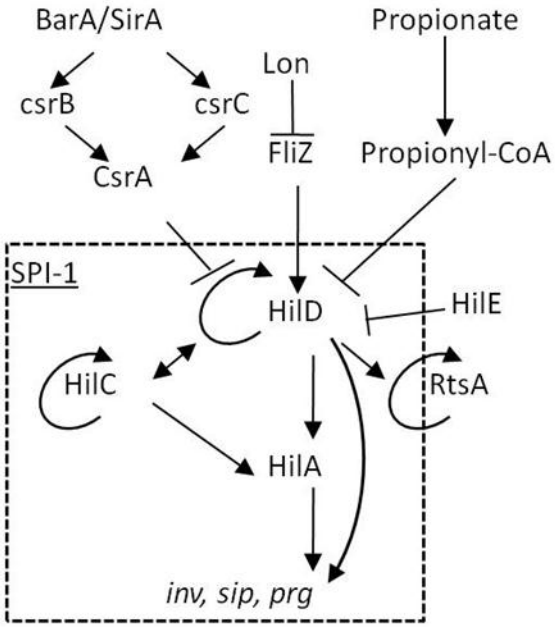

Figure 3.10. A model for the control of SPI1 by propionate and genetic regulators

that propionate must penetrate the bacterium to induce its effects.

Propionate and other short chain fatty acids have long been known to affect the physiology and behavior of bacteria. Propionate, along with acetate and butyrate, act as potent repellents of bacterial chemotaxis (106). Short chain fatty acids, being weak acids, can also enter bacteria and concentrate within the cytoplasm under the conditions of mildly acidic pH used in this study. Cytoplasmic weak acids then reduce the internal pH, affecting transmembrane potential and reducing the proton motive force (92). In light of these generic effects, one might expect propionate to elicit global changes on *Salmonella* gene expression. The effects of propionate, however, showed a surprising specificity for the genes of SPI1 and those controlled by regulators within this island; a majority of the most highly repressed genes were those involved in invasion. In addition, the failure of acetate to induce similar expression changes demonstrates that propionate affects invasion genes by a mechanism independent of the generic effects that might be caused by weak acid accumulation. As the experimental conditions chosen here were designed to approximate those of the mammalian intestinal tract in their propionate concentration and pH, these results suggest that under physiologically relevant conditions the primary effect of intestinal propionate on *Salmonella* is on the repression of invasion.

The results of genetic studies conducted here strongly suggest that propionyl-CoA is the metabolite essential to the repression of invasion by propionate. Upon deletion of the genes necessary for metabolism of exogenous propionate*, prpE*, *acs*, and *pta*, we found a significant increase in invasion gene expression in the presence of propionic acid when compared to that of the wild type strain under the same conditions (Fig. 3.4). The mutant deleted for all of the known pathways for production of propionyl-CoA from both endogenous and exogenous sources, a *prpE, acs, pta, pflB, tdcE* mutant, demonstrated an every larger increase in invasion gene expression in the presence of propionic acid (Fig. 3.4). In both of these cases, however, there was not full restoration of invasion gene expression, suggesting that there may be other,

uncharacterized routes of propionyl-CoA metabolism, thus preventing complete effects by the mutants tested. If further metabolism of propionyl-CoA is required for its repressive effect, it is not accomplished by the known route through the 2-methyl citrate cycle, as blocking this pathway failed to prevent repression of invasion gene expression. Although our results indicate that propionate must be metabolized to propionyl-CoA to have the observed repressive effect on invasion genes, one other model that cannot be excluded is that acetyl-CoA, rather than propionyl-CoA, is responsible for this negative effect. Many of the genes necessary for propionate metabolism are also involved in acetate metabolism. Phosphotransacetylase, encoded by *pta*, reversibly interconverts acetyl phosphate and acetyl-CoA. Acetyl-CoA synthetase, encoded by *acs*, can metabolize acetate directly to acetyl-CoA (102). Thus, it is possible that the effects we observe in our deletion mutants of *acs* and *pta* could be the result of changes in the metabolism of acetyl-CoA and acetyl phosphate. However, if so, it would be expected that the addition of exogenous acetate would produce the same repressive effect as is seen with propionate. Evidence presented here and previous work in our laboratory, however, has shown this not to be the case, with acetate failing to repress SPI1 gene expression or invasion (69). Also, the only known pathway for the metabolism of propionate to acetyl-CoA is through the 2-methyl citric acid cycle. The results in this work show that deletion of *prp*C, the gene necessary for entry into the 2-methyl citric acid cycle, does not affect the repression of propionate on invasion genes. Thus, although we cannot completely rule out this model, our results suggest that propionyl-CoA, and not acetyl-CoA, is the important metabolic intermediate.

It has previously been shown that butyrate, another intestinal short chain fatty acid, similarly represses SPI1 genes, although the specificity of this effect for the invasion regulon was not described (31, 32, 43, 69). Both of these short chain fatty acids exist in high concentrations in the large intestine of mammals, and thus likely play important roles in the modulation of *Salmonella* virulence in the animal host. Based upon the results of this study, however, it is apparent that these two closely related compounds (differing by only a single

carbon atom) exert their effects by different routes, at least in terms of the metabolic products required. Although very little is known about butyrate metabolism in *Salmonella*, it is clear that the metabolic pathways required for the effects of propionate are not solely required for those of butyrate. The elimination of propionate metabolism through the mutation of *prpE, acs, pta, pflB,* and *tdcE* did not significantly restore invasion and SPI1 expression in the presence of butyric acid (Fig. 3.4). Thus the metabolism of butyrate might also be required for its effects, but by routes that are as yet uncharacterized. It also remains to be tested whether butyrate manifests its effects through the same genetic pathways used by propionate.

This work demonstrates that the repression of invasion genes by propionate functions specifically through HilD. This transcriptional regulator occupies a position at the apex of the regulatory cascade within SPI1 (Fig. 3.10), and thus changes in its level or function exert large effects on its target effector genes. Although much is known about the control of *hilD* itself, and a number of regulators of this gene have been identified, none of those tested in this study was shown to be important for the effects of propionate. Additionally, regulators under the control of HilD were also not required for propionate to repression invasion genes. The transcriptional regulator *hilC*, which has only weak effects on invasion (95), was not repressed by propionate, and *hilA* was dispensable for its activity. These findings thus strongly suggest that HilD is the direct target of propionate action. The effects on HilD also appear to be manifested at the post-translational level: although *hilD* was the only invasion gene required for the effects of propionate, neither transcriptional nor translational control was apparent.

To verify the mechanism by which propionate affects HilD at the post-translational level, we will create a strain constitutively expressing the single-copy chromosomal *hilD* by using the suicide vector gene exchange stratagem (48) to integrate a consensus promoter followed by a synthetic ribosome binding site (Shine-Dalgarno sequence) based upon that of *lacZ* in front of the *hilD* ORF. Thus, expression of *hilD* in this bacterial strain will be not be controlled by transcriptional and post-transcriptional (affecting the mRNA stability or the translation of *hilD*)

means. Once the bacterial strain is constructed, we will transform the *sopB::luxABCD* plasmid or transduce the *sipC::lacZY* into it. We expect that if propionate post-translationally controls HilD, adding propionate will affect the stability or the activity of HilD and consequently decrease *sopB::luxABCD* or *sipC::lacZY* expression. If the expression of *sopB::ABCD* or *sipC::lacZ* is reduced in the above conditions, we will transform the *hilD*::*luxABCD* plasmid into the strain carrying the consensus promoter and *lacZ* SD sequence fused to *hilD*. We expect that the expression of *hilD*::*luxABCD* will also be reduced by propionate as are *sopB::luxABCD* or *sipC::lacZ* fusions in this strain background. As a control, we will use the bacterial strain possessing the native promoter sequence and the ribosomal binding site of *hilD*, placing at upstream of the *hilD* ORF using the same suicide vector gene exchange stratagem. We expect that in this control strain, adding propionate will reduce the expression of the *hilD*::*luxABCD* , *sopB::luxABCD*, or *sipC::lacZY* due to the negative effect of propionate on the chromosomal *hilD*. To further determine whether the control of propionate on HilD is accomplished by affecting HilD protein stability or activity, we will create a strain expressing HilD protein with a 6-His tag at the C-terminus by using the suicide vector gene exchange to integrate the DNA sequence encoding 6 histidines fused to *hilD*. We will grow this bacterial strain with or without propionate and assess the level of HilD under these two growth conditions by performing western blotting with anti-His antibody. We expect that if propionate controls HilD by affecting the stability of HilD, a reduction in the amount of HilD will be observed with propionate present. In contrast, if the level of HilD is not affected by propionate, it will suggest that propionate controls HilD by affecting protein activity.

The mechanism by which propionyl-CoA might exert its negative effects on invasion genes is as yet unknown, but the results presented here suggest a possible model. Propionyl-CoA may function by the inactivation of a protein or proteins required for invasion through the addition of a propionyl moiety donated by propionyl-CoA. Propionyl-CoA has previously been reported to inactivate PrpE, the propionyl-CoA synthetase, via N-Lysine propionylation (45). For

this to occur, protein acetyltransferase, encoded by Pat, transfers a propionyl group from propionyl-CoA to a lysine residue on PrpE, thus inactivating the protein (45, 101). Other examples exist in bacteria of N-lysine acylation through acyl-CoA intermediates as a means to alter protein structure and function. In *Salmonella*, Acs can be reversibly acetylated using acetyl-CoA as the acyl group donor, also through Pat (101). CheY of *E. coli* is also acetylated, and can additionally be auto-acetylated *in vitro*, in the absence of any catalyzing enzyme (9-11, 73). If such a mechanism operates to control invasion genes, HilD remains the most likely target of regulation. Our genetic results demonstrate that the effects of propionate center on HilD, and that they are post-translational, as would be expected in the case of protein inactivation secondary to chemical modification. In addition, HilD in *Salmonella* has been shown to undergo acetylation of two lysine residues (112), and work in our laboratory suggests that additional residues are similarly acetylated *in vivo* (not shown). There remains the possibility however, that propionyl-CoA might exert its repressive effect by modification of other, uncharacterized proteins that control invasion through effects on *hilD*. To examine this possibility, we will use the strain expressing the chromosomal copy of HilD with 6 histidines fused to its C-terminus. We will grow this strain with or without propionate present. HilD-His protein derived from bacteria grown under these conditions will be individually purified by using histidine affinity resin, and purified HilD will be used for mass spectrometry analysis to identify post-translational modification of HilD. We expect that if propionyl-CoA causes its repressive effect by direct modification of HilD, propionylation or acetylation of certain lysine residues or a higher percentage of acyl-modification of specific lysines may be observed in the HilD sample prepared from the strain grown with propionate present.

The invasion of the intestinal epithelium in an animal host is clearly essential for productive infection by *Salmonella*. The fact then that propionate, a common constituent of that organ, exhibits such pronounced repression of invasion functions may at first seem incongruous. It is likely, however, that *Salmonella* uses this fatty acid, as well as others, as an

environmental cue to differentiate regions of the intestinal tract. Although propionate can be present in the small intestine, its concentration is much higher in the colon and cecum, regions in which *Salmonella* invasion is repressed in the presence of the resident microbiota (49, 103). Thus, as *Salmonella* passes into the large intestine, propionate may be one important signal to define for this pathogen that the possibility of productive infection has passed, and thus allow *Salmonella* to shift its energies to those required for survival within the intestinal lumen and passage to new hosts.

ACKNOWLEDGMENTS

This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, award number 2005-35201-16270.

REFERENCES

1. **Ahmer, B. M., J. van Reeuwijk, P. R. Watson, T. S. Wallis, and F. Heffron.** 1999. Salmonella SirA is a global regulator of genes mediating enteropathogenesis. Molecular Microbiology **31:**971-82.
2. **Altier, C., M. Suyemoto, and S. D. Lawhon.** 2000. Regulation of Salmonella enterica serovar typhimurium invasion genes by csrA. Infection and Immunity **68:**6790-7.
3. **Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer.** 2000. Characterization of two novel regulatory genes affecting Salmonella invasion gene expression. Molecular Microbiology **35:**635-46.
4. **Ardawi, M. S., and E. A. Newsholme.** 1985. Fuel utilization in colonocytes of the rat. Biochem J **231:**713-9.
5. **Argenzio, R. A., and M. Southworth.** 1975. Sites of organic acid production and absorption in gastrointestinal tract of the pig. Am J Physiol **228:**454-60.

6. **Argenzio, R. A., M. Southworth, and C. E. Stevens.** 1974. Sites of organic acid production and absorption in the equine gastrointestinal tract. Am J Physiol **226:**1043-50.

7. **Bajaj, V., C. Hwang, and C. A. Lee.** 1995. hilA is a novel ompR/toxR family member that activates the expression of Salmonella typhimurium invasion genes. Molecular Microbiology **18:**715-27.

8. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Molecular Microbiology **22:**703-14.

9. **Barak, R., and M. Eisenbach.** 2001. Acetylation of the response regulator, CheY, is involved in bacterial chemotaxis. Mol Microbiol **40:**731-43.

10. **Barak, R., M. Welch, A. Yanovsky, K. Oosawa, and M. Eisenbach.** 1992. Acetyladenylate or its derivative acetylates the chemotaxis protein CheY in vitro and increases its activity at the flagellar switch. Biochemistry **31:**10099-107.

11. **Barak, R., J. Yan, A. Shainskaya, and M. Eisenbach.** 2006. The chemotaxis response regulator CheY can catalyze its own acetylation. J Mol Biol **359:**251-65.

12. **Barthel, M., S. Hapfelmeier, L. Quintanilla-Martinez, M. Kremer, M. Rohde, M. Hogardt, K. Pfeffer, H. Russmann, and W. D. Hardt.** 2003. Pretreatment of mice with streptomycin provides a Salmonella enterica serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun **71:**2839-58.

13. **Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones.** 2003. HilE interacts with HilD and negatively regulates hilA transcription and expression of the Salmonella enterica serovar Typhimurium invasive phenotype. Infection and Immunity **71:**1295-305.

14. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells. Journal of Bacteriology **175:**4475-84.

15. **Blottiere, H. M., B. Buecher, J. P. Galmiche, and C. Cherbut.** 2003. Molecular analysis of the effect of short-chain fatty acids on intestinal cell proliferation. Proc Nutr Soc **62:**101-6.

16. **Boddicker, J. D., and B. D. Jones.** 2004. Lon protease activity causes down-regulation of Salmonella pathogenicity island 1 invasion gene expression after infection of epithelial cells. Infection and Immunity **72:**2002-13.

17. **Boddicker, J. D., B. M. Knosp, and B. D. Jones.** 2003. Transcription of the Salmonella invasion gene activator, hilA, requires HilD activation in the absence of negative regulators. Journal of Bacteriology **185:**525-33.

18. **Bohnhoff, M., C. P. Miller, and W. R. Martin.** 1964. Resistance of the Mouse's Intestinal Tract to Experimental Salmonella Infection. Ii. Factors Responsible for Its Loss Following Streptomycin Treatment. J Exp Med **120:**817-28.

19. **Boyen, F., F. Haesebrouck, A. Vanparys, J. Volf, M. Mahu, F. Van Immerseel, I. Rychlik, J. Dewulf, R. Ducatelle, and F. Pasmans.** 2008. Coated fatty acids alter virulence properties of Salmonella Typhimurium and decrease intestinal colonization of pigs. Vet Microbiol **132:**319-27.

20. **Brown, A. J., S. M. Goldsworthy, A. A. Barnes, M. M. Eilert, L. Tcheang, D. Daniels, A. I. Muir, M. J. Wigglesworth, I. Kinghorn, N. J. Fraser, N. B. Pike, J. C. Strum, K. M. Steplewski, P. R. Murdock, J. C. Holder, F. H. Marshall, P. G. Szekeres, S. Wilson, D. M. Ignar, S. M. Foord, A. Wise, and S. J. Dowell.** 2003. The Orphan G protein-coupled receptors GPR41 and GPR43 are activated by propionate and other short chain carboxylic acids. J Biol Chem **278:**11312-9.

21. **Carter, P. B., and F. M. Collins.** 1974. The route of enteric infection in normal mice. J Exp Med **139:**1189-203.

22. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol **134:**1141-56.

23. **Chavez, R. G., A. F. Alvarez, T. Romeo, and D. Georgellis.** 2010. The physiological stimulus for the BarA sensor kinase. J Bacteriol **192:**2009-12.

24. **Chubiz, J. E., Y. A. Golubeva, D. Lin, L. D. Miller, and J. M. Slauch.** 2010. FliZ regulates expression of the Salmonella pathogenicity island 1 invasion locus by controlling HilD protein activity in Salmonella enterica serovar typhimurium. J Bacteriol **192:**6261-70.

25. **Cummings, J. H.** 1981. Short chain fatty acids in the human colon. Gut **22:**763-79.

26. **Cummings, J. H., E. W. Pomare, W. J. Branch, C. P. Naylor, and G. T. Macfarlane.** 1987. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut **28:**1221-7.

27. **Darwin, A., P. Tormay, L. Page, L. Griffiths, and J. Cole.** 1993. Identification of the formate dehydrogenases and genetic determinants of formate-dependent nitrite reduction by Escherichia coli K12. J Gen Microbiol **139:**1829-40.

28. **Darwin, K. H., and V. L. Miller.** 1999. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in Salmonella typhimurium. Journal of Bacteriology **181:**4949-54.

29. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-5.

30. **De Keersmaecker, S. C., K. Marchal, T. L. Verhoeven, K. Engelen, J. Vanderleyden, and C. S. Detweiler.** 2005. Microarray analysis and motif detection reveal new targets of the Salmonella enterica serovar Typhimurium HilA regulatory protein, including hilA itself. J Bacteriol **187:**4381-91.

31. **Durant, J. A., D. E. Corrier, and S. C. Ricke.** 2000. Short-chain volatile fatty acids modulate the expression of the hilA and invF genes of Salmonella typhimurium. J Food Prot **63:**573-8.

32. **Durant, J. A., V. K. Lowry, D. J. Nisbet, L. H. Stanker, D. E. Corrier, and S. C. Ricke.** 1999. Short-chain fatty acids affect cell-association and invasion of HEp-2 cells by Salmonella typhimurium. J Environ Sci Health B **34:**1083-99.

33. **Ehrbar, K., S. Mirold, A. Friebel, S. Stender, and W. D. Hardt.** 2002. Characterization of effector proteins translocated via the SPI1 type III secretion system of Salmonella typhimurium. Int J Med Microbiol **291:**479-85.

34. **Eichelberg, K., and J. E. Galan.** 1999. Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA. Infection and Immunity **67:**4099-105.

35. **Ellermeier, C. D., J. R. Ellermeier, and J. M. Slauch.** 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. Mol Microbiol **57:**691-705.

36. **Ellermeier, C. D., A. Janakiraman, and J. M. Slauch.** 2002. Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290:**153-61.

37. **Ellermeier, J. R., and J. M. Slauch.** 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. Curr Opin Microbiol **10:**24-9.

38. **Ernst, R. K., D. M. Dombroski, and J. M. Merrick.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by Salmonella typhimurium. Infect Immun **58:**2014-6.

39. **Fortune, D. R., M. Suyemoto, and C. Altier.** 2006. Identification of CsrC and characterization of its role in epithelial cell invasion in Salmonella enterica serovar Typhimurium. Infect Immun **74:**331-9.

40. **Galan, J. E.** 2001. Salmonella interactions with host cells: type III secretion at work. Annu Rev Cell Dev Biol **17:**53-86.

41. **Galan, J. E., and R. Curtiss, 3rd.** 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc Natl Acad Sci U S A **86:**6383-7.

42. **Galan, J. E., and R. Curtiss, 3rd.** 1990. Expression of Salmonella typhimurium genes required for invasion is regulated by changes in DNA supercoiling. Infection and Immunity **58:**1879-85.

43. **Gantois, I., R. Ducatelle, F. Pasmans, F. Haesebrouck, I. Hautefort, A. Thompson, J. C. Hinton, and F. Van Immerseel.** 2006. Butyrate specifically down-regulates salmonella pathogenicity island 1 gene expression. Appl Environ Microbiol **72:**946-9.

44. **Garner, C. D., D. A. Antonopoulos, B. Wagner, G. E. Duhamel, I. Keresztes, D. A. Ross, V. B. Young, and C. Altier.** 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a Salmonella enterica serovar typhimurium murine model of infection. Infect Immun **77:**2691-702.

45. **Garrity, J., J. G. Gardner, W. Hawse, C. Wolberger, and J. C. Escalante-Semerena.** 2007. N-lysine propionylation controls the activity of propionyl-CoA synthetase. J Biol Chem **282:**30239-45.

46. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. Embo J **12:**3779-87.

47. **Hague, A., A. J. Butt, and C. Paraskeva.** 1996. The role of butyrate in human colonic epithelial cells: an energy source or inducer of differentiation and apoptosis? Proc Nutr Soc **55:**937-43.

48. **Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner.** 1989. New method for generating deletions and gene replacements in Escherichia coli. J Bacteriol **171:**4617-22.

49. **Hapfelmeier, S., and W. D. Hardt.** 2005. A mouse model for S. typhimurium-induced enterocolitis. Trends Microbiol **13:**497-503.

50. **Hesslinger, C., S. A. Fairhurst, and G. Sawers.** 1998. Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in Escherichia coli that degrades L-threonine to propionate. Mol Microbiol **27:**477-92.

51. **Hinnebusch, B. F., S. Meng, J. T. Wu, S. Y. Archer, and R. A. Hodin.** 2002. The effects of short-chain fatty acids on human colon cancer cell phenotype are associated with histone hyperacetylation. J Nutr **132:**1012-7.

52. **Horswill, A. R., A. R. Dudding, and J. C. Escalante-Semerena.** 2001. Studies of propionate toxicity in Salmonella enterica identify 2-methylcitrate as a potent inhibitor of cell growth. J Biol Chem **276:**19094-101.

53. **Horswill, A. R., and J. C. Escalante-Semerena.** 2001. In vitro conversion of propionate to pyruvate by Salmonella enterica enzymes: 2-methylcitrate dehydratase (PrpD) and aconitase Enzymes catalyze the conversion of 2-methylcitrate to 2-methylisocitrate. Biochemistry **40:**4703-13.

54. **Horswill, A. R., and J. C. Escalante-Semerena.** 1999. The prpE gene of Salmonella typhimurium LT2 encodes propionyl-CoA synthetase. Microbiology **145 (Pt 6):**1381-8.

55. **Horswill, A. R., and J. C. Escalante-Semerena.** 1999. Salmonella typhimurium LT2 catabolizes propionate via the 2-methylcitric acid cycle. J Bacteriol **181:**5615-23.

56. **Huang, Y., M. Suyemoto, C. D. Garner, K. M. Cicconi, and C. Altier.** 2008. Formate acts as a diffusible signal to induce Salmonella invasion. J Bacteriol **190:**4233-41.

57. **Hueck, C. J., M. J. Hantman, V. Bajaj, C. Johnston, C. A. Lee, and S. I. Miller.** 1995. Salmonella typhimurium secreted invasion determinants are homologous to Shigella Ipa proteins. Molecular Microbiology **18:**479-90.

58. **Johnston, C., D. A. Pegues, C. J. Hueck, A. Lee, and S. I. Miller.** 1996. Transcriptional activation of Salmonella typhimurium invasion genes by a member of the phosphorylated response-regulator superfamily. Molecular Microbiology **22:**715-27.

59. **Jones, B. D., and S. Falkow.** 1994. Identification and characterization of a Salmonella typhimurium oxygen-regulated gene required for bacterial internalization. Infection and Immunity **62:**3745-52.

60. **Jones, B. D., N. Ghori, and S. Falkow.** 1994. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med **180:**15-23.

61. **Joseph, J., G. Mudduluru, S. Antony, S. Vashistha, P. Ajitkumar, and K. Somasundaram.** 2004. Expression profiling of sodium butyrate (NaB)-treated cells: identification of regulation of genes related to cytokine signaling and cancer metastasis by NaB. Oncogene **23:**6304-15.

62. **Kaniga, K., J. C. Bossio, and J. E. Galan.** 1994. The Salmonella typhimurium invasion genes invF and invG encode homologues of the AraC and PulD family of proteins. Molecular Microbiology **13:**555-68.

63. **Kessler, D., and J. Knappe.** 1996. Anaerobic dissimilation of pyruvate. *In* F. C. Neidhardt, R. Curtiss, 3rd, L. Ingraham, E. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), Escherichia coli and Salmonella: cellular and molecular biology, 2 ed. ASM Press, Washington, D.C.

64. **Kinoshita, M., Y. Suzuki, and Y. Saito.** 2002. Butyrate reduces colonic paracellular permeability by enhancing PPARgamma activation. Biochem Biophys Res Commun **293:**827-31.

65. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science **280:**602-5.

66. **Laerke, H. N., and B. B. Jensen.** 1999. D-tagatose has low small intestinal digestibility but high large intestinal fermentability in pigs. J Nutr **129:**1002-9.

67. **Laerke, H. N., B. B. Jensen, and S. Hojsgaard.** 2000. In vitro fermentation pattern of D-tagatose is affected by adaptation of the microbiota from the gastrointestinal tract of pigs. J Nutr **130:**1772-9.

68. **Lawhon, S. D., J. G. Frye, M. Suyemoto, S. Porwollik, M. McClelland, and C. Altier.** 2003. Global regulation by CsrA in Salmonella typhimurium. Molecular Microbiology **48:**1633-45.

69. **Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier.** 2002. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Molecular Microbiology **46:**1451-64.

70. **Lee, C. A., and S. Falkow.** 1990. The ability of Salmonella to enter mammalian cells is affected by bacterial growth state. Proc Natl Acad Sci U S A **87:**4304-8.

71. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a Salmonella typhimurium invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci U S A **89:**1847-51.

72. **Levison, M. E.** 1973. Effect of colon flora and short-chain fatty acids on growth in vitro of Pseudomonas aeruginsoa and Enterobacteriaceae. Infect Immun **8:**30-5.

73. **Liarzi, O., R. Barak, V. Bronner, M. Dines, Y. Sagi, A. Shainskaya, and M. Eisenbach.** 2010. Acetylation represses the binding of CheY to its target proteins. Mol Microbiol **76:**932-43.

74. **Lostroh, C. P., V. Bajaj, and C. A. Lee.** 2000. The cis requirements for transcriptional activation by HilA, a virulence determinant encoded on SPI-1. Mol Microbiol **37:**300-15.

75. **Luhrs, H., T. Gerke, J. G. Muller, R. Melcher, J. Schauber, F. Boxberge, W. Scheppach, and T. Menzel.** 2002. Butyrate inhibits NF-kappaB activation in lamina propria macrophages of patients with ulcerative colitis. Scand J Gastroenterol **37:**458-66.

76. **Lundberg, U., U. Vinatzer, D. Berdnik, A. von Gabain, and M. Baccarini.** 1999. Growth phase-regulated induction of Salmonella-induced macrophage apoptosis correlates with transient expression of SPI-1 genes. J Bacteriol **181:**3433-7.

77. **Macfarlane, G. T., G. R. Gibson, and J. H. Cummings.** 1992. Comparison of fermentation reactions in different regions of the human colon. J Appl Bacteriol **72:**57-64.

78. **Martinez, L. C., H. Yakhnin, M. I. Camacho, D. Georgellis, P. Babitzke, J. L. Puente, and V. H. Bustamante.** 2011. Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the Salmonella SPI-1 and SPI-2 virulence regulons through HilD. Mol Microbiol **80:**1637-56.

79. **McHan, F., and E. B. Shotts.** 1993. Effect of short-chain fatty acids on the growth of Salmonella typhimurium in an in vitro system. Avian Dis **37:**396-8.

80. **Meynell, G. G.** 1963. Antibacterial mechanisms of the mouse gut. II. The role of Eh and volatile fatty acids in the normal gut. Br J Exp Pathol **44:**209-19.

81. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

82. **Mills, D. M., V. Bajaj, and C. A. Lee.** 1995. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol Microbiol **15:**749-59.

83. **Nilsson, N. E., K. Kotarsky, C. Owman, and B. Olde.** 2003. Identification of a free fatty acid receptor, FFA2R, expressed on leukocytes and activated by short-chain fatty acids. Biochem Biophys Res Commun **303:**1047-52.

84. **Olekhnovich, I. N., and R. J. Kadner.** 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of Salmonella enterica serovar Typhimurium. Journal of Bacteriology **184:**4148-60.

85. **Ono, S., S. Karaki, and A. Kuwahara.** 2004. Short-chain fatty acids decrease the frequency of spontaneous contractions of longitudinal muscle via enteric nerves in rat distal colon. Jpn J Physiol **54:**483-93.

86. **Pajak, B., A. Orzechowski, and B. Gajkowska.** 2007. Molecular basis of sodium butyrate-dependent proapoptotic activity in cancer cells. Adv Med Sci **52:**83-8.

87. **Palacios, S., and J. C. Escalante-Semerena.** 2004. 2-Methylcitrate-dependent activation of the propionate catabolic operon (prpBCDE) of Salmonella enterica by the PrpR protein. Microbiology **150:**3877-87.

88. **Palacios, S., and J. C. Escalante-Semerena.** 2000. prpR, ntrA, and ihf functions are required for expression of the prpBCDE operon, encoding enzymes that catabolize propionate in Salmonella enterica serovar typhimurium LT2. J Bacteriol **182:**905-10.

89. **Palacios, S., V. J. Starai, and J. C. Escalante-Semerena.** 2003. Propionyl coenzyme A is a common intermediate in the 1,2-propanediol and propionate catabolic pathways needed for expression of the prpBCDE operon during growth of Salmonella enterica on 1,2-propanediol. J Bacteriol **185:**2802-10.

90. **Pfeifer, C. G., S. L. Marcus, O. Steele-Mortimer, L. A. Knodler, and B. B. Finlay.** 1999. Salmonella typhimurium virulence genes are induced upon bacterial invasion into phagocytic and nonphagocytic cells. Infect Immun **67:**5690-8.

91. **Rakeman, J. L., H. R. Bonifield, and S. I. Miller.** 1999. A HilA-independent pathway to Salmonella typhimurium invasion gene transcription. Journal of Bacteriology **181:**3096-104.

92. **Repaske, D. R., and J. Adler.** 1981. Change in intracellular pH of Escherichia coli mediates the chemotactic response to certain attractants and repellents. J Bacteriol **145:**1196-208.

93. **Roediger, W. E.** 1982. Utilization of nutrients by isolated epithelial cells of the rat colon. Gastroenterology **83:**424-9.

94. **Sanderson, K. E., A. Hessel, and K. E. Rudd.** 1995. Genetic map of Salmonella typhimurium, edition VIII. Microbiol Rev **59:**241-303.

95. **Schechter, L. M., S. M. Damrauer, and C. A. Lee.** 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the hilA promoter. Molecular Microbiology **32:**629-42.

96. **Schiemann, D. A., and S. R. Shope.** 1991. Anaerobic growth of Salmonella typhimurium results in increased uptake by Henle 407 epithelial and mouse peritoneal cells in vitro and repression of a major outer membrane protein. Infect Immun **59:**437-40.

97. **Segain, J. P., D. Raingeard de la Bletiere, A. Bourreille, V. Leray, N. Gervois, C. Rosales, L. Ferrier, C. Bonnet, H. M. Blottiere, and J. P. Galmiche.** 2000. Butyrate inhibits inflammatory responses through NFkappaB inhibition: implications for Crohn's disease. Gut **47:**397-403.

98. **Sheu, C. W., D. Salomon, J. L. Simmons, T. Sreevalsan, and E. Freese.** 1975. Inhibitory effects of lipophilic acids and related compounds on bacteria and mammalian cells. Antimicrob Agents Chemother **7:**349-63.

99. **Shin, R., M. Suzuki, and Y. Morishita.** 2002. Influence of intestinal anaerobes and organic acids on the growth of enterohaemorrhagic Escherichia coli O157:H7. J Med Microbiol **51:**201-6.

100. **Slonczewski, J. L., B. P. Rosen, J. R. Alger, and R. M. Macnab.** 1981. pH homeostasis in Escherichia coli: measurement by 31P nuclear magnetic resonance of methylphosphonate and phosphate. Proc Natl Acad Sci U S A **78:**6271-5.

101. **Starai, V. J., I. Celic, R. N. Cole, J. D. Boeke, and J. C. Escalante-Semerena.** 2002. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. Science **298:**2390-2.

102. **Starai, V. J., and J. C. Escalante-Semerena.** 2004. Acetyl-coenzyme A synthetase (AMP forming). Cell Mol Life Sci **61:**2020-30.

103. **Stecher, B., A. J. Macpherson, S. Hapfelmeier, M. Kremer, T. Stallmach, and W. D. Hardt.** 2005. Comparison of Salmonella enterica serovar Typhimurium colitis in germfree mice and mice pretreated with streptomycin. Infect Immun **73:**3228-41.

104. **Tedelind, S., F. Westberg, M. Kjerrulf, and A. Vidal.** 2007. Anti-inflammatory properties of the short-chain fatty acids acetate and propionate: a study with relevance to inflammatory bowel disease. World J Gastroenterol **13:**2826-32.

105. **Teplitski, M., R. I. Goodier, and B. M. Ahmer.** 2003. Pathways leading from BarA/SirA to motility and virulence gene expression in Salmonella. Journal of Bacteriology **185:**7257-65.

106. **Tsang, N., R. Macnab, and D. E. Koshland, Jr.** 1973. Common mechanism for repellents and attractants in bacterial chemotaxis. Science **181:**60-3.

107. **Van Immerseel, F., J. De Buck, I. De Smet, F. Pasmans, F. Haesebrouck, and R. Ducatelle.** 2004. Interactions of butyric acid- and acetic acid-treated Salmonella with chicken primary cecal epithelial cells in vitro. Avian Dis **48:**384-91.

108. **Van Immerseel, F., V. Fievez, J. de Buck, F. Pasmans, A. Martel, F. Haesebrouck, and R. Ducatelle.** 2004. Microencapsulated short-chain fatty acids in feed modify colonization and invasion early after infection with Salmonella enteritidis in young chickens. Poult Sci **83:**69-74.

109. **Van Immerseel, F., J. B. Russell, M. D. Flythe, I. Gantois, L. Timbermont, F. Pasmans, F. Haesebrouck, and R. Ducatelle.** 2006. The use of organic acids to

combat Salmonella in poultry: a mechanistic explanation of the efficacy. Avian Pathol **35:**182-8.

110. **Voravuthikunchai, S. P., and A. Lee.** 1987. Cecectomy causes long-term reduction of colonization resistance in the mouse gastrointestinal tract. Infect Immun **55:**995-9.

111. **Wallis, T. S., and E. E. Galyov.** 2000. Molecular basis of Salmonella-induced enteritis. Mol Microbiol **36:**997-1005.

112. **Wang, Q., Y. Zhang, C. Yang, H. Xiong, Y. Lin, J. Yao, H. Li, L. Xie, W. Zhao, Y. Yao, Z. B. Ning, R. Zeng, Y. Xiong, K. L. Guan, S. Zhao, and G. P. Zhao.** 2010. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science **327:**1004-7.

113. **Wolfe, A. J.** 2005. The acetate switch. Microbiol Mol Biol Rev **69:**12-50.

114. **Zhang, Y., L. Zhou, Y. L. Bao, Y. Wu, C. L. Yu, Y. X. Huang, Y. Sun, L. H. Zheng, and Y. X. Li.** 2010. Butyrate induces cell apoptosis through activation of JNK MAP kinase pathway in human colon cancer RKO cells. Chem Biol Interact **185:**174-81.

CHAPTER 4

Pat and CobB regulate *Salmonella* invasion gene expression via HilD and the Lon protease

ABSTRACT

Salmonella is an important food-borne pathogen that causes disease ranging from mild enteritis to severe septicemia. Expression of invasion genes encoded on *Salmonella* Pathogenicity island 1 (SPI1) is essential for this pathogen to first penetrate the host epithelial cells and further leads to systemic infection. In our previous work, we have shown that short chain fatty acids present in the host intestinal environment are able to regulate invasion gene expression. Additionally, previous studies have shown that Pat and CobB act as post-translational regulators and control the activity of their targets, PrpE and Acs, through N-lysine acyl-modification. As PrpE and Acs are enzymes utilized in fatty acid (propionate and acetate) metabolism, and they are known targets of Pat and CobB, in this work we thus examined whether Pat and CobB affect *Salmonella* invasion. We found that Pat and CobB repressed and induced SPI1 gene expression, respectively, when they were over-expressed. Surprisingly, using genetic approaches, we demonstrated that the effects of Pat and CobB on invasion were not through their known targets, PrpE and Acs. Instead, their control was through HilD and the Lon protease, while other SPI1 regulators, SirA, FliZ, H-NS and HilE, were dispensable. We also found that the amount of HilD was significantly reduced by over-production of Pat. In addition, using mass spectrometry analysis, we found three lysine residues (K131, K263 and K296) on HilD were acetylated. These results, taken together, suggest that Pat and CobB may work through the modification of HilD and its subsequent degradation by Lon protease, and consequently may affect invasion gene expression.

INTRODUCTION

Salmonella is among the most important of the bacterial entero-pathogens, and the process of invasion is essential to its virulence. Most of the genes required for this invasion process are located on a 40 kb gene cluster, the *Salmonella* Pathogenicity Island I (SPI1). The genes on SPI1 encode proteins required for assembling a type III secretion apparatus, the needle complex, which spans the bacterial inner and outer membranes and translocates secreted effector proteins into the host cell cytoplasm (8, 26, 32, 36, 39, 42) . When these proteins are delivered into a targeted cell, they evoke actin cytoskeleton rearrangement and further cause membrane ruffling, leading to engulfment of *Salmonella* (13, 25, 33, 55).

The expression of SPI1 genes is regulated through complex pathways and includes regulators both inside and outside this pathogenicity island. SPI1 contains seven known operons, and their expression is controlled by four regulators within the SPI1 (HilD, HilC, HilA and InvF) and one regulator outside SPI1 (RtsA) (14, 17, 20, 39, 45, 48). HilD was thought to be at the top of the hierarchy within SPI1 and positively regulates RtsA and HilC. HilD, RtsA, and HilC have been shown to form a feed-forward regulatory loop by auto-regulating their own gene expression and inducing expression of *hilD*, *rtsA* and *hilC* independently of each other (18, 21, 29). Each regulator in this regulatory loop can positively regulate *hilA,* which induces the *prg/org*, *inv/spa*, and *sic/sip* operons and another transcriptional regulator, *invF* (14, 17, 29). InvF induces the expression of downstream SPI1 effector proteins encoded by the *sipBCDA* operon and other effectors outside SPI1, such as SopB, SopE and SigD (14, 15, 17). In addition, HilD and HilC have been demonstrated to induce the expression of the *sipBCDA* operon by directly controlling *invF,* where HilD and HilC bind to a second promoter of *invF* not used by HilA (1).

Several genetic regulators outside SPI1 have also been shown to control invasion gene expression thorough HilD. SirA, the response regulator of the BarA/SirA two-component system (TCS), has been shown to positively control invasion gene expression through the control of the

Csr (carbon storage regulator) system. The Csr system is composed of an RNA-binding protein, CsrA, that binds to the *hilD* mRNA and prevents the translation of this message, and two small regulatory RNAs, CsrB and CsrC, that bind to CsrA and titrate it from the *hilD* message. SirA positively regulates SPI1 gene expression by inducing CsrB and CsrC, both of which inhibit the activity of CsrA, and subsequently increases the level of HilD and downstream invasion gene expression (3, 4, 24, 37, 40). FliZ, a class 2 flagellar gene, has been demonstrated to positively regulate SPI1 gene. The mechanism by which FliZ positively regulates invasion genes is thorough post-transcriptional control of HilD (12, 35). Additionally, HilE and Lon have been shown to negatively control invasion genes by affecting the activity or the level of HilD (7, 9, 22, 51, 52). HilE was suggested to interact with HilD and prevent it from inducing the expression of downstream invasion genes (7, 22). Lon is an ATP–dependent protease and has been suggested to be responsible for the degradation of HilD and HilC (51, 52). Besides the above regulators that affect invasion gene expression through HilD, the global regulator H-NS, a histone-like protein, has been shown to negatively control SPI1 gene expression. H-NS has been suggested to bind to multiple sites on the AT-rich SPI1 DNA, bending the DNA and silencing the transcription of SPI1 genes (23, 43, 44).

In prokaryotic and eukaryotic cells, post-translational modification of proteins is an important way to manipulate the function of regulatory proteins and the activity of enzymes. Pat, which functions as a propionylase or an acetylase, has been show to manipulate the activity of PrpE and Acs through N-Lysine propionylation or acetylation. In this process, Pat utilizes propionyl-CoA or acetyl-CoA as its substrates and transfers a propionyl group or an acetyl group, respectively, to a specific lysine residue of target proteins and thus inhibits their activities (28, 50). In contrast to Pat, CobB, a Sir2 family protein, acts as a depropionylase or a deacetylase. CobB reversely removes the acyl group from the propionylated or acetylated lysine of the same target proteins and restores their functions (28, 49).

Interestingly, the known targets of Pat and CobB in *Salmonella* are PrpE and Acs that play roles in propionate and acetate metabolic pathways (28, 47, 49). Since our previous studies showed that these short chain fatty acids affect SPI1 gene expression (27, 38), in this work, we thus examined whether Pat and CobB affect *Salmonella* invasion. We found that over-expression of *pat* or *cobB* is able to affect invasion gene expression. In particular, Pat represses invasion gene expression, but CobB induces it. However, using genetic approaches, we demonstrated that the effects of Pat and CobB on invasion were not through the known targets PrpE and Acs. Instead, we found that two regulators involved in the invasion gene regulatory cascade, *hilD* and *lon*, were required for Pat and CobB to control SPI1 gene expression. These results thus suggest that Pat and CobB may work through the modification of HilD and its subsequent degradation by Lon.

MATERIALS AND METHODS

β-galactosidase assays. Triplicate cultures of tested bacterial strains were grown standing overnight at 37°C in LB broth buffered to pH 6.7 with 100 mM MOPS and with 100 μg/ml ampicillin as needed to maintain plasmids. β-galactosidase activity was measured as described previously (41).

Construction of mutant strains. *Salmonella enterica* serovar Typhimurium strain ATCC 14028s and isogenic mutants were used throughout this study and are shown in Table 4.1. Gene deletions were made using the previously reported one-step inactivation method (16). In brief, PCR reactions were performed to amplify the fragments containing the FRT sequences flanking the antibiotic resistance markers from plasmids pKD3 or pKD4 using primers carrying 40 bases of homologous sequence flanking the coding region of the target gene. The resulting PCR product was purified and transformed into a *Salmonella* strain carrying the plasmid pKD46, which expresses the Red λ recombinase, allowing allelic exchange. The resulting deletion

mutants were cultured at 42°C to remove the temperature-sensitive pKD46 plasmid, and the loss of the target gene was determined by PCR.

Table 4.1. List of strains and plasmids in this study

Strain or Plasmid	Genotype	Source or reference
Strains		
ATCC 14028s	wild type	American Type Culture Collection
CA412	*sipC::lacZY*	(5)
CA2039	*fljB::lacZ*	This study
CA2184	Δ*preE* Δ*acs sipC::lacZY*	This study
CA1983	Δ*hilD sipC::lacZY*	This study
CA921	Δ*sirA sipC::lacZY*	This study
CA2122	Δ*filZ sipC::lacZY*	This study
CA504	Δ*hilA sipC::lacZY*	This study
CA749	Δ*hilE sipC::lacZY*	This study
CA2294	Δ*lon sipC::lacZY*	This study
CA1601	Δ*hilD::cam*	(48)
Plasmids		
pACYC177	Cloning vector	(10)
pPat (pCA 175)	*pat* ORF on pACYC177	This study
pCobB (pCA 176)	*cobB* ORF on pACYC177	This study
pQE60	His tag fusion protein cloning vector	Qiagen (Hilden, Germany)
pHilD^{-His} (pCA 180)	*hilD* ORF on pQE60	This study
pPat-HilD^{-His} (pCA181)	*pat* and *hilD* ORF on pQE60	This study
pHilD (pCA 177)	*hilD* ORF on pQE60	This study
pPat-HilD (pCA 178)	*pat* and *hilD* ORF on pQE60	This study

***fljB::lacZ* strain construction.** To create a single-copy chromosomal *lacZ* transcriptional fusion to *fljB*, we used the methods published by Ellermeier *et al* (19). In brief, we first constructed a marked deletion of *fljB* with the pKD13 plasmid and designed primers

TCCCAGTCCGCACTGGGCACCGCTATCGAGCGTCTGTCTTATTCCGGGGATCCGTCGACC

and

AAAAGCCCCGAATTCACGGGGCTGAATAAAACGAAATAAATGTAGGCTGGAGCTGCTTC

using the previously described a one-step inactivation method. Next, the antibiotic marker was removed by transducing the temperature-sensitive plasmid pCP20, which encodes the FLP protein that specially recognizes the FRT sequences. The resulting transductants were selected on LB agar pates with 100 µg/ml ampicillin and 10 mM EGTA at 30º C, and the colonies were purified twice and grown under the same conditions. Colonies losing the antibiotic marker by the FLP recombinase created an FRT scar at the site of *fljB*, and this scar allowed site-specific integration of *lacZ* by further transforming a *lacZ* fusion plasmid, pCE37 (19). The resulting transformants were selected on a 100 µg/ml kanamycin plate at 37º C and the presence of the *fljB::lacZ* fusion was confirmed by PCR.

Plasmid construction. To create the *pat* (pPat) and *cobB* (pCobB) expression plasmids, a PCR product was produced that included a synthetic ribosome binding site, based on that of *lacZ*, and the ORF of *pat* (*yfiQ* of *Salmonella enterica* serovar Typhimurium LT2) or *cobB*. The PCR product was then cloned into the unique *Xho*I and *Sma*I sites of pACYC177 to place *pat* or *cobB* under the control of the kanamycin resistance gene (*npt*) promoter on this vector.

To make the pHilD and pHilD^{-His} plasmids, a PCR product was produced that included a synthetic ribosome binding site, based upon that of *lacZ*, and the ORF of *hilD* with (for pHilD) or without (for pHilD^{-His}) the stop codon. The PCR product was then cloned into the unique *Nco*I and *Bgl*II sites of pQE60 to place *hilD* under the control of an IPTG-inducible promoter (a T5 promoter followed by the *lacO* operators) on this vector. For pPat-HilD^{-His} and pPat-HilD plasmid construction, a PCR product containing the ORF of *pat* was amplified and further cloned into the *Nco*I and *BamH*I sites of the pHilD^{-His} and pHilD plasmids to place *pat* in front of *hilD*.

HilD-His protein purification. The *hilD* mutant strains carrying the pHilD^{-His} or pPat-HilD^{-His} plasmid and the *lacI* expression plasmid pMS421 (31) were grown in 10 ml LB broth with 100 µg/ml ampicillin, 100 µg/ml spectinomycin and 20 µg/ml streptomycin at 37º C overnight with aeration. Ten ml of each overnight was subcultured into 1L of the same media with an

additional 1 mM IPTG and grown for another 16 hr at 37º C without aeration. The His-tagged HilD protein from each bacterial strain was extracted using the TALON His-Tag Purification Resin (Clontech, Mountain View, CA). The eluted protein solutions were further concentrated using Amicon Ultra-5 centrifugal filter devices (Millipore, Billerica, MA), and concentrated proteins from each sample were separated by 12% SDS-PAGE. The SDS-PAGE was sent to the Cornell University Life Science Core Laboratories Center for further SYPRO RUBY staining and mass spectrometry analysis.

Statistical analysis. Results from β-galactosidase assays were analyzed using a one-way analysis of variance to determine if the mean of at least one strain or condition differed from any of the others. The Tukey-Kramer HSD multiple comparison test was then used to determine which means were statistically different. A p-value <0.05 was considered significant. Statistical analysis was performed using Jmp 9.0 software (SAS, Cary, NC).

RESULTS

Over-expression of Pat or CobB alters *sipC* expression. Our previous studies have demonstrated that propionate, a short chain fatty acid predominant in the large intestine, represses SPI1 gene (invasion gene) expression (27, 38). Our data also suggested that propionyl-CoA, a high energy compound produced in the propionate metabolic pathway, is essential for the negative control of propionate on invasion (data unpublished). In *Salmonella,* it has been shown that Pat, which functions as a propionase, is able to utilize propionyl-CoA as a substrate to transfers the propionyl group on to the lysine residue of a PXXXXGK motif of target proteins, and subsequently inactivate the target protein's activity (28, 49, 50). Based on these results, we hypothesized that Pat might affect invasion gene expression by inactivating invasion gene regulators via N-Lysine propionylation using propionyl CoA as substrate. Therefore, we first asked whether *pat* plays any role in the control of *Salmonella* invasion. To answer this question, we cloned the *pat* open reading frame (ORF) onto a low copy-number plasmid on which *pat* was constitutively expressed under the control of an exogenous promoter. We transformed this plasmid (pPat) into a wild type strain carrying a *lacZY* fusion to a downstream invasion gene *sipC*, and examined *sipC::lacZY* expression by β-galactosidase assay. We found that the expression of *sipC* was decreased 5-fold compared to the wild type strain carrying the control plasmid (Fig.4.1A), indicating that *pat* negatively controlled the expression of invasion genes.

Although Pat is able to inactivate target proteins by propionylation, this effect can be reversed by the depropionylase CobB, which removes the propionyl group from the propionylated protein, allowing the protein to regain its function (28, 49, 50). Since CobB acts counter to Pat, we expected that over-expressing CobB would have the opposite effect on invasion. To test the effect of CobB, we used the same stratagem used for *pat.* As we expected, over-expressing *cobB* using a *cobB* constitutive-expression plasmid pCobB induced *sipC* expression 2-fold compared to the wild type strain carrying the control plasmid,

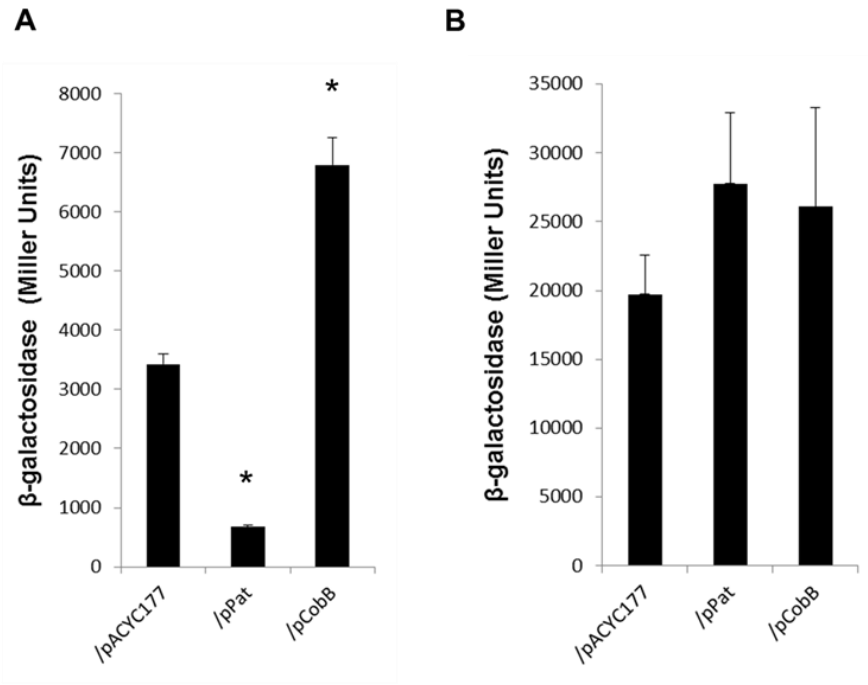

Figure 4.1. Over-expression of *pat* or *cobB* alters expression of invasion genes, but not flagella genes. The expression of A) *sipC::lacZY* or B) *fljB::lacZ* was measured in strains carrying a low copy-number plasmid expressing *pat* (pPat), *cobB* (pCobB), or their vector control (pACYC177). Bacterial strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration. *sipC::lacZY* and *fljB::lacZ* expression were measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the expression plasmid pPat or pCobB as compared to the strain carrying the control plasmid pACYC177 at $p < 0.05$.

demonstrating that *cobB* positively controls invasion (Fig.4.1A). Based upon these results, we conclude that *pat* and *cobB* both can regulate the expression of invasion genes. However, they act oppositely; Pat represses invasion gene expression, but CobB induces it. In addition, our results may also suggest that propionylation and depropionylation of target proteins by Pat and CobB are important to regulate invasion gene expression.

In order to understand whether the effects of *pat* and *cobB* on invasion are specific, we also examined their effects on flagellar genes. We over-expressed *pat* or *cobB* using the pPat or the pCobB plasmid in the wild type strain carrying a *lacZ* fusion to a flagellar gene *fljB*, which encodes the *Salmonella* type II flagellin (11). Again using β-galactosidase assays, we found

that there was no significant difference in *fljB::lacZ* expression in the strain carrying the *pat* or the *cobB* expression plasmid compared to the same strain carrying the control plasmid (Fig. 4.1B). These results, taken together, demonstrated that over-expression of *pat* or *cobB* does not affect flagellar gene expression and that their effects on invasion are specific. Although we have shown the effects *of pat* and *cobB* on invasion gene expression, these effects were seen only when they were over-expressed, since the expression of the downstream SPI1 effector *sopB* was not changed by deletion of *pat* or *cobB* (Data not shown).

***prpE* and *acs*, encoding the propionyl-CoA synthesis, are not required for Pat and CobB to control SPI1 gene expression.** Our work suggests that propionyl-CoA may be a key component in the propionate-induced repression of SPI1 gene expression. In *Salmonella*, PrpE and Acs are two enzymes known to mediate the production of propionyl-CoA from propionate (47). It has been shown that the activity of PrpE is altered by Pat and CobB via propionyl modification of the lysine within a PXXXXGK motif (28). Additionally, because Acs also possesses the PXXXXGK motif (49), we surmised that the activity of Acs could be modulated by Pat and CobB through N-lysine propionyl modification as well. Based up on these results and our assumption, we thus hypothesized that Pat and CobB could modulate PrpE and Acs protein activity, changing the level of propionyl-CoA, and subsequently reducing SPI1 gene expression. To test this hypothesis, we separately introduced the pPat or the pCobB plasmid into a *prpE acs* double mutant carrying a *sipC::lacZY* fusion. We expected that if PrpE and Acs were important for the effects of *pat* or *cobB* on invasion, *sipC* expression would not be changed in the *prpE acs* double mutant strain by over-expression of *pat* or *cobB.* However, the results showed that in the *prpE acs* double mutant strain, *sipC::lacZY* expression was still repressed by *pat* (4.6-fold) and induced by *cobB* (2-fold) compared to the same strain with the

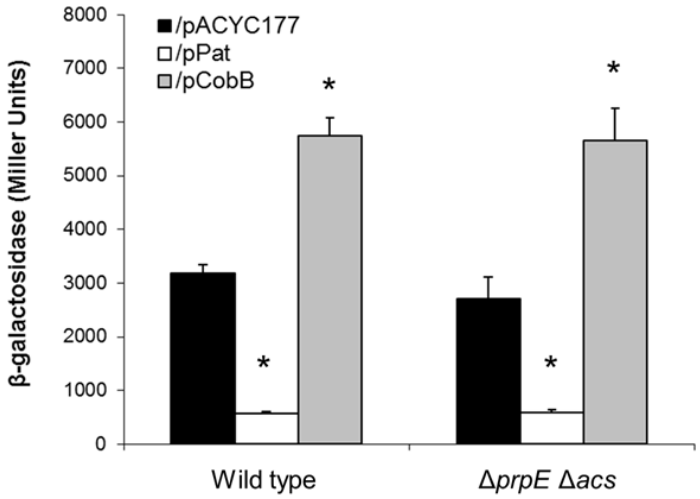

Figure 4.2. The propionyl CoA synthases, PrpE and Acs, are not required for the effects of *pat* or *cobB* on invasion. The plasmid expressing *pat* (pPat; white bars) or *cobB* (pCobB; gray bars) and its control plasmid (pACYC177; black bars) were tested in the wild type, and the *prpE acs* double mutant carrying the *sipC::lacZY* fusion. Strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration and *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the expression plasmid pPat or pCobB as compared to the same strain carrying the control plasmid pACYC177 at $p < 0.05$.

control plasmid, by β-galactosidase assays (Fig. 4.2). In addition, the expression level of *sipC::lacZY* in the *prpE acs* double mutant strain carrying the pPat and pCobB was almost identical to that in the wild type carrying the same plasmid (Fig. 4.2). Therefore, these results indicate that PrpE and Acs, the only known targets of Pat and CobB, are not essential for effects of *pat* and *cobB* on SPI1 gene expression.

***hilD* is required for Pat and CobB control of SPI1 gene expression.** In *Salmonella,* the expression of SPI1 genes is hierarchically controlled by several regulators located inside or outside this pathogenicity island (2, 21). As we have shown that *pat* and *cobB* affect the most downstream invasion gene *sipC*, we further asked whether any known regulator involved in the

invasion regulon was required for the effects of *pat* and *cobB*. Among many identified invasion regulators, we first choose *hilD* since this gene has been placed in the center of the invasion regulatory cascade; it governs other regulators inside SPI1 but also is affected by regulators outside the island (2, 18, 21). To test whether *hilD* was required, we used the *hilD* mutant strain carrying *a sipC::lacZY* fusion and over-expressed *pat* or *cob* by using the pPat or pCobB plasmid. The expression of *sipC::lacZY* was examined by β-galactosidase assays. We found that there was no significant difference in *sipC::lacZY* expression in the *hilD* mutants carrying the pPat, the pCobB or the control plasmid, suggesting that *hilD* plays an important role in the effect of *pat* and *cobB* on invasion genes (Fig. 4.3). Further, we examined several regulators

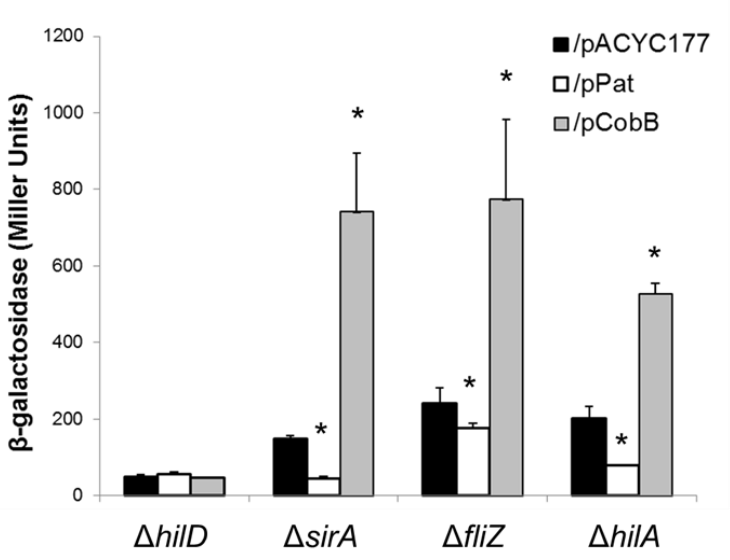

Figure 4.3. *hilD* is essential for *pat* and *cobB* to control invasion gene expression. The *pat* expression plasmid (pPat; white bars) or the *cobB* expression plasmid (pCob; gray bars) and its control plasmid (pACYC177; black bars) were tested in the *hilD* mutant, the *sirA* mutant, the *filZ* mutant, and the *hilA* mutant carrying the *sipC::lacZY* fusion. Strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration and *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the expression plasmid pPat or pCobB as compared to the same strain carrying the control plasmid pACYC177 at $p < 0.05$.

either upstream or downstream of *hilD.* For upstream regulators, we choose *sirA* and *fliZ*, which are located outside SPI1 and have been shown to positively control invasion gene expression through *hilD* (12, 40). Using the same approach by deletion of *sirA* or *fliZ*, we found that in *sirA* mutant or the *fliZ* mutant, the expression of *sipC::lacZY* was still altered by over-expressing *pat* or *cobB* compared to the same strain carrying the control plasmid (Fig. 4.3). These results demonstrated that *sirA* and *fliZ* are not essential for *pat* and *cobB* to affect *Salmonella* invasion (Fig. 4.3). Further, we examined a regulator downstream from *hilD*, *hilA.* We expected that in the *hilA* mutant over-expression of *pat* or *cobB* would no longer show their effects on *sipC*, similar to the *hilD* mutant. Surprisingly, we found that in the *hilA* mutant, *sipC::lacZY* expression was still repressed 2.8-fold by over-expressing *pat* and was induced 2.6-fold by over-expressing *cobB*. Since it has been shown that *hilD* is able to control *sipC* independent of *hilA* by directly controlling another SPI1 regulator *invF* (1), thus, the effects of over-production of *pat* and *cobB* on *sipC* in the *hilA* mutant may be caused by their control on *hilD*, and subsequently on *sipC* through *invF.*

Besides the above regulators, we also examined the importance of *hns* in control by *pat* and *cobB* of invasion. H-NS has been shown to negatively regulate invasion gene expression by binding to several sites of the AT-rich DNA sequence in SPI1 and preventing transcription (23, 43, 44). Previous studies have shown that mutation of *hns* is lethal to *Salmonella*, unless the strain carries an additional mutation in *phoP* (46). To address the role of *hns*, we over-expressed *pat* or *cobB* in a *phoP hns* double mutant strain carrying the *sipC::lacZY* fusion. To understand whether the additional mutation in *phoP* affected the invasion gene *sipC*, we examined *sipC::lacZY* expression in the *phoP* mutant carrying the pPat, pCobB or control plasmid. The *sipC::lacZY* expression level in the *phoP* mutant carrying the pPat, pCobB or control plasmid was similar to the wild type carrying the corresponding plasmid, indicating that mutation of *phoP* did not affect *sipC* (Fig. 4.3). Further, we tested *sipC::lacZY* expression in the *hns phoP* mutant strain. We found that in the *hns phoP* double mutant the expression of

sipC::lacZY was still repressed by over-expression of *pat*, suggesting that *hns* is not important for the control of *pat* on invasion (Fig. 4.4). Surprisingly, we found that over-expression of *cobB*reduced the *sipC::lacZY* expression by 2-fold as compared to the same strain carrying the control plasmid (Fig. 4.4). This result was contradictory to our previous finding that invasion gene expression was induced by over-expression of *cobB.* Apparently, a second effect of over-expression of *cobB* on invasion existed when *hns* was missing. Based on these results, taken together, we conclude that *hilD* is required for *pat* or *cobB* to control invasion gene expression. In addition, over-production of *pat* or *cobB* may affect the message or protein level of *hilD*, and consequently affect downstream invasion gene expression.

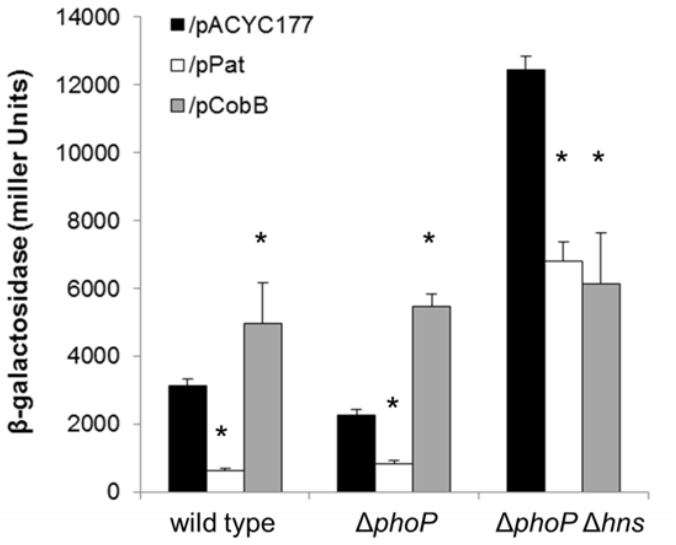

Figure 4.4. Effects of over-expression of *pat* and *cobB* in the *phoP* and the *phoP hns* double mutant. The *pat* expression plasmid (pPat; white bars) or the *cobB* expression plasmid (pCob; gray bars) and its control plasmid (pACYC177; black bars) were tested in the wild type, the *phoP* mutant, and the *phoP hns* double mutant carrying the *sipC::lacZY* fusion. Strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration and *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the expression plasmid pPat or pCobB as compared to the same strain carrying the control plasmid pACYC177 at $p < 0.05$.

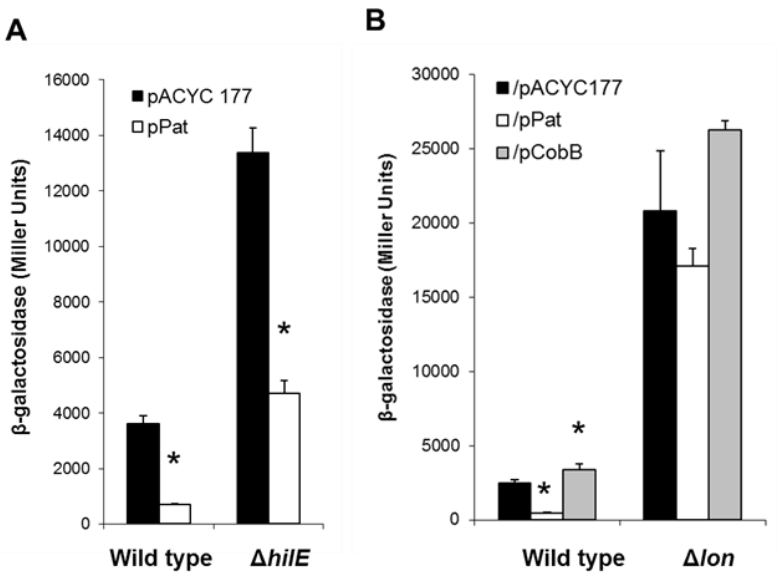

Figure 4.5. The Lon protease is essential for *pat* and *cobB* to affect invasion genes. The *pat* expression plasmid (pPat; white bars) or the *cobB* expression plasmid (pCob; gray bars) and its control plasmid (pACYC177; black bars) were tested in A) the *hilE* mutant and B) the *lon* mutant carrying the *sipC::lacZY* fusion. Strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS overnight without aeration and *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the expression plasmid pPat or pCobB as compared to the same strain carrying the control plasmid pACYC177 at $p < 0.05$.

Lon protease is essential for *pat* and *cobB* to regulate invasion gene expression.

Previous studies have shown that HilD is directly regulated by two negative regulators, HilE and Lon (7, 9, 22, 51, 52). HilE is able to control HilD by directly interacting with HilD and preventing HilD from binding to promoters of target genes (7). Lon functions as a protease that degrades HilD (51). Thus, we further asked whether these two HilD-regulators were also important for *pat* and *cobB* to affect invasion genes through HilD. In order to address this question, we applied the same genetic approach that we used in the previous experiments. Measuring *sipC::lacZY* expression by β-galactosidase assays, we found that there was still a 2.8-fold decrease in *sipC* expression in the *hilE* mutant strain with over-expression of *pat* (Fig. 4.5A), indicating that *hilE* is not important for the control of *pat* on invasion. Since this showed that *hilE* is not essential, we did not further examine the effect of *hilE* on the control of *sipC* by *cobB*. In contrast to the result

using *hilE*, we found that in the *lon* mutant, *sipC* expression was not significantly changed by over-expression of *pat* or *cob*B (Fig. 4.5B). Additionally, we noticed that the strains with mutation in *lon* showed higher *sipC::lacZY* expression in compared to the wild type strain carrying the control plasmid, as expected. These results, thus, suggest that *lon* is also required for *pat* and *cobB* to control invasion gene expression by working through HilD.

Over-expression of Pat reduces the level of HilD. The above results show that Lon protease and HilD are important for Pat and CobB to control invasion genes. It is possible that Pat and CobB post-translationally regulate HilD by propionyl modification of specific lysine residues or by changing the proportion of HilD molecules that are propionylated. Propionylation of HilD might then alter its sensitivity to Lon protease, and subsequently affected downstream invasion gene expression. To address this possibility, we first asked whether the HilD protein amount was affected by over-expression of Pat. Second, we asked if the propionylation profile (the sites and proportion of propionylation) of HilD was also modified by Pat. To address these questions simultaneously, we used a high copy Pat-HilD co-expression plasmid, pPat-HilD^{-His}. In this plasmid, a tag of six histidines was attached to the carboxyl terminus of the HilD protein, and the expression of Pat and HilD was driven by an IPTG inducible promoter. As a control, we used the plasmid pHilD^{-His} that only expressed the His-tagged HilD (HilD^{-His}) protein. These two plasmids were introduced into the *hilD* mutant strain individually, and the production of Pat and the HilD^{-His} protein was induced by adding IPTG. The HilD^{-His} proteins produced from individual strains were affinity purified, and the eluted protein samples were separated by SDS-PAGE and stained with SYPRO Ruby staining. The 36 kDa protein band that showed the equivalent molecular weight to HilD^{-His} from each sample was cut from the gel, and the containing peptides were analyzed by mass spectrometry. The results showed that only a little HilD^{-His} protein prepared from the strain carrying the Pat-HilD^{-His} co-expression plasmid could be observed on SDS-PAGE. In addition, only 0.82% of peptides in this band possessed the sequence of HilD

by mass spectrometry analysis. However, the sample prepared from the strain carrying the HilD^{-His} only expression plasmid showed a noticeable 36 kDa band, which represents HilD^{-His} protein, and 99.2% of peptides showed the sequence of HilD (Fig. 4.6A). These results, taken together, suggest that the amount of HilD was reduced by over-production of Pat.

As the amount of HilD^{-His} protein was low using the Pat-HilD^{-His} co-expression plasmid, this low yield of HilD^{-His} could result from the interference of expression of *hilD* from the plasmid

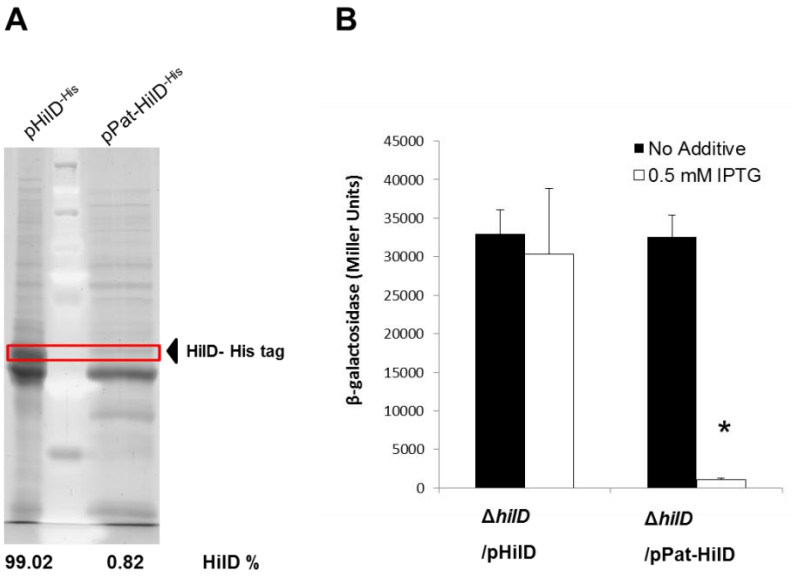

Figure 4.6. Over-expression of Pat reduces the level of HilD protein. A) The *hilD* mutant strains carrying the pHilD^{-His} or pPat-HilD^{-His} plasmid were grown to mid log phase. The plasmid expression was induced by adding 1mM IPTG for 16 hr. The HilD^{-His} proteins from individual stains were purified by using His-tagged protein affinity column. The purified protein was separated by 12% SDS-PAGE and stained with SYPRO Ruby staining. The band of HilD^{-His} protein (36 KDa) produced from each strain was excised form the gel and the peptide sequence from each sample was determined by Mass Spectrometry, and the peptides showing the HilD sequence of each sample in is shown in % and labeled at the bottom. B) The HilD expression plasmid and the Pat-HilD co-expression plasmid were examined in the *hilD* mutant carrying the *sipC::lacZY* fusion. Strains were cultured in LB broth buffered to pH 6.7 with 100 mM MOPS under no additive (black bars) or 0.5 mM IPTG (white bars) induction conditions overnight without aeration and *sipC::lacZY* expression was measured using β-galactosidase assays. The value of individual bars represents means for samples tested in triplicate, and the error bars represent standard deviations. An asterisk (*) indicates a statistically significant difference due to the expression overproduction of Pat by adding IPTG as compared to the same strain under no additive condition at $p < 0.05$.

carrying both genes. To rule out this possibility, we used a similar plasmid, pPat-HilD, that co-expressed Pat and native HilD by placing the *pat* and *hilD* OFRs on to the same expression vector and using the same cloning sites that we used for the construction of the Pat-HilD^{-His} co-expression plasmid. We introduced this plasmid into the *hilD* mutant carrying the *sipC::lacZY* fusion. As a control, we used the plasmid pHilD that only expressed native HilD. We found that this high-copy Pat-HilD co-expression plasmid was able to strongly induce *sipC::lacZY* expression without IPTG being added, as did the control plasmid that only expressed native HilD, due to the leaky expression of HilD from the IPTG promoter. Additionally, we found that by adding IPTG to induce the expression of *pat* and *hilD*, *sipC::lacZY* expression was significantly reduced in this strain (Fig. 4.6B). These results suggested that Pat *and* HilD were co-expressed from the expression plasmid that we used (Fig. 4.6B). Since this Pat-HilD co-expression plasmid was constructed similar to the co-expression plasmid used in the above experiment, we infer that the low yield of HilD^{-His} protein in Figure 4.6A resulted from over-expression of Pat, not from the low transcription of *hilD.*

Acetylation of HilD occurs on three lysine residues. To address whether HilD can be propionylated by Pat, we further analyzed the propionylation of lysines on HilD by mass spectrometry. We expected that HilD would be propionylated or show a higher ratio of propionylation by over-expression of Pat. The result showed that no propionylated lysines on HilD were found from the strain carrying the HilD^{-His} expression plasmid. Since the HilD^{-His} protein obtained from the strain carrying the Pat-HilD^{-His} co-expression plasmid was small, we were not able to assess whether HilD was propionated by over-expression of Pat.

Previous studies have shown that Pat and CobB also function as an acetylase and a deacetylase, respectively, to control the activity of the target proteins PrpE and Acs by post-

Figure 4.7. Acetylated lysine residues in HilD. The acetylated lysine residues of HilD are labeled with the bold K and an asterisk * (**K***). The acetylated lysine residues of HilD have been reported are labeled with the underline K (K)(53).

translational acetyl modification of the lysine with a PXXXXGK motif (28, 49). Thus, it is possible that HilD might be acetylated by Pat, rather than propionylated. To test this hypothesis, we analyzed the acetylation profile of purified HilD^{-His} protein obtained from the *hilD* mutant carrying the pPat-HilD^{-His} or the pHilD^{-His} plasmids. Interestingly, we found that three lysines had been acetylated in the strain carrying the the pHilD^{-His} plasmid using mass spectrometry (Fig 4.7). Additionally, the peptides containing the acetylated lysines were about 2% of the total peptides derived from HilD. However, we were not able to assess whether the acylation profile of HilD was changed by over-production of Pat due to the low yield of HilD^{-His} in the *hilD* mutant carrying the pPat-HilD^{-His} plasmid (Fig. 4.6).

Based on the results, we showed that over-production of Pat led to a reduction of HilD protein. Additionally, we showed that HilD can be acetylated under the growth conditions that we applied. Since the acetylation of HilD does occur in *Salmonella*, this result leaves the possibility that HilD can be chemically modified by Pat. Once modified, HilD may become more sensitive to Lon, degrading HilD and subsequently reducing invasion gene expression.

DISCUSSION AND FUTURE DIRECTIONS

Post-translational modification of proteins is an important means for both prokaryotic cells and eukaryotic cells to manipulate the activity of functional proteins, enzymes, and regulatory proteins for gene expression. In this work, we demonstrated that the Pat and CobB, which have been shown to function as an acylase and a deacylase, respectively, affected the expression of *Salmonella* invasion genes; over-expression of Pat repressed invasion gene expression, but over-expression CobB induced it (Fig. 4.1). In addition, we found that the SPI1 regulator HilD and Lon protease that functions to degrade HilD are required for Pat and CobB to control invasion genes (Fig. 4.3 and Fig. 4.5B). Our results also showed that the amount of HilD was significantly reduced by over-production of Pat (Fig. 4.6A). As Pat and CobB have been shown to post-translationally modify proteins, we suggest that Pat and CobB could possibly modify the important SPI1 regulatory protein HilD by N-lysine acylation and deacylation. If the acylation profile of HilD were altered by Pat and CobB, it might then change the susceptibility of HilD to Lon and subsequently affected downstream invasion gene expression.

In the model that we propose here, we suggest that HilD is the target of Pat and CobB. However, there is a possible alternative mechanism by which Pat and CobB control invasion gene expression, by directly or indirectly affecting the protease activity of Lon to HilD. If this hypothesis is correct, other genes or phenotypes regulated by Lon should be affected by overexpression of Pat or CobB as well. In *Salmonella*, the production of the polysaccharide colonic acid is negatively regulated by the Lon protease. Thus, the *lon* mutant strain showed a mucoid phenotype due to over-production of colonic acid (12, 30). We expect that if over-expression of Pat induces the protease activity of Lon, the *pat* mutant would show a mucoid phenotype due to the loss of the negative effect of *lon* on the production of colonic acid. Additionally, since *pat* and *cobB* act oppositely, this mucoid phenotype should be also observed in the CobB over-expression strain. However, in our experiments the *pat* mutant strain and wild type strain carrying the CobB over-expression plasmid did not shown a mucoid phenotype.

These results suggest that Lon activity is not affected by Pat and CobB, and further suggest that Pat and CobB affect invasion gene expression through means other than the direct control on Lon.

Our results showed that over-production of *pat* or *cobB* could affect invasion gene expression (Fig. 4.1). However, the effects of these two genes on invasion gene expression could be seen only when they were over-expressed, since mutations of neither *pat* nor *cobB* change the expression of the downstream SPI1 gene, *sopB* (data not shown). There are three possibilities that could explain these phenomena: 1) A single copy of *pat* or *cobB* has very little effect on invasion, but the effect is amplified when either is over-expressed. 2) In *Salmonella*, there are redundant enzymes that function like Pat and CobB. Thus, when *pat* or *cobB* is deleted, these functional analogues can compensate for the loss of Pat or CobB, and show no change in the phenotype. 3) Over-production of Pat or CobB may cause these two enzymes to falsely use HilD as their substrate and further change the acylation profile of HilD by acyl-modification.

In this work, we surmise that propionyl-CoA could function as a substrate and provide the propionyl group to Pat to inactivate a target protein through N-lysine propionylation. However, we still lack the direct evidence to show that the propionyl-CoA is also essential for the negative effect of *pat* on invasion. One approach to answer this question is to use a bacterial strain that cannot produce propionyl-CoA. We expect that in this strain SPI1 gene expression would not be affected by over-expression of *pat* due to the absence of propionyl-CoA as its substrate. In contrast, if propionyl-CoA is not important, over-production of *pat* would still affect SPI1 gene expression in this mutant. In *Salmonella*, propionyl-CoA can be produced from exogenous propionate through PrpE, Acs, Pta, and from endogenously produced 2-ketobutyrate via TdcE and PflB (34, 47). We expect that in a *prpE acs pta tdcE pflB* mutant strain the production of propionyl-CoA would be greatly reduced or completely abolished. Thus,

we can over-express *pat* or *cobB* in this strain, then examine whether invasion genes are still affected by Pat and CobB.

Since Pat has been shown to post-translationally modify target proteins through N-lysine acylation, we surmise that HilD could be chemically modified by Pat. To answer this question, we simply asked whether the acylation profile of lysine residues on HilD was changed by over-expression of Pat. Unfortunately, we were not able to assess this hypothesis since only a small amount of HilD was obtained with Pat over-production (Fig, 4.6A), and this small amount of HilD was not sufficient to assess additional acylation by mass spectrometry. We thought that this low yield of HilD might result from the degradation of chemically modified HilD by the Lon protease. If this assumption is correct, we may be able to isolated sufficient HilD from the *lon* mutant strain. Thus, we can alternatively use the *lon* mutant carrying the pHiD^{-His} or the pPat-HilD^{-His} plasmid and perform the same experiment shown in Fig. 4.6A to address our hypothesis.

In this work, we also found three acetylated lysine residues (K131, K263 and K296) on HilD, and two (K131, K263) of the three have not been reported before (Fig. 4.7). Adding this information to the previous studies reported by Wang and coworkers (53), there are four acetylated lysine residues found on HilD (K131, K263 and K296, K305). Pat and CobB have been shown to modify their target proteins by acetylation and deacetylation, the same as by propionylation and depropionylation. This result leads to the possibility that Pat and CobB may modulate HilD activity by changing the acetylation profile of HilD, rather than by propionylation. To examine this hypothesis, we can simply substitute an individual lysine residue, or a combination of residues, with arginine and examine whether over-production of Pat or CobB affects invasion gene expression when these lysine residues are substituted. Previous studies have also shown that some regulators function by auto-acetylation. One example is the *Salmonella* chemotaxis regulator CheY, which directly utilizes acetyl-CoA as an acetyl donor to acetylate itself (6, 54). Thus, one possible alternative model is that Pat and CobB over-production may indirectly affect the production of Acetyl-CoA, leading HilD to use acetyl-CoA as

a substrate to acetylate itself on various lysine resides that we have previously described. Any change in the acetylation profile of HilD may subsequently affect invasion gene expression.

ACKNOWLEDGEMENTS

We thank Zach Dwyer for the *sopB* experiment and Julie Siler for the critical review of this manuscript. This project was supported by the National Research Initiative of the USDA Cooperative State Research, Education and Extension Service, award number 2005-35201-16270.

REFERENCES

1. **Akbar, S., L. M. Schechter, C. P. Lostroh, and C. A. Lee.** 2003. AraC/XylS family members, HilD and HilC, directly activate virulence gene expression independently of HilA in Salmonella typhimurium. Molecular Microbiology **47:**715-28.
2. **Altier, C.** 2005. Genetic and environmental control of salmonella invasion. J Microbiol **43 Spec No:**85-92.
3. **Altier, C., M. Suyemoto, and S. D. Lawhon.** 2000. Regulation of Salmonella enterica serovar typhimurium invasion genes by csrA. Infection and Immunity **68:**6790-7.
4. **Altier, C., M. Suyemoto, A. I. Ruiz, K. D. Burnham, and R. Maurer.** 2000. Characterization of two novel regulatory genes affecting Salmonella invasion gene expression. Molecular Microbiology **35:**635-46.
5. **Bajaj, V., R. L. Lucas, C. Hwang, and C. A. Lee.** 1996. Co-ordinate regulation of Salmonella typhimurium invasion genes by environmental and regulatory factors is mediated by control of hilA expression. Molecular Microbiology **22:**703-14.
6. **Barak, R., J. Yan, A. Shainskaya, and M. Eisenbach.** 2006. The chemotaxis response regulator CheY can catalyze its own acetylation. J Mol Biol **359:**251-65.

7. **Baxter, M. A., T. F. Fahlen, R. L. Wilson, and B. D. Jones.** 2003. HilE interacts with HilD and negatively regulates hilA transcription and expression of the Salmonella enterica serovar Typhimurium invasive phenotype. Infection and Immunity **71:**1295-305.

8. **Behlau, I., and S. I. Miller.** 1993. A PhoP-repressed gene promotes Salmonella typhimurium invasion of epithelial cells. Journal of Bacteriology **175:**4475-84.

9. **Boddicker, J. D., and B. D. Jones.** 2004. Lon protease activity causes down-regulation of Salmonella pathogenicity island 1 invasion gene expression after infection of epithelial cells. Infect Immun **72:**2002-13.

10. **Chang, A. C., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol **134:**1141-56.

11. **Chilcott, G. S., and K. T. Hughes.** 2000. Coupling of flagellar gene expression to flagellar assembly in Salmonella enterica serovar typhimurium and Escherichia coli. Microbiol Mol Biol Rev **64:**694-708.

12. **Chubiz, J. E., Y. A. Golubeva, D. Lin, L. D. Miller, and J. M. Slauch.** 2010. FliZ regulates expression of the Salmonella pathogenicity island 1 invasion locus by controlling HilD protein activity in Salmonella enterica serovar typhimurium. J Bacteriol **192:**6261-70.

13. **Collazo, C. M., and J. E. Galan.** 1997. The invasion-associated type-III protein secretion system in Salmonella--a review. Gene **192:**51-9.

14. **Darwin, K. H., and V. L. Miller.** 1999. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in Salmonella typhimurium. Journal of Bacteriology **181:**4949-54.

15. **Darwin, K. H., and V. L. Miller.** 2001. Type III secretion chaperone-dependent regulation: activation of virulence genes by SicA and InvF in Salmonella typhimurium. EMBO J **20:**1850-62.

16. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-5.

17. **Eichelberg, K., and J. E. Galan.** 1999. Differential regulation of Salmonella typhimurium type III secreted proteins by pathogenicity island 1 (SPI-1)-encoded transcriptional activators InvF and hilA. Infection and Immunity **67:**4099-105.

18. **Ellermeier, C. D., J. R. Ellermeier, and J. M. Slauch.** 2005. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. Mol Microbiol **57:**691-705.

19. **Ellermeier, C. D., A. Janakiraman, and J. M. Slauch.** 2002. Construction of targeted single copy lac fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. Gene **290:**153-61.

20. **Ellermeier, C. D., and J. M. Slauch.** 2003. RtsA and RtsB coordinately regulate expression of the invasion and flagellar genes in Salmonella enterica serovar Typhimurium. Journal of Bacteriology **185:**5096-108.

21. **Ellermeier, J. R., and J. M. Slauch.** 2007. Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. Curr Opin Microbiol **10:**24-9.

22. **Fahlen, T. F., N. Mathur, and B. D. Jones.** 2000. Identification and characterization of mutants with increased expression of hilA, the invasion gene transcriptional activator of Salmonella typhimurium. FEMS Immunol Med Microbiol **28:**25-35.

23. **Fang, F. C., and S. Rimsky.** 2008. New insights into transcriptional regulation by H-NS. Curr Opin Microbiol **11:**113-20.

24. **Fortune, D. R., M. Suyemoto, and C. Altier.** 2006. Identification of CsrC and characterization of its role in epithelial cell invasion in Salmonella enterica serovar Typhimurium. Infect Immun **74:**331-9.

25. **Fu, Y., and J. E. Galan.** 1998. Identification of a specific chaperone for SptP, a substrate of the centisome 63 type III secretion system of Salmonella typhimurium. Journal of Bacteriology **180:**3393-9.

26. **Galan, J. E., and R. Curtiss, 3rd.** 1989. Cloning and molecular characterization of genes whose products allow Salmonella typhimurium to penetrate tissue culture cells. Proc Natl Acad Sci U S A **86:**6383-7.

27. **Garner, C. D., D. A. Antonopoulos, B. Wagner, G. E. Duhamel, I. Keresztes, D. A. Ross, V. B. Young, and C. Altier.** 2009. Perturbation of the small intestine microbial ecology by streptomycin alters pathology in a Salmonella enterica serovar typhimurium murine model of infection. Infect Immun **77:**2691-702.

28. **Garrity, J., J. G. Gardner, W. Hawse, C. Wolberger, and J. C. Escalante-Semerena.** 2007. N-lysine propionylation controls the activity of propionyl-CoA synthetase. J Biol Chem **282:**30239-45.

29. **Golubeva, Y. A., A. Y. Sadik, J. R. Ellermeier, and J. M. Slauch.** 2011. Integrating Global Regulatory Input into the Salmonella Pathogenicity Island 1 Type III Secretion System. Genetics.

30. **Gottesman, S., and V. Stout.** 1991. Regulation of capsular polysaccharide synthesis in Escherichia coli K12. Mol Microbiol **5:**1599-606.

31. **Grana, D., T. Gardella, and M. M. Susskind.** 1988. The effects of mutations in the ant promoter of phage P22 depend on context. Genetics **120:**319-27.

32. **Groisman, E. A., and H. Ochman.** 1993. Cognate gene clusters govern invasion of host epithelial cells by Salmonella typhimurium and Shigella flexneri. Embo J **12:**3779-87.

33. **Hardt, W. D., L. M. Chen, K. E. Schuebel, X. R. Bustelo, and J. E. Galan.** 1998. S. typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. Cell **93:**815-26.

34. **Hesslinger, C., S. A. Fairhurst, and G. Sawers.** 1998. Novel keto acid formate-lyase and propionate kinase enzymes are components of an anaerobic pathway in Escherichia coli that degrades L-threonine to propionate. Mol Microbiol **27:**477-92.

35. **Kage, H., A. Takaya, M. Ohya, and T. Yamamoto.** 2008. Coordinated regulation of expression of Salmonella pathogenicity island 1 and flagellar type III secretion systems by ATP-dependent ClpXP protease. J Bacteriol **190:**2470-8.

36. **Kubori, T., Y. Matsushima, D. Nakamura, J. Uralil, M. Lara-Tejero, A. Sukhan, J. E. Galan, and S. I. Aizawa.** 1998. Supramolecular structure of the Salmonella typhimurium type III protein secretion system. Science **280:**602-5.

37. **Lawhon, S. D., J. G. Frye, M. Suyemoto, S. Porwollik, M. McClelland, and C. Altier.** 2003. Global regulation by CsrA in Salmonella typhimurium. Molecular Microbiology **48:**1633-45.

38. **Lawhon, S. D., R. Maurer, M. Suyemoto, and C. Altier.** 2002. Intestinal short-chain fatty acids alter Salmonella typhimurium invasion gene expression and virulence through BarA/SirA. Molecular Microbiology **46:**1451-64.

39. **Lee, C. A., B. D. Jones, and S. Falkow.** 1992. Identification of a Salmonella typhimurium invasion locus by selection for hyperinvasive mutants. Proc Natl Acad Sci U S A **89:**1847-51.

40. **Martinez, L. C., H. Yakhnin, M. I. Camacho, D. Georgellis, P. Babitzke, J. L. Puente, and V. H. Bustamante.** 2011. Integration of a complex regulatory cascade involving the SirA/BarA and Csr global regulatory systems that controls expression of the Salmonella SPI-1 and SPI-2 virulence regulons through HilD. Mol Microbiol **80:**1637-56.

41. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for Escherichia coli and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

42. **Mills, D. M., V. Bajaj, and C. A. Lee.** 1995. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol Microbiol **15:**749-59.

43. **Navarre, D. A., and T. J. Wolpert.** 1995. Inhibition of the glycine decarboxylase multienzyme complex by the host-selective toxin victorin. Plant Cell **7:**463-471.

44. **Navarre, W. W., M. McClelland, S. J. Libby, and F. C. Fang.** 2007. Silencing of xenogeneic DNA by H-NS-facilitation of lateral gene transfer in bacteria by a defense system that recognizes foreign DNA. Genes Dev **21:**1456-71.

45. **Olekhnovich, I. N., and R. J. Kadner.** 2002. DNA-binding activities of the HilC and HilD virulence regulatory proteins of Salmonella enterica serovar Typhimurium. Journal of Bacteriology **184:**4148-60.

46. **Olekhnovich, I. N., and R. J. Kadner.** 2007. Role of nucleoid-associated proteins Hha and H-NS in expression of Salmonella enterica activators HilD, HilC, and RtsA required for cell invasion. J Bacteriol **189:**6882-90.

47. **Palacios, S., V. J. Starai, and J. C. Escalante-Semerena.** 2003. Propionyl coenzyme A is a common intermediate in the 1,2-propanediol and propionate catabolic pathways needed for expression of the prpBCDE operon during growth of Salmonella enterica on 1,2-propanediol. J Bacteriol **185:**2802-10.

48. **Schechter, L. M., S. M. Damrauer, and C. A. Lee.** 1999. Two AraC/XylS family members can independently counteract the effect of repressing sequences upstream of the hilA promoter. Molecular Microbiology **32:**629-42.

49. **Starai, V. J., I. Celic, R. N. Cole, J. D. Boeke, and J. C. Escalante-Semerena.** 2002. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. Science **298:**2390-2.

50. **Starai, V. J., and J. C. Escalante-Semerena.** 2004. Identification of the protein acetyltransferase (Pat) enzyme that acetylates acetyl-CoA synthetase in Salmonella enterica. J Mol Biol **340:**1005-12.

51. **Takaya, A., Y. Kubota, E. Isogai, and T. Yamamoto.** 2005. Degradation of the HilC and HilD regulator proteins by ATP-dependent Lon protease leads to downregulation of Salmonella pathogenicity island 1 gene expression. Mol Microbiol **55:**839-52.

52. **Takaya, A., T. Tomoyasu, A. Tokumitsu, M. Morioka, and T. Yamamoto.** 2002. The ATP-dependent lon protease of Salmonella enterica serovar Typhimurium regulates invasion and expression of genes carried on Salmonella pathogenicity island 1. Journal of Bacteriology **184:**224-32.

53. **Wang, Q., Y. Zhang, C. Yang, H. Xiong, Y. Lin, J. Yao, H. Li, L. Xie, W. Zhao, Y. Yao, Z. B. Ning, R. Zeng, Y. Xiong, K. L. Guan, S. Zhao, and G. P. Zhao.** 2010. Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. Science **327:**1004-7.

54. **Yan, J., R. Barak, O. Liarzi, A. Shainskaya, and M. Eisenbach.** 2008. In vivo acetylation of CheY, a response regulator in chemotaxis of Escherichia coli. J Mol Biol **376:**1260-71.

55. **Zhou, D., M. S. Mooseker, and J. E. Galan.** 1999. Role of the S. typhimurium actin-binding protein SipA in bacterial internalization. Science **283:**2092-5.