IDENTIFYING FUNCTIONS OF RPON-DEPENDENT GENES IN *SALMONELLA*

By

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(Under the Direction of Timothy Hoover)

Abstract

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) is a significant food borne pathogen causing gastroenteritis in humans. Although there has been extensive research conducted on *S.* Typhimurium's pathogenicity islands and effector proteins, the potential role of *S.* Typhimurium's metabolic genes in colonization has been widely overlooked until recently. In recent studies, a number of metabolic genes have been implicated as important for successful colonization of chickens, pigs, and calves. RpoN is an alternative sigma factor required for transcription of genes involved in a range of metabolic pathways including nitrogen metabolism, H_2 evolution and uptake, carbohydrate transport and degradation of aromatic compounds. The RpoN regulon in *Salmonella* is not fully characterized. Many RpoN-dependent genes are of unknown function, all of which appear to be involved in metabolism. The goal of my research has been to characterize genes within the RpoN regulon. *S.* Typhimurium possesses three sugar phosphotransferase (PTS) permeases which are absent in most strains of closely related bacteria and whose expression is controlled by the transcription factor RpoN. *S.*

Typhimurium strains lacking all three of these PTS permeases or the activators responsible for their transcription weredeficient in colonization of the chicken ileum, jejunum, and ceca. I identified the substrates for two of the three PTS permeases. The permease and associated enzymes encoded with the *dga* locus are responsible for the transport and utilization of D-glucosaminate. The permease and associated enzymes encoded within the *gfr* locus are responsible for the transport and utilization of fructoselysine and glucoselysine. It is possible that D-glucosaminate, fructoselysine, and/or glucoselysine may be encountered by *S.* Typhimurium in the gastrointestinal tract of chickens and other animals. Two other RpoN-dependent operons have been looked at that also seem to be involved in metabolism. The first may be involved in arginine transport or utilization and may have broader effects such as involvement in acid stress. The other operon appears to be important for gluconate utilization during anaerobic conditions. Taken together, the five RpoN-dependent operons discussed here are an example of the diversity of metabolic genes in the RpoN regulon.

INDEX WORDS: *Salmonella*, PTS permease, RpoN, Animal Colonizarion

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DEDICATION

This work is dedicated to all of the people who have helped me during my college and doctoral career. I want to dedicate this to Dr. Michael Lawson for first exposing me to Microbiology, to Dr. Margie Lee for allowing me to work in her lab as an undergraduate which changed my career path entirely, to Dr. Timothy Hoover for his patients, understanding, and willingness to impart his knowledge towards making me an independent scientist. I especially want to dedicate this to my parents, Richard and Francis, for their never ending support and love- without them I would have never made it this far.

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CHAPTER 1: INTRODUCTION

No-typhoidal almonellosis is one of the most prevalent foodborne diseases in the world, accounting for tens of millions of cases and over one hundred thousand deaths annually (http://www.who.int/mediacentre/factsheets/fs139/en/). In the United States alone, non-typhoidal *Salmonella* cause an estimated 1.4 million infections in humans in the United States annually (1), with costs associated with medical care and loss of productivity ranging from $0.5-2.3 billion (2). Non-typhoidal salmonellosis is characterized by gastroenteritis accompanied with intestinal inflammation and diarrhea. *Salmonella* Typhimurium is the serotype most commonly associated with the diarrheal disease (3). Symptoms of gastroenteritis include diarrhea, nausea, vomiting, intestinal cramping, and fever, which develop after approximately one day post ingestion of *S.* Typhimurium (4). Although patients infected with *S*. Typhimurium usually only develop such acute gastrointestinal disease, ~5% of infections result in bacteremia which can lead to septic shock, pneumonia, meningitis and other disseminated diseases (5). Patients exhibiting symptoms of invasive disease are treated with antimicrobials, but antibiotic resistance in *Salmonella* is rising at an alarming rate (11).

Salmonella is usually transmitted to humans through the food chain, with 95% of salmonellosis cases resulting from consumption of contaminated meat, eggs or poultry (6). Infection of poultry with *Salmonella* serovars generally results in asymptomatic intestinal colonization that leads to carcass contamination and entry into the food supply. Understanding the strategies *Salmonella* employs for intestinal colonization of food animals is critical for

reducing the entry of this significant pathogen into the food supply. Salmonella and other enteropathogens are “scavengers” or secondary degraders that are dependent on nutrients provided by the enzymatic action of primary degraders which digest complex carbohydrates and intestinal glycoproteins. Most enteropathogens lack the array of secreted enzymes needed to liberate amino acids and sugars present in complex macromolecules and they are therefore dependent on saccharides (7-9) and amino acids (10, 11) liberated by primary degraders such as *Bacteroides* and *Clostridium*.

One of the ongoing frontiers of *Salmonella* control in food animals is colonization of the intestine. While in some food animals, invasion of the intestinal epithelial cells is essential for colonization, there are many *Salmonella* serotypes that colonize poultry and appear to compete quite well within the intestinal bacterial community without the requirement for cellular invasion. A considerable body of work is available on *Salmonella* pathogenicity genes and the mechanisms by which they facilitate infection (12-20), but there is a paucity of data regarding nutritional requirements of *Salmonella* that enable them to establish a niche within the intestinal microbiome (16-17, 21-24). Studies that have examined how *Salmonella*’s nutritional requirements impact colonization have typically focused on global regulators. For example, mutations targeting catabolite repression, specifically *crp* and *cya*, significantly attenuate *Salmonella* without affecting epithelial invasion (22, 25). Catabolite repression is a regulatory mechanism used by bacteria to prioritize expression of transporters and catabolic enzymes to enable the hierarchical usage of different carbon sources. *Salmonella* crp/cya mutants are reduced in the ability to colonize the chicken cecum, yet we do not understand the mechanism by which *Salmonella* prioritizes the utilization of available carbohydrates enabling it to compete for colonization niches with the intestine. There has been considerable emphasis on understanding

this ability in *Escherichia coli* infection and colonization (26-31). In contrast, only a few *Salmonella* transporters have been examined as to their role in infection (32-33), and those few studies have focused primarily on ion transport (34-38). Identification of the full complement of transporter genes present in a bacterial genome can provide insight into the organism's metabolic potential and inferences as to nutrients that the organism may encounter and utilize within its niche.

RpoN (σ^{54}) is an alternative sigma factor required for the transcription of genes involved in an array of metabolic processes including nitrogen assimilation and metabolism, H_2 metabolism, propionate catabolism and carbohydrate transport (6,7). Given the range of metabolic processes in bacteria w are regulated by RpoN, it is reasonable to postulate a significant role for RpoN in colonization of food animals. The goal of my dissertation research has been to identify functions of RpoN-dependent genes in *S.* Typhimurium that remain uncharacterized. Transcription by σ^{54}-RNA polymerase holoenzyme is absolutely dependent upon an activator which stimulates formation of a closed complex between polymerase and the promoter to an open complex that is component to initiate transcription (8). *S.* Typhimurium strains whose genomes have been sequenced to date have 13 different RpoN-dependent activators. These activators and the processes which they regulate are: NtrC (nitrogen assimilation and catabolism), ZraR (zinc tolerance), GlrR (glucosamine-6-phosphate homeostasis), FhlA (pH homeostasis via formate-hydrogen lyase), YgaA (nitric oxide reduction), PrpR (propionate catabolism), PspF (cell envelope stress response), STM2361 (function unknown), STM0652 (function unknown), RtcR (presumed RNA repair and degradation), GfrR (transport and catabolism of fructoselysine and glucoselysine), DgaR (transport and catabolism of D-glucosaminate) and STM0571 (function unknown).

Three of the RpoN-dependent activators in *S.* Typhimurium (GfrR, DgaR and STM0571) appear to regulate expression of operons which encode sugar phosphotransferase system (PTS) permeases and enzymes involved in the catabolism of the substrates of these permeases. PTS permeases are major routes for carbohydrate transport in bacteria and *S.* Typhimurium possesses over 20 different PTS permeases. PTS-mediated transport involves the coupled phosphorylation of sugar substrates with their translocation across the cell membrane. The general mechanism of PTS-mediated transport is as follows. A phosphoryl group from phosphoenolpyruvate is transferred to enzyme I (EI) which in turn transfers it to a phosphoryl carrier, the histidine protein (HPr). From HPr, the phosphoryl group is transferred to a sugar-specific permease, a membrane-bound complex known as enzyme 2 (EII), which phosphorylates the sugar as it is transported across the cell membrane. EII complexes (PTS permeases) are associated with the cell membrane and are specific for a single substrate or set of structurally related substrates. EII complexes consists of at least three distinct domains (IIA, IIB and IIC) which are fused into a single polypeptide or exist as multiple, interacting polypeptides. Enzyme I and HPr are cytoplasmic proteins used in conjunction with most PTS permeases, although some permeases (e.g., FruA and FrwBCD fructose-specific PTS permeases) have dedicated Enzyme I and/or HPr homologs (39).

The three RpoN-dependent PTS permeases belong to the mannose family of PTS permeases. Mannose family PTS permeases are characterized by an addition domain, EIID (40, 41). The best characterized member of the mannose family of PTS permeases is *E. coli* ManXYZ, which transports D-mannose as well as D-glucose, D-glucosamine and N-acetylglucosamine.

When I began my work to identify functions for RpoN-dependent genes the substrates for the RpoN-dependent mannose family PTS permeases were unknown. I identified D-glucosaminate

as the substrate for one of these PTS permeases as well as elucidated the pathway by which this compound is catabolized (Chapter 4, 42). The genes required for D-glucosaminate utilization were designated as *dgaABCDEF* and the RpoN-dependent activator which regulates expression of this operon was designated as *dgaR*.

I identified fructoselysine and glucoselysine as substrates for a second RpoN-dependent PTS permease and showed that the other genes in this second PTS operon encode two deglycases that are required for utilization of these compounds (Chapter 5). The operon encoding the genes required for utilization of fructoselysine and glucoselysine were designated as *gfrABCDEF* and the RpoN-dependent activator which regulates expression of this operon was designated as *gfrR*. Fructoselysine is formed by the reaction of glucose with the ε-amine of lysine followed by a spontaneous isomerization of the sugar referred to as an Amadori rearrangement (43). Glucoselysine is similarly formed from the reaction of fructose with the ε-amine of lysine followed by the spontaneous isomerization of the sugar (referred to as Heyns rearrangements) which lead to the formation of 2-amino-aldose derivative or 2-amino-3-hexulose derivatives, each of which can be in either a glucosamine or mannosamine configuration (44). The spontaneous reactions of primary amines with glucose and fructose are referred to as glycation and fructation, respectively. Fructosamines are common in nature and can result from the modification of free amino acids as well as proteins. Fructoselysine occurs in dehydrated fruits, grains and vegetables, including raisins, prunes, dates, figs, apricots, onion, cereal and carrots (45). Other fructosamines are found in rotting fruits and vegetables, accounting for as much as 7% of the fresh mass (46).

I recently found that *S*. Typhimurium utilizes another fructosamines, including fructosemethionine and fructosevaline. Although I have not yet identified the genetic locus

responsible for the transport and catabolism of these compounds, I postulate that the remaining RpoN-dependent PTS permease of unknown function is involved in the transport and utilization of one or both of these fructosamines. This hypothesis is based on the observation that there are two genes in the operon encoding this PTS permease are predicted to encode enzymes that share homology with deglycases involved in fructosamine catabolism.

The results of my studies have reveal unexpected aspects of *Salmonella* physiology. Recent findings suggest that fructosamine utilization in *Salmonella* plays an important role in the physiology and ecology of *Salmonella* in the intestinal microbiome. Ali and co-workers identified a genetic locus in *S.* Typhimurium required for catabolism of fructose-asparagine (*fra* locus), and showed that this locus is essential for fitness of *S.* Typhimurium in an inflamed mouse intestine model (47). In addition, Chaudhuri et al. (48) reported results from a high throughput screen of random insertion mutants in *S.* Typhimurium that transposon insertions within the *fra* locus generally resulted in colonization defects in chickens, pigs and cattle. Moreover, Chaudhuri et al found that insertions in genes within the *gfr* locus typically resulted in colonization defects in food animals (48). Taken together, these findings indicate that fructosamine catabolism is an unexplored area of *Salmonella* physiology that may be important of colonization of food animals. Knowledge of the role of fructosamine catabolism in *Salmonella* could result in a better understanding of how *Salmonella* is able to carve out a niche within the extremely competitive environment of the host intestine. Such new information could lead to new approaches for reducing growth of *Salmonella* during the food animal production period and thereby mitigate the entry of *Salmonella* into the human food chain.

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CHAPTER 2: LITERATURE REVIEW: ROLE OF *SALMONELLA* METABOLIC GENES IN THE COLONIZATION OF FOOD ANIMALS

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Abstract

Salmonella colonize the intestinal tract of a wide variety of animals. Within the intestinal tract, *Salmonella* tend to be scavengers or secondary degraders that are dependent upon nutrients released by the enzymatic action of the more abundant primary degraders which digest intestinal glycoproteins and complex carbohydrates. *Salmonella* are catalytically robust and capable of using a wide variety of carbon and nitrogen sources. While a considerable amount of information is available on *Salmonella* pathogenicity genes and how they facilitate infection, we have only recently begun to appreciate the nutritional requirements of *Salmonella* within the context of the intestinal microbiome. Several recent studies have used –omics driven (i.e., genomics, transcriptomics, proteomics, metabolomics) approaches to investigate possible contributions of metabolic processes to *Salmonella* colonization and virulence. We review here recent research literature on the roles of metabolic genes in *Salmonella* colonization in various host systems including mice, swine, calves and poultry, as well as metabolic genes implicated in the persistence of *Salmonella* on produce. Understanding the nutritional requirements of *Salmonella* in the host intestinal tract is important for identifying potential targets and designing new approaches for reducing growth of *Salmonella* during the food animal production period and thereby mitigating the entry of *Salmonella* into the human food supply.

Introduction

Salmonella are Gram-negative, rod-shaped, flagellated bacteria belonging to the family *Enterobacteriaceae*. There are currently two species of *Salmonella*, *S. bongeri* and *S. enterica*, with *S. enterica* divided into six subspecies. *Salmonella* are classified further by three distinct types of antigens which have been used to distinguish over 2500 serotypes. Thus, the nomenclature for *Salmonella* is complex and includes both the subspecies and serovar designations (1). For example, the *Salmonella* laboratory strain commonly used for laboratory studies is *S. enterica* subspecies *enterica* serovar Typhimurium, which is shortened to *Salmonella* Typhimurium or *S*. Typhimurium.

Salmonella is a major foodborne pathogen worldwide. A 2011 report estimated that in the United States alone there were approximately 1 million cases of salmonellosis, resulting in 19,000 hospitalizations and 378 deaths (2). In the United States costs associated with *Salmonella* are estimated to be between 2.3 and 3.6 billion dollars annually (3). *Salmonella* is usually transmitted to humans through the food chain. Although there have been reports of salmonellosis cases being linked to vegetables and fruits, 95% of salmonellosis cases result from consumption of meat, eggs or poultry (4). Infection of poultry with most *Salmonella* serovars results in asymptomatic intestinal colonization so reliance on birds appearing to be sick is an ineffective indicator of *Salmonella* colonization. Egg shells can be contaminated with *Salmonella* from infected laying hens, and unless bacterial numbers exceed 10^9 colony forming units per egg no obvious changes in appearance or odor can be observed for eggs which are contaminated with *Salmonella* (5).

One of the ongoing frontiers of *Salmonella* control in food animals is colonization of the intestine. While in some food animals, invasion of the intestinal epithelial cells is essential for colonization, there are many *Salmonella* serotypes that colonize poultry and other food animals which appear to compete quite well within the intestinal bacterial community without the requirement for cellular invasion. A considerable body of work is available on *Salmonella* pathogenicity genes and the mechanisms by which they facilitate infection (6-14). Until recently, however, there has been relatively little work done on defining nutritional requirements of *Salmonella* that enable them to establish a niche within the intestinal microbiome (10, 11, 15-18). Studies that have examined how *Salmonella'*s nutritional requirements impact colonization have often focused on global regulators. For example, mutations targeting catabolite repression, specifically *crp* and *cya*, significantly attenuate *Salmonella* without affecting epithelial invasion (16, 19). Catabolite repression is a regulatory mechanism used by bacteria to prioritize expression of transporters and catabolic enzymes to enable the hierarchical usage of different carbon sources. *Salmonella crp*/*cya* mutants are reduced in the ability to colonize the chicken cecum, yet we do not understand the mechanism by which *Salmonella* prioritizes the utilization of available carbohydrates enabling it to compete for colonization niches within the intestine.

Successful colonization of the intestinal tract of food animals by *Salmonella* is a complex process that involves not only interactions of *Salmonella* with the host, but also interactions with the gut microbiota. We review here some of the approaches used to identify *Salmonella* genes that appear to have a role in host colonization. We focus on results from these studies that identify *Salmonella* metabolic genes that may be important for colonization of the GI tract of various animals. In addition, we examine the research literature on metabolic genes implicated in the persistence of *Salmonella* on produce.

Use of –omics driven methods to identify *Salmonella* metabolic genes important for colonization and virulence

The application of –omics driven approaches (i.e., genomics, transcriptomics, proteomics and metabolomics) has contributed tremendously to our understanding of metabolic processes that are important for *Salmonella* colonization and virulence. For purposes of this review, we will restrict the definition of metabolic processes to those which are involved in nutrient acquisition (e.g., carbon, nitrogen, sulfur, metal ions) and energy generation. We will highlight some approaches which have been used to identify *Salmonella* genes which have roles in colonization or virulence, and indicate metabolic genes that were identified in these studies as having such roles.

Identification of Salmonella metabolic genes needed for colonization using signature-tagged mutagenesis. Signature-tagged mutagenesis (STM) is a powerful negative selection method used to identify bacterial genes that are potentially important for the successful colonization of a host animal. The method was developed about twenty years ago by David Holden and co-workers to identify mutants in *S.* Typhimurium attenuated for virulence in a murine model of typhoid fever (20). STM allows for the identification of attenuated mutants from a large pool of mutants by combining transposon-mutagenesis and negative selection with a detection system. Short individual DNA sequences introduced within the transposons (i.e., signature-tags) are used to individually mark the mutants. Pools of mutants with distinct signature-tags are assembled and the mutant pools are subject to a negative selection by inoculating them into an animal host. Mutants that survive the screen are recovered from the animal host (output pool) for detection of the individual signature-tags. Mutants that fail to survive the screen are identified from cultures of the mutant pools used to inoculate the animals (input pool) using the same detection system.

The original STM method used hybridization analysis to identify signature-tags, but this detection system has since been supplanted by less laborious methods, including polymerase chain reaction (PCR)-amplification, real-time PCR and DNA microarrays (21-23).

Roles for genes in colonization which have been identified by STM need to be verified by reconstructing the relevant mutations in the original parental strain and then testing the resulting mutants for colonization defects. A drawback of STM is that it is not possible to confirm colonization phenotypes for all (or even most) of the mutant alleles identified from screens that yield large numbers of potential colonization mutants. The colonization phenotype of a reconstructed mutant strain can be examined by inoculating the host animal with a pure culture of the strain or by using a competitive index (CI) assay in which a mixture of the mutant and parental strain is used to inoculate the host animal. In the CI assay, a mutant strain is typically mixed in a 1:1 ratio with its parental strain and the resulting mixture is used to inoculate the host animal. Bacteria recovered from the host post-inoculation are analyzed to determine the ratio of mutant to wild type. Mutations which decrease fitness for colonization result in the recovery of substantially fewer mutants compared to wild type.

Several studies have used STM to identify *Salmonella* genes which have potential roles in colonization or virulence in a variety of animal hosts, including mice, chicks, pigs and calves (8, 14, 20, 24-35). While most of the work in these studies focused on virulence genes within *Salmonella* pathogenicity islands (SPI), in some cases metabolic genes were identified as having potential roles in colonization. Two of the most comprehensive STM-based studies to identify *Salmonella* genes important for colonization of food animals were done by Morgan *et al*. (14) and Carnell *et al*. (8). Morgan and co-workers identified approximately 150 *S*. Typhimurium genes that appeared to be important for colonization of the intestinal tracts of calves, 2-week-old

chicks, or both animal species (14); while Carnell and colleagues identified 95 S. Typhimurium genes which appear to be needed for intestinal colonization of the pig (8). Several of the genes identified in these studies are involved in carbohydrate catabolism, sulfur metabolism, amino acid degradation or nutrient transport (Table 2.1), although the colonization phenotypes were not confirmed for any of these mutants.

Given the caveat that not all of the genes listed in Table 2.1 may ultimately prove to be important for colonization, a couple of observations are notable. First, some genes appear to be specifically required in one animal host but not others. For example, the carnitine/γ-butyrobetaine antiporter CaiT is required for efficient intestinal colonization of the chick, but not for colonization of the calf or pig. L-carnitine is a commonly found trimethylammonium compound which *Salmonella* and other members of the *Enterobacteriaceae* are able to reduce to γ-butyrobetaine under anaerobic conditions (36). The reduction of L-carnitine stimulates fermentative growth of *S.* Typhimurium by allowing the bacterium to regenerate NAD^+ (36). Given that the intestinal tract is an anaerobic environment, the ability of *S.* Typhimurium to utilize L-carnitine might be expected to be an important colonization factor. That L-carnitine utilization does not appear to play an important role in *S.* Typhimurium colonization of the pig or calf suggests that other electron acceptors are available to *S.* Typhimurium for fermentative growth or anaerobic respiration in these host animals.

Another notable observation regarding the data presented in Table 2.1 is that many of the metabolic genes appear to be required for efficient colonization of a range of animal hosts. For example, the ferric enterobactin receptor FepA was found to be an important colonization factor in all three host animals. During iron starvation, bacteria often utilize high-affinity iron chelators known as siderophores to scavenge Fe^{2+} from the surrounding medium. Enterobactin is a

siderophore produced by *S.* Typhimurium which it uses for iron acquisition. Enterobactin synthetase (EntF) appears to be important for intestinal colonization of the chick and calf, but not the pig intestinal tract. This observation does not necessarily indicate that enterobactin is not required for iron acquisition by *S.* Typhimurium in pigs as the *entF* mutant could have utilized enterobactin produced by other *S.* Typhimurium mutants or other bacteria in the pig intestinal microbiota.

Despite the wealth of available information on *Salmonella* physiology, functions for several of the genes listed in Table 2.1 are unknown. Identifying functions for these genes will lead to a better understanding of *Salmonella* physiology. Moreover, it seems reasonable to assume that *Salmonella* encounters a different array of nutrients in the various host animals which it colonizes, and identifying activities for these genes of unknown function will provide valuable information on the variety of nutrients *Salmonella* encounters and is able to utilize within various host animals.

Use of transposon-directed insertion-site sequencing to identify Salmonella metabolic genes needed for colonization. While STM has proven to be a powerful method for identifying genes with potential roles in colonization, the task of assigning genotypes with colonization phenotypes is somewhat time consuming. Transposon-directed insertion-site sequencing (TraDIS) circumvents this problem by simultaneously identifying the location of the transposon within a mutant and the relative fitness of the mutant. As with STM, a negative selection method is used in TraDIS to identify bacterial genes important for colonization by inoculating pools of transposon-insertion mutants in a host animal and then recovering colonization-proficient bacteria from the animal. The locations of transposons in the mutants are identified by parallel sequencing of transposon-flanking regions using DNA isolated from the input and output pools.

DNA sequences from transposon insertions that result in colonization attenuation are underrepresented in the output pool relative to the input pool. Chaudhuri and co-workers used the TraDIS method to screen pools of random insertion mutants of *S.* Typhimurium in chicks, pigs, and calves (37). Over 7,700 transposon-insertion mutants were mapped and assigned colonization phenotypes to 2,715 different genes in the study. Fitness scores were defined as the $\log_2$-fold change in the number of sequence reads obtained across the boundaries of each transposon insertion between the input and output pools. The researchers estimated *P* values using biological replicates (duplicates or triplicates of each mutant pool), and defined attenuated mutants as those with a negative fitness score and $P \leq 0.05$ (37). Colonization phenotypes for twelve of the mutants identified from the TraDIS assay were tested by creating null mutants with defined deletions and using a CI assay in chicks to verify the predicted colonization defect. At day 4 post-inoculation, which corresponded to the time at which mutants were recovered for the TraDIS analysis, statistically significant differences in the ratio of mutant to wild-type strains recovered from the chicks compared to the ratio in the inocula were observed for eight of the null mutants, while significant differences were detected at later time points for two additional mutants (37). These data suggest that the reliability of the TraDIS assay in predicting colonization phenotypes of individual mutants within a large mutant pool is fairly good. Further evidence supporting the reliability of TraDIS assay in predicting colonization phenotypes was the observation that multiple independent mutations for a given gene or pathway often resulted in attenuation (see Table 2.2 and reference (37)).

A somewhat unexpected result from the study by Chaudhuri *et al*. (37) was the seemingly high number of transposon-insertion mutants which were attenuated. Of the 7,700 transposon-insertion mutants that were screened and mapped in the TraDIS assays in the food-producing

animals, 25%, 28% and 36% were identified as being attenuated in chicks, pigs and calves, respectively. Most of the genes identified as being potentially involved in colonization were required in all three animals, but smaller subsets of genes appeared to be host-specific. Many of the transposon insertions were in SPI genes or other genes which were known previously to be important for colonization. Many of the mutations, however, were in genes involved in nutrient acquisition or energy generation and had not been shown previously to be important for colonization. Examples of genes involved in catabolism, nutrient acquisition or energy generation that appear to be highly relevant for colonization of chicks, pigs or calves are listed in Table 2.2. To generate the gene list, we picked a set of 30 mutants (within unique genes) with the lowest *P*-values from the statistical analysis of the TraDIS data for each animal host, reasoning that these should be some of the most highly attenuated mutants.

As seen in Table 2.2, mutations within a given gene did not always result in the same colonization phenotype in the TraDIS assay. These variances might result from the location or orientation of the transposon within the gene. The location of the transposon could affect the degree to which the insertion exerts polar effects on downstream genes. Alternatively, some transposon insertions within a given gene could result in the complete loss of activity of the product of the gene while other insertions might result in the expression of truncated proteins that retain most or all of their function. It is possible that some such truncated proteins have unregulated activities that are deleterious.

From the information presented in Table 2.2, we can make some generalizations which have been noted already. For instance, while most of the genes listed in Table 2.2 appear to be important for efficient colonization in all three of the food-producing animals there do appear to be some genes which are host specific. Two such examples of host-specific genes are *pgi* and

pfkA, which encode the glycolytic enzymes glucose-6-phosphate isomerase (catalyzes the reversible isomerization of glucose-6-phosphate to fructose-6-phosphate) and 6-phosphofructokinase (catalyzes the irreversible phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate). These glycolytic enzymes appear to be important for efficient colonization in the chick (and pig to a lesser degree), but not in the calf. Since glucose levels are presumably quite low in the chick cecum (12), *S.* Typhimurium most likely needs glycolysis for chick colonization in order to generate precursor metabolites for biosynthesis rather than energy generation. Consistent with this hypothesis, mutants with transposon-insertions in genes required for the biosynthesis of branched chain amino acids (isoleucine, leucine and valine) or histidine tended to be more attenuated for chick colonization than calf colonization in the TraDIS assays (37). Biosynthesis of these amino acids requires precursor metabolites derived from glycolysis, pyruvate for branched chain amino acids and fructose-6-phosphate for histidine. This suggests that branched chain amino acids and histidine are limiting in the chick cecum (but not the calf intestinal tract), and that *Salmonella* must be able to synthesize these amino acids to colonize the chick cecum. Such differences in the availability of these amino acids could be attributed to differences in the hosts' diets, physiologies or microbiota.

As noted for the data presented in Table 2.1, functions are not known for many of the genes listed in Table 2.2. There are a couple of possible reasons for failure to assign known or predicted functions to these genes. The most obvious reason is that the products of the genes have not been analyzed biochemically. A second reason is that the genes were not carefully annotated in the genome sequence databases. Examples of potential gene assignments which have gone unnoticed in automated gene annotations are found in the genes *gfrC* (SL1344_4468), *gfrE* (SL1344_4470) and *grfF* (SL1344_4471) in Table 2.2. In 2005, Wiame and co-workers

reported that the *Enterococcus faecium grfABCDEF* operon encode a novel phosphotransferase system (PTS) permease and two deglycases involved in fructoselysine and glucoselysine catabolism (38). These researchers identified a homologous operon in *S.* Typhimurium and predicted that it was similarly involved in fructoselysine and glucoselyine catabolism (38). We constructed null mutants for homologs of *gfrAB*, *gfrE* and *gfrF* in *S.* Typhimurium and confirmed that these genes are indeed required for utilization of fructoselysine and glucoselyine (K. A. Miller, unpublished data). Fructoselysine is formed by the reaction of glucose with the ε-amine of lysine followed by a spontaneous isomerization reaction (Amadori rearrangement) (39). Glucoselysine is similarly formed from the reaction of fructose with the ε-amine of lysine, but the resulting product can undergo a couple of possible rearrangements, one of which is the 2-amino-aldose derivative with either a glucosamine (i.e., glucoselysine) or a mannosamine (i.e., mannoselysine) configuration (40). The spontaneous reactions of amines with glucose or fructose are designated as glycation and fructation, respectively. We postulate that *Salmonella* encounters fructoselysine and glucoselysine in the intestinal tracts of food-producing animals when proteins modified by glycation or fructation are degraded by proteases derived from the host or primary degraders in the gut microbiota. These modified proteins could come from feed, the host or members of the microbiota.

Once a metabolic gene has been identified as having a potential role in colonization using a transposon-based screening method such as STM or TraDIS, one still has the tasks of first verifying that it is disruption of the gene containing the transposon insertion which is responsible for the colonization phenotype, and second, determining why inactivation of the gene interferes with colonization. With regard to verifying genotype-phenotype assignments, it is important to rule out polar effects on genes located downstream of the transposon insertion as being

responsible for the colonization defect. This is typically done by constructing an unmarked deletion of the gene using the lambda Red recombination (referred to as recombineering) system (41) and then determining the colonization phenotype of the resulting null mutant. With regard to understanding of how a mutation affects colonization, there are a couple of scenarios which could account for how loss of a metabolic gene interferes with colonization. First, the mutation might directly block a pathway or process that is necessary for growth of the bacterium in the animal host. The colonization defects of strains with mutations in the genes listed under the classification of 'Energy generation' in Table 2.2 are most likely explained by this scenario. A second scenario is the mutation prevents the synthesis of an essential building block (e.g., amino acid, nucleotide, sugar) by preventing formation of a precursor metabolite. As discussed previously, we postulate that this scenario may account for the colonization defects for *S.* Typhimurium strains with mutations in *pgi* and *pfkA.* A third scenario is the mutation results in an accumulation of a toxic metabolic intermediate. Precedence for this hypothesis comes from the observation that elevated levels of sugar phosphates in *E. coli* can lead to the cessation of cell growth or even cell death (42-44). Disruption of *mtlD*, which encodes mannitol-1-phosphate-5-dehydrogenase, resulted in attenuation for colonization of *S.* Typhimurium in the chick, pig and calf in the TraDIS assay (37). We postulate that the *mtlD* mutants are attenuated due to the accumulation of mannitol-1-phosphate since strains with transpospon insertions in *mtlA*, which encodes a mannitol-specific PTS permease, display wild-type levels of colonization in all three animal hosts. MtlA phosphorylates mannitol as it is transported across the membrane, and MtlD subsequently converts mannitol-1-phosphate to fructose-6-phosphate. Thus, knocking out *mtlD* would be expected to result in an intracellular accumulation of mannitol-1-phosphate when mannitol is available extracellularly.

Transcriptome analysis to identify Salmonella metabolic genes involved in colonization. Transcriptomics, also referred to as expression profiling, examines the expression levels of mRNAs in a cell population using high-throughput methods such as DNA microarrays or next-generation sequencing technology (RNA-Seq). Harvey and co-workers used a whole-genome DNA microarray to examine gene expression of *S*. Typhimurium harvested from chick ceca, comparing the expression pattern with that of bacteria grown in broth culture (12). The researchers identified 282 *S*. Typhimurium genes which were up-regulated >2-fold in bacteria recovered from the luminal contents of the chick ceca compared to bacteria grown in broth culture (12). Genes required for utilization of propionate (*prp* operon), ethanolamine (*eut* operon), 1,2-propanediol (*pdu* operon), melibiose (*melA*) and L-ascorbate (*yiaM*, *yiaN*, *lyxK* and *sbgH*) were all up-regulated in *S*. Typhimurium from the chick ceca, suggesting that these are carbohydrates that are utilized by *Salmonella* in the chick cecum. Other metabolic genes which were up-regulated in *S*. Typhimurium isolated from the chick ceca included genes required for the transport or catabolism of putrescine (*potFG*) and arginine (*artJ*, *speA*, *adi*, *argA*), again suggesting that *S*. Typhimurium encounters and utilizes these compounds in the chick ceca.

Harvey and co-workers' identification of propionate, enthanolamine and 1,2-propanediol as carbon sources for *S*. Typhimurium in the chick cecum is an important observation and is consistent with results from similar studies in other host animals. Their results, however, represent only a brief snapshot of the metabolic processes *Salmonella* uses for efficient and long-term colonization of the intestinal tract. In their study, Harvey and co-workers inoculated chicks which were less than one-day old and sacrificed the chicks 16 hours post-inoculation. In addition, the chicks were given sterile water but were not fed during this period, with their only nourishment coming from the unabsorbed yolk sac. Under such conditions, the chicks would not

have developed the normal intestinal microflora and the bacteria within the intestinal tract would not receive the variety of nutrients derived from the feed. Thus, based on the results from the study by Harvey *et al*. (12) it is impossible to rule out roles for metabolic pathways in *S*. Typhimurium that might be needed for colonization in the presence of the normal intestinal microflora or as the birds mature.

Jay Hinton and co-workers recently published an extensive analysis of the *S*. Typhimurium transcriptome using RNA-Seq based technology (45). While this study did not examine the *S*. Typhimurium transcriptional profile in an animal host, the researchers did use 22 distinct growth conditions which mimicked environmental conditions relevant to infection. Some of the conditions were anaerobic growth, growth at 25^{o}C, and shock to a variety of insults, including acid, bile, NaCl, low iron, nitric oxide, and peroxide. As expected, the various conditions resulted in characteristic transcription profiles, and 86% of all of the *S*. Typhimurium genes were induced under one or more of the conditions (45). The study is a valuable resource for the *Salmonella* research community as it provides a comprehensive report on how infection-relevant environment conditions affect global transcription in *S*. Typhimurium. In addition, the Hinton lab has provided a web-based site where the expression profile data can be easily browsed (http://bioinf.gen.tcd.ie/cgi-bin/salcom.pl).

Identification of Salmonella metabolic genes involved in colonization using proteomic approaches. Proteomics is the large-scale study of proteins and it generally involves the identification, and sometimes the quantification, of the entire set of proteins produced by an organism. A common proteomic procedure is liquid chromatograph-tandem mass spectrometry (LC-MS/MS). In LC-MS/MS, protein samples from an organism are digested with trypsin. The resulting peptides are separated by liquid chromatography and then analyzed by tandem mass

spectrometry to identify the peptides based on their predicted atomic masses. We are unaware of any reports of proteomic approaches used to examine the *Salmonella* proteome for bacteria isolated from the intestinal tract of any host animal. Steeb and co-workers (46), however, used proteomics to examine the *Salmonella* proteome in a mouse systemic infection model that mimics human enteric fever. For these studies, the researchers used a cell sorter to isolate *S.* Typhimurium from infected mouse spleen. The *S.* Typhimurium strain used to infect the mice expressed a green fluorescent protein which facilitated the sorting. The researchers were able to estimate copy numbers for 477 metabolic enzymes, which included 102 transporters and enzymes involved in the 24 pathways for the catabolism of various carbohydrates, amino acids, nucleosides and lipids (46). To determine which of these pathways were important for intracellular growth and survival of *Salmonella* in host tissue, Steeb and co-workers used a CI assay to analyze a panel of mutants disrupted in the various pathways. Transport proteins were preferentially targeted for deletion rather than enzymes in catabolic pathways to avoid the potential for the accumulation of toxic metabolic intermediates. The researchers found that *Salmonella* colonization of the mouse spleen was dependent on the ability of the bacterium to utilize glycerol, fatty acids, N-acetylglucosamine, gluconate, glucose, lactate and arginine (46). These findings indicate that in systemic infections, *Salmonella* is dependent on a complex diet with a variety of nutrients which appear to be available in only scarce amounts.

Acquisition of divalent metal ions by *Salmonella* in the intestinal tract

In addition to –omics driven approaches to identify genes with potential roles in host colonization, researchers have often examined how inactivation of specific metabolic processes or genes impacts colonization. Researchers have used this later approach to determine potential roles for *Salmonella* metal acquisition in host colonization. To establish a niche within the gut

microbial community, *Salmonella* must be able to scavenge essential metal ions from the surrounding environment. S. Typhimurium uses a variety of specialized transporters to acquire essential metals in the metal-limited environment of the gut.

One of the most important metals that *Salmonella* requires is iron. Iron is limited in the gut because of sequestration of iron by host proteins like heme, ferretin, transferrin, and lactoferrin (47). All *Enterbacteriaceae,* including *Salmonella* and commensal *Eschericia coli,* secrete enterochelin, a siderophore which allows the bacteria to scavenge iron in a non-inflamed host (48). During an inflammatory response induced by*Salmonella* during infection, however, the host secretes lipocalin-2, an antimicrobial peptide that sequesters ferric enterochelin which can limit growth of bacteria that only use enterochelin for iron acquisition (49, 50). To circumvent the iron limitation during inflammation, *Salmonella* produces salmochelin, a C-glycosylated derivative of enterochelin that is too large to be bound by lipocalin-2 (51, 52). Thus, salmochelin enhances *Salmonella* colonization in the inflamed intestine (53). *E. coli* Nissle 1917 is a commensal bacterium that can establish a persistent colonization of the murine intestinal tract and has been used to treat acute enteritis (54). Interestingly, the *E. coli* Nissle 1917 genome encodes for multiple iron uptake systems including one for salmochelin (55). This probiotic bacterium reduces *S.* Typhimurium's ability to colonize the intestinal tract by competing for iron (56). When *Escherichia coli* strain Nissle 1917 is co-inoculated with *S.* Typhimurium, it is able to outcompete and reduce *S.* Typhimurium colonization in the mouse models of acute colitis and chronic persistent infection (56).

Salmonella induces inflammation through its interactions with host cells including epithelial cells and antigen-presenting cells such as macrophages and dendritic cells. Antigen-presenting cells infected by *S.* Typhimurium secrete cytokines that induce a tissue response leading to an

influx of neutrophils to the gut mucosa. Neutrophil protein calprotectin is induced during infection with *S.* Typhimurium. Calprotectin, which is the most abundant antimicrobial protein secreted by neutrophils, chelates zinc and inhibits microbial growth by limiting zinc availability (57). Although calprotectin has antimicrobial properties against many bacteria, it does not inhibit growth of *S.* Typhimurium as the bacterium is able to acquire zinc by expressing a high-affinity zinc transporter (ZnuABC). Mutants that lack ZnuABC are impaired in colonization of the intestinal tract suggesting that the zinc transport system is required for *Salmonella* to compete effectively with the resident microbiota (58).

Salmonella utilizes liberated host sugars to promote expansion

The gut microbiota is made up of primary and secondary degraders like *Bacteroides* and Firmicutes (59). The primary degraders break down polysaccharides, oligosaccharides and glycoproteins ingested by the host as well as host mucin to release sugars which they, as well as other bacteria in the gut, can utilize. During normal conditions, where the host is not challenged by a pathogen or treated with antibiotics, most of the nutrients that are released are utilized by the normal microflora, making it difficult for an invading pathogen to find the nutrients it needs to successfully colonize the host. Treatment of the host with antibiotics disrupts the normal intestinal microflora which allows pathogens such as *Salmonella* to establish a niche within the host. Sialic acid and fucose commonly occur as terminal sugars of the oligosaccharides attached to mucin. Although *Salmonella* lacks extracellular enzymes for removing sialic acid and fucose from host glycoproteins, it does possess genes encoding enzymes that enable it to utilize sialic acid (*nan* genes) and fucose (*fuc* genes) freed by primary degraders.

Ng and co-workers (60) explored the interaction that *S.* Typhimurium has with a representative member of the intestinal microflora and primary degrader, *Bacteroides thetaiotaomicron*. *B. thetaiotaomicron* is a common colonizer of the human gut which produces extracellular enzymes to degrade both dietary and mucin-derived polysaccharides. Ng *et al.* (60) observed that levels of free sialic acid were increased in mice inoculated with *B. thetaiotaomicron* 6-fold compared to germ-free mice or mice that were colonized with a sialidase-deficient mutant of *B. thetaiotaomicron*. *B. thetaiotaomicron* does not use the liberated sialic acid, but does release and utilize the sugars that are interior to the terminal sialic acid. In a mouse colitis model, *S*. Typhimurium *nan* and *fuc* genes were shown to be upregulated in a *B. thetaiotaomicron*-dependent manner (60). Removing the pathways for fucose and sialic acid utilization reduced the competitiveness of *S.* Typhimurium in mice in the presence of *B. thetaiotaomicron* showing that these systems are important for *Salmonella* to grow during infection.

To determine the role of sialic acid in mice containing a complex microflora, Ng *et al*. treated conventional mice with antibiotics. Before antibiotic treatment, the levels of free sialic acid were low suggesting that the sialic acid that is present is quickly used up by the resident bacteria. After antibiotic treatment, however, there was a spike in the amount of free sialic acid, presumably due to the disruption of the microbiota (60). It should be noted that high doses of streptomycin treatment results in a mild inflammatory response in mice (61), and *Salmonella* presumably takes advantage of both the disruption of the microflora as well as the host inflammatory response.

Host inflammation can promote growth of *Salmonella*

Studies of *S.* Typhimurium in the streptomycin-treated mouse colitis model have shown that certain virulence factors induce inflammation resulting in the production of compounds that can serve as terminal electron receptors to support anaerobic respiration in *Salmonella* during infection (61). In the 1980s, a multidrug-resistent *S.* Typhimurium strain that contained a phage encoding type III effector SopE caused an epidemic among cattle and humans (62, 63). SopE induces intestinal inflammation in the host (64). *S.* Typhimurium strains that possess the phage-encoded SopE stimulate the host to produce nitric oxide synthetase (iNOS) in the intestine. Nitric oxide generated under these conditions can be converted to nitrate which is a desirable electron acceptor for *Salmonella* and results in an outgrowth of *Salmonella* in the intestinal lumen (65). In addition to stimulating nitric oxide production in the host, SopE also suppresses the expression of *S.* Typhimurium genes involved in the utilization of less favorable electron acceptors such as tetrathionate (65).

Tetrathionate utilization is common in *Salmonella* and is used to differentiate it from other *Enterobacteriaceae*. The *ttrSR ttrBCA* gene cluster in *Salmonella* is required for utilization of tetrathionate as a terminal electron acceptor. Colonic bacteria produce high amounts of hydrogen sulfide (H_2S), which is extremely toxic. To circumvent this toxicity, the cecal mucosa converts hydrogen sulfide to thiosulfate ($S_2O_3^{2-}$) (66). Thiosulfate cannot be used as an electron receptor by the *ttrSR ttrBCA* gene cluster (67). However, thiosulfate can be oxidized during intestinal inflammation to tetrathionate ($S_4O_6^{2-}$) (68), which can be used as a respiratory electron acceptor for *Salmonella*. Under the anaerobic conditions of the intestinal lumen, bacteria compete for high-energy nutrients for fermentation. Using tetrathionate as an electron acceptor for anaerobic respiration allows *Salmonella* to use products that can only be respired, which

provides the bacterium with a growth advantage. Using the mouse colitis model, Winter *et al.* showed that a *ttrA* mutant, which is defective in tetrathionate utilization, is attenuated for colonization of the mouse intestinal tract (68). By analyzing the microbiota composition, it was shown that the wild-type strain, but not the *ttrA* mutant, was able to out-compete the resident microbiota in the cecum (68). Ethanolamine is a major non-fermentable carbon source found in the intestinal tract. *S.* Typhimurium's ability to respire ethanolamine in the presence of tetrathionate gives it an advantage to successfully colonize the inflamed gut (69).

Although inflammation from the host allows *Salmonella* to thrive, too much inflammation can result in tissue damage which can trigger a severe immune response. To overcome this, *Salmonella* keeps host inflammation in check through GogB. Host ubiquitiniation systems regulate the host inflammatory response (70). GogB is an effector protein in *Salmonella* that targets the host SCF E3 type ubiquitin ligase via an interaction with Skp1 and the human F-box only 22 (FBXO22) protein. SCF complexes of E3 ubiquitin ligase catalyze the ubiquination of proteins for degradation. The E3-type SCF ligase complex regulates the NFKB (nuclear transcription factor KB) pathway (71). It has been shown that *Salmonella* lacking a functional GogB are unable to limit NFKB activation which causes an increase in the proinflammatory response in mice. This proinflammatory response is accompanied by extensive tissue damage and enhanced colonization of the gut in long-term chronic infection models (72). GogB, then, is able to regulate the amount of inflammation-enhanced colonization by limiting the amount of tissue damage during infection (72).

Metabolic genes involved in *Salmonella* plant colonization

In addition to being a problematic contaminant in meat and eggs, *Salmonella* contamination has also been an issue in produce where it has been linked to the presence of soft rot disease. Goudeau *et al.* showed that the population of *S.* Typhimurium increases 56-fold when inoculated by itself on to cilantro leaves, compared to a 2,884-fold increase in population size when co-inoculated with *Dickeya dadantii*, a pathogen that causes soft rot lesions in plants by macerating the plant tissue (73). Transcriptome analysis of *D. dadantii* and *S.* Typhimurium indicate that *S.* Typhimurium uses anaerobic metabolism while infecting lettuce and cilantro and that it catabolizes substrates that are made available by the plant tissue degradation. Twenty-nine percent of the genes that were up-regulated in *S.* Typhimurium upon plant tissue infection had previously been shown to be involved in the colonization of the chick intestine (73). Many of the genes identified in these studies were also shown to be involved in the colonization of mice, pigs, and cattle. The transcriptome data revealed that some of the most highly expressed genes in *S.* Typhimurium were genes involved in the utilization of 1,2-propanediol (*pdu* operon) and ethanolamine (*eut* operon*)* in both lettuce and cilantro (73). Utilization of 1,2-propanediol and ethanolamine are dependent on cobalamin, and expression of genes involved in cobalamin synthesis are also highly expressed in *S.* Typhimurium upon colonization of produce. A *S.* Typhimurium mutant which was unable to utilize 1,2-propanediol or ethanolamine did not colonize lettuce and cilantro as efficiently as wild type indicating that these compounds represent significant carbon and energy sources for the bacterium when it colonizes produce (73).

It has also been shown that siderophore biosynthesis is required for efficient colonization of alfalfa by *Salmonella* (74). Through screening deletion mutants, Hao *et al*. found that an *aroA* mutant was defective in alfalfa root colonization (74). AroA is part of the chorismic acid

biosynthesis pathway which is a metabolic part of aromatic amino acid and siderophore production. This growth defect was not complemented with the addition of tryptophan or phenylalanine, but growth and alfalfa colonization were restored with the addition of ferrous sulfate indicating that siderophore biosynthesis is important in the colonization of alfalfa roots. In support of this hypothesis, loss of *entB*, which is required for siderophore biosynthesis, interfered with the ability of *S*. Typhimurium to colonize alfalfa (74).

Future directions

Salmonella continues to be a major foodborne pathogen and is transmitted primarily to humans through the consumption of meat, eggs or poultry. Infection of poultry and other food-producing animals with most *Salmonella* serovars generally results in asymptomatic intestinal colonization that leads to carcass contamination and entry into the food supply. If we are to begin to make progress on reducing the prevalence of *Salmonella* in food-producing animals and thereby reduce the incidence of salmonellosis in humans, we need to develop a better understanding of the metabolic processes in *Salmonella* which promote intestinal colonization of food animals by the pathogen. Results from recent –omics driven approaches have begun to close this critical gap in our knowledge by identifying a number of metabolic genes as having possible roles in *Salmonella* colonization of the chick, pig and calf. While roles for these metabolic genes in colonization will need to be verified, it seems likely that a significant number of these genes will prove to be important for efficient intestinal colonization of food-producing animals. Studies on how specific metabolic processes in *Salmonella* impact intestinal colonization have already yielded exciting discoveries, such as how *Salmonella* exploits the host's inflammation response to gain access to electron acceptors (e.g., nitrate and tetrathionate) which it can use for anaerobic respiration (65, 68). We anticipate that future studies to

characterize metabolic genes and their roles in colonization will similarly lead to exciting new discoveries regarding *Salmonella* physiology within the intestinal tract.

Table 2.1. *S*. Typhimurium metabolic genes identified from STM studies as having possible roles in intestinal colonization of the chick, calf or pig.

Classification	Gene name	Known or predicted function	[a]chick	[b]calf	[c]pig
Carbon compound degradation / central metabolism	*kduD*	2-deoxy-D-gluconate 3-dehydrogenase	- (50)	wt	wt
	STM3600		- (50)	wt	wt
	STM3793	putative sugar kinase	wt	-	wt
	rbsK	putative carbohydrate kinase	- (33)	wt	wt
	citC	ribokinase	- (50)	-	-
	celF	citrate lyase synthetase	n.d.	n.d.	-
	kduI	cellobiose-6-phosphate hydrolase	n.d.	n.d.	-
	pflA	5-keto-4-deoxyuronate isomerase pyruvate formate lyase activating enzyme I	n.d.	n.d	-
Sulfur metabolism	STM0036	putative arylsulfatase regulator	-	wt	-
	STM0084	putative sulfatase	(100)	wt	wt
			- (100)		
Amino acid degradation	STM2196	putative L-serine dehydratase	- (100)	wt	wt
Transport/binding	*caiT*	carnitine transport	-	wt	wt
	STM0328	putative permease	(100)	-	-
	sfbB	ABC transporter	-	wt	wt
	fepA	ferric enterobactin receptor	(100)	-	-
	entF	enterobactin synthetase	-	-	wt
	STM0765	putative cation transporter	(100)	-	-
	potH	putrescine transporter	- (50)	-	wt
	focA	probable formate transporter	-	wt	wt
	hpaX	4-hydroxyphenylacetate permease	(100)	-	wt
	chaA	sodium-calcium/proton antiporter	-	wt	wt
	hisM	histidine transport system permease	(100)	-	wt
	gabP	gamma-aminobutyrate permease	wt	wt	wt
	yneA	ABC transporter	-	-	wt
	cadB	cadaverine-lysine antiporter	(100)	n.d.	-
	STM2574	putative permease	- (100)	n.d.	-
			- (50)		
			- (50)		

			- (50) - (50) n.d. n.d.		

[a]Data from Morgan *et al*. (14). The '-' denotes mutant showing attenuated colonization; 'wt' indicates wild-type colonization phenotype for the mutant; and 'n.d.' indicates the colonization phenotype for the mutant was not determined. Number in parentheses indicates the percent of birds in which the mutant was attenuated.

[b]Data from Morgan *et al*. (14).

[c]Data from Carnell *et al*. (8).

Table 2.2. Examples of *S*. Typhimurium metabolic genes that appear to have roles in intestinal colonization of the chick, pig or calf.

			[d]Number of attenuated mutants versus total number of mutants		
Classification	**Gene name**	**Known or predicted function**	**chick**	**pig**	**calf**
Carbohydrate metabolism	[a]*mtlD*	mannitol-1-phosphate dehydrogenase	5/5	5/5	4/5
	[a]*tktA*	transketolase	5/5	5/5	5/5
	[a]*pgi*	glucose-6-phosphate isomerase	2/2	1/2	0/2
	[a]*pfkA*	6-phosphofructokinase	2/2	2/2	0/2
	[a,c]*gfrE*	glucoselysine-6-phosphate deglycase	2/3	2/3	2/2
	[a,b]*aceF*	E2 component of pyruvate dehydrogenase	2/2	2/2	2/2
	[a]SL1344_3746	hypothetical fructose-1,6-bisphosphate aldolase	2/2	2/2	2/2
	[a]*rpe*	ribulose-phosphate-3-epimerase	3/3	3/3	3/3
	[a]*pflB*	formate acetyltransferase 1	5/6	2/6	2/6
	[a]SL1344_3565	hypothetical carbohydrate kinase	3/4	3/3	4/4
	[b]*nagA*	N-acetylglucosamine-6-phosphate deacetylase	2/2	2/2	2/2
	[b,c]SL1344_3106	putative xylanase/chitin deacetylase	3/6	4/5	4/4
	[b]*eda*	KHG/KDPG aldolase	1/1	1/1	0/0
	[b]SL1344_3059	putative mannitol dehydrogenase	1/3	1/3	1/3
	[b]*gfrF*	fructoselysine-6-phosphate deglycase	5/6	5/6	5/5
	[c]*idnD*	L-idonate 5-dehydrogenase	3/4	3/4	4/4
	[c]SL1344_2738	hypothetical hexulose 6-phosphate synthase	3/3	3/3	3/3
	[c]*iolG2*	myo-inositol 2-dehydrogenase	1/2	1/1	2/2
	[c]SL1344_2242	putative dehydratase	4/4	3/3	4/4
		putative zinc-binding			

	[c]SL1344_1471	dehydrogenase	2/2	2/2	2/2
	[c]*iolG1*	myo-inositol 1-dehydrogenase	1/8	2/6	4/6
Nitrogen metabolism	[a,b]SL1344_3563	L-asparaginase	3/7	5/6	3/7
	[a,b]*tdcB*	catabolic threonine dehydratase	6/6	6/6	4/4
	[b]*tdcD*	proprionate kinase	1/3	2/3	3/3
Sulfur metabolism	[a]*trmE*	thiophene/furan oxidation	3/3	3/3	3/3
	[a]SL1344_0084	hypothetical sulfatase	6/7	7/7	7/7
	[a]SL1344_3959	possible sulfatase	9/13	11/12	11/12
	[a]*yejM*	hypothetical sulfatase	2/2	2/2	2/2
	[a]*yheM*	putative oxidation of intracellular sulfur	2/2	2/2	2/2
	[a]SL1344_0033	possible sulfatase	2/2	1/1	2/2
	[b]*assT*	probable arylsulfate sulfotransferase	1/4	2/2	3/3
	[c]SL1344_4047	hypothetical arylsulfate sulfotransferase	5/9	7/9	7/8
	[c]*asrA*	anaerobic sulfite reductase subunit A	2/3	2/3	2/3
Transport/ binding	[a]*pstC*	phosphate transport	3/3	3/3	2/3
	[a]*pstB*	phosphate transport	3/3	3/3	2/2
	[a,c]*yjeM*	putative amino acid transporter	4/8	3/7	6/8
	[a,c]*ycaM*	putative amino acid transporter	4/5	4/4	5/5
	[a]SL1344_3748	IIC component of sugar-PTS permease	5/7	6/6	6/6
	[a,c]SL1344_3799	hypothetical transporter	8/12	10/12	9/11
	[a]*znuB*	high-affinity zinc transporter, membrane protein	1/1	1/1	1/1
	[a,c]*mgtC*	magnesium transporter, ATPase protein C	1/2	2/2	2/2

[a]*focA*	probable formate transporter	3/3	2/3	2/3
[a]*ydeZ*	putative sugar transport protein	3/6	5/6	6/6
[b]*gfrC*	IIC component of fructose-/glucoselysine PTS permease	1/1	1/1	1/1
[b]*yrbF*	putative ABC transporter, ATP-binding protein	1/1	1/1	1/1
[b,c]*ptsI*	phosphotransferase system (PTS), enzyme I	3/3	3/3	3/3
[b]SL1344_0860	possible transport protein	1/2	1/2	2/2
[b]*pstA*	phosphate transport system permease protein	3/3	2/3	2/3
[b]*iroN*	TonB-dependent siderophore receptor protein	4/7	5/7	3/6
[b]*ybbL*	putative ABC transporter, ATP-binding protein	1/1	1/1	1/1
[b]*fepG*	ferric enterobactin transport protein	1/1	1/1	1/1
[b]*fepC*	ferric enterobactin transport ATP-binding protein	1/1	1/1	1/1
[b]SL1344_3750	EIIA component of PTS permease	4/4	4/4	3/3
[b]SL1344_2536	putative permease	1/1	1/1	1/1
[b]*mglB*	D-galactose-binding periplasmic protein precursor	1/4	2/3	1/2
[c]SL1344_4000	oligogalacturonate-specific porin (KdgM) family member	1/2	1/2	2/2
[c]*yiaM*	TRAP-type transporter, small permease protein	1/1	1/1	1/1
[c]*yhjV*	hypothetical amino acid permease	5/9	7/7	9/9
[c]SL1344_4001	TRAP-type transporter, large permease component	2/4	1/2	2/4
[c]SL1344_0940	hypothetical ion:amino acid symporter	2/2	2/2	2/2
[c]SL1344_3100	possible amino acid transport protein	2/6	2/6	5/6
[c]*ybbY*	putative purine permease	3/4	4/4	4/4
[c]SL1344_3000	magnesium transporter, ATPase protein C	6/12	5/9	9/12
[c]*proV*	glycine betaine/l-proline transport ATP-binding protein	1/5	5/5	5/5
	probable permease			

	[c]SL1344_3758		1/3	1/3	2/3
Regulatory protein for catabolic pathway(s) or nutrient acquisition	[a,b,c]*fruR*	fructose repressor	4/4	4/4	4/4
	[b]*crp*	cyclic AMP receptor protein	2/2	2/2	1/1
	[b]*cyaA*	adenylate cyclase	2/2	2/2	2/2
	[b]*phoU*	phosphate transport system regulatory protein	2/2	2/2	1/1
	[b]*citC*	activates citrate lyase by acetylation	0/4	1/2	4/4
Energy generation	[a]*hybD*	hydrogenase-2 component	2/2	0/2	0/1
	[b]*nuoC*	NADH dehydrogenase I, chain C	5/7	5/5	5/5
	[b]*nuoA*	NADH dehydrogenase I, chain A	2/2	1/1	2/2
	[b]*atpD*	ATP synthase beta subunit	3/3	1/3	3/3
	[c]*cydA*	cytochrome d ubiquinol oxidase subunit I	1/1	1/1	1/1
	[c]*atpA*	ATP synthase alpha subunit	5/5	3/3	4/4
	[c]*nuoJ*	NADH dehydrogenase I, chain J	0/2	2/2	2/2
	[c]*hypO*	hydrogenase-2 small chain protein	4/5	1/5	2/5

[a]Transposon insertions in *S.* Typhimurium SL1344 metabolic genes that displayed the most statistically significant colonization defect (i.e., lowest E value) in the chick colonization model. The top 30 genes are indicated.

[b] Transposon insertions in *S.* Typhimurium SL1344 metabolic genes that displayed the most statistically significant colonization defect (i.e., lowest E value) in the pig colonization model. The top 30 genes are indicated.

[c] Transposon insertions in *S*. Typhimurium SL1344 metabolic genes that displayed the most statistically significant colonization defect (i.e., lowest E value) in the calf colonization model. The top 30 genes are indicated.

[d]Number of different insertion mutations in gene which displayed a statistically significant colonization defect (E<0.05) versus total number of insertion mutations for gene. Data presented in table are from Chaudhuri *et al*. (37).

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CHAPTER 3: DISRUPTION OF THREE MANNOSE-FAMILY PTS PERMEASES AFFECTS COLONIZATION OF THE CHICKEN GASTROINTESTINAL TRACT

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Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a source of contamination in meat, poultry, and other food animal products, and is a leading cause of food-borne illness in humans (1). An aspect of *S.* Typhimurium persistence that has been largely overlooked is the impact of metabolic genes in virulence or colonization, especially in poultry and other food animals. *S.* Typhimurium possesses three mannose family phosphotransferase (PTS) permeases that are generally absent in most strains of closely related bacteria such as *Escherichia coli*. Expression of the genes encoding these mannose family PTS permeases was predicted from bioinformatics to be under control of the alternative sigma factor RpoN (σ^{54}) (2). This prediction was confirmed in a study of the *S.* Typhimurium RpoN regulon which employed DNA microarrays in combination with a promiscuous RpoN-dependent activator (3). Transcription initiation by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) requires an activator which stimulates the isomerization of a closed complex between σ^{54}-holoenzyme and the promoter to an open complex (4, 5). Genes encoding RpoN-dependent activators are often closely associated with the genes they regulate, and adjacent to each of the PTS permease operons is a gene encoding an RpoN-dependent activator (Fig. 3.1).

During the course of my studies, I identified substrates for two of the PTS permeases. One of these mannose family PTS permeases and associated enzymes are required for the transport and catabolism of D-glucosaminate (Chapter 4;(6)). A second of these mannose family PTS permeases and associated enzymes are required for the transport and catabolism of fructoselysine and glucoselysine (Chapter 5). We have reason to believe that the third mannose family PTS permease and associated enzymes have a role in the transport and catabolism of fructosemethionine and other α-glycated amino acids (Chapter 6).

PTS permeases catalyze the phosphorylation and translocation of carbohydrate substrates across the cell membrane (Fig. 3.2). A phosphorylation cascade occurs with phosphate originating from phosphoenolpyruvate (PEP) that is transferred sequentially to EI, HPr, EIIA, EIIB, and finally to the sugar as it is transported across the cell membrane. All PTS permeases consist of at least 3 domains (EIIA, EIIB, EIIC), while mannose family PTS permeases have a fourth domain (EIID). The EII domains are arranged in 1 to 4 polypeptides (7).

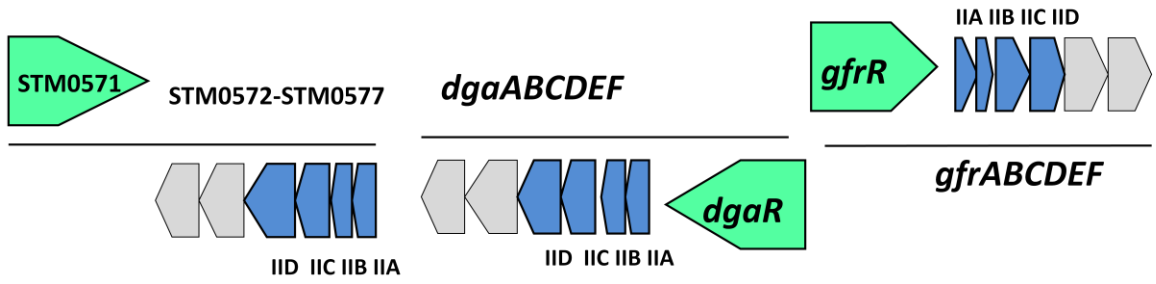

Figure 3.1. Organization of the RpoN-dependent PTS permease operons in *S.* Typhimurium. Genes encoding PTS permease components (EIIA, EIIB, EIIC, and EIID) are shown in blue. Genes encoding activators for the permease operons are displayed in green. The remaining genes are shown in grey. In the *dga* operon, *dgaE* encodes a pyridoxal phosphate (PLP)-dependent dehydratase; while *dgaF* encodes a 2-keto-3-deoxy-D-gluconate-6-P (KDPG) aldolase. In the *gfr* operon, *gfrE* encodes a glucoselysine-6-phosphate deglycase; while *gfrF* encodes a fructoselysine-6-phosphate deglycase. Locus tags displayed are from *S.* Typhimurium LT2, but gene organization and sequence for all three permeases is conserved in all sequenced strains of *S.* Typhimurium. Gene spacing in figure is based on IMG Neighborhood viewer (http://img.jgi.doe.gov).

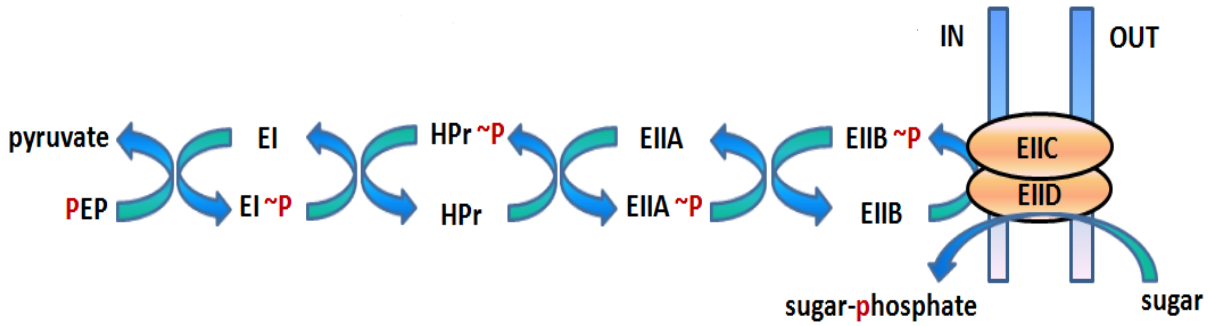

Figure 3.2. The bacterial sugar phosphotransferase system (PTS).

We wished to address two very important questions regarding the importance of these mannose family PTS permeases in the colonization of chickens. The first question was, are these PTS permeases important in a nutrient rich environment? This question was addressed by comparing colonization of the jejunum and ileum by wild-type and the mutant strains of *S.* Typhimurium. The second question was, are these PTS permeases important in a nutrient poor environment? This question was addressed by comparing colonization of the ceca by the wild-type and mutant strains of *S.* Typhimurium.

The chicken ileum is rich with nutrients as it is the site where most of digestion occurs. The ileum is also the site where feed utilization is most efficient (http://www.extension.org/pages/65376/avian-digestive-system#.VRF-LvnF_dc). The ileum of chicks fed a vegetarian corn-soy broiler diet devoid of feed additives consists of approximately 70% *Lactobacillus,* 11% *Clostridiaceae,* 6.5% *Streptococcus,* and 6.5% *Enterococcus* (8). During the first 72 hours after hatching, the chick acquires most of its nutrients from the yolk rather than the feed (http://www.mypetchicken.com/catalog/helper/dayoldchicks.aspx).

The ceca are a blind-end paired cecum. Bacteria persist here longer than they do in the ileum. Differences in the nutrient content and microbiota composition can occur between each cecum. Birds are able to extract some nutrients from the ceca, especially fatty acids and B

vitamins, but most of the nutrients in the ceca cannot be utilized by the host. The cecal contents are rich in complex carbohydrates (http://www.extension.org/pages/65376/avian-digestive-system#.VRF-LvnF_dc). The ceca of chicks fed a vegetarian corn-soy broiler diet devoid of feed additives consists of approximately 65% *Clostidiaceae*, 14% *Fusobacterium*, 8% *Lactobacillus*, and 5% *Bacteroides* (8).

At the start of my studies, my central hypothesis was that the substrates for these three mannose family PTS permeases were carbohydrates encountered by *S.* Typhimurium in the chick intestinal tract and that the ability of *S.* Typhimurium to utilize these carbohydrates is an important colonization determinant. We present evidence in this chapter to support this hypothesis.

Materials and Methods

Bacterial strains, growth conditions and reagents. The *S.* Typhimurium strains and plasmids used in this study are listed in Table 3.1 and Table 3.2. Strains were maintained in Luria-Bertani (LB) broth or agar supplemented as needed with 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin.

Construction of plasmids and mutant strains. *S.* Typhimurium mutants indicated in Table 3.1 were constructed using the λ Red Recombineering method as described (9). Plasmid pKD4, which contains a kanamycin resistance (*kan)* cassette flanked by two Flp recognition targets (FRT sites), was used as a PCR template to create amplicons which contained sequences at their ends that were homologous to the genes targeted for deletion. Primer sets used to generate the amplicons for the targeted mutagenesis are listed in Table 3.S1. Amplified DNA was introduced by electroporation into *S.* Typhimurium 14028s bearing plasmid pKD46 which carries the λ phage recombinase genes under control of the *araBAD* promoter. Genomic DNA isolated from

resulting kanamycin-resistant colonies was checked by PCR to confirm that the target gene had been knocked out using PCR primers that flanked the target deletion gene (primers are listed in Table 3.S1). Mutant alleles in which the target genes had been replaced with the *kan* cassette were moved into a naive genetic background in either *S.* Typhimurium 14028s or SL1344 by P22 transduction. The *kan* cassette was then removed by introducing plasmid pCP20 into the mutant strains. Plasmid pCP20 expresses the Flp recombinase which recognizes the FRT sites and excises the *kan* cassette. Loss of the *kan* cassette was confirmed by susceptibility to kanamycin and PCR using the same flanking primers listed in Table 3.S1.

For all transformations involving *S.* Typhimurium 14028s, DNA was introduced into the bacterium by electroporation using the parameters 2.4 kV, 25 μF and 400Ω. Following electroporation, cells were allowed to recover in SOC broth (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) for 1 h at 37^{o}C.

Chicken Colonization. Two-day old SPF White Leghorn broiler chicks were orally administered ~2 x 10^7 colony forming units (cfu) of wild-type *S.* Typhimurium SL1344 or a mutant strain in which the genes encoding all three of the RpoN-dependent mannose family PTS permeases (permease mutant) or all three of the putative RpoN-dependent activators of the PTS operons (activator mutant) had been deleted. Each treatment group contained 48 chicks. For each sampling, 6-9 chicks were sacrificed, the small intestine and cecal contents were removed, serially diluted in PBS and plated on XLT4 agar (Difco).

Table 3.1. Bacterial strains used for this study.

species	strain	relevant genotype	source
S. Typhimurium	SL1344	wild type	ATCC
S. Typhimurium	KAM58	ΔSTM0571	this study
S. Typhimurium	KAM57	ΔSTM0576-STM0577	this study
S. Typhimurium	KAM60	Δ*dgaR*	this study
S. Typhimurium	KAM55	Δ*dgaAB*	this study
S. Typhimurium	KAM61	Δ*gfrR*	this study
S. Typhimurium	KAM41	Δ*gfrAB*	this study
S. Typhimurium	KAM99	ΔSTM0571, Δ*dgaR*, Δ*gfrR*	this study
S. Typhimurium	KAM98	ΔSTM0576-STM0577, Δ*dgaAB*, Δ*gfrAB*	this study

Table 3.2. Plasmids used in this study.

plasmid	relevant characteristics	Ref.
pKD46	expresses phage λ recombinase genes γ, β and *exo* from arabinose-inducible P_{araB} promoter; temperature-sensitive replicon	(9)
pKD4	template plasmid used to generate amplicons for inactivation of target genes in the λ Red system	(9)
pCP20	expresses FLP enzyme for removal of kan gene flanked by FRT sites; temperature-sensitive replicon	(10)

Table 3.S1. Primers used in this study.

Sequence	Experiment
5'-ATG AGA CGT ATT GAG ATC GTA CTG GGA GAG TGT GTA GGC TGG AGC TGC TTC-3'	*dgaR* knock out λ-Red
5'-TTA ACT ATA GAG CAG TTC GTA GAT ATA AAC ATA TGA ATA TCC TCC TTA-3'	*dgaR* knock out λ-Red
5'-CAT TTT TCG CCA GGC CTT TAA TCA GGA AGG CGA CAC CAT ATG AAT ATC CTC CTT A-3'	*dgaAB* knock out λ-Red

5’-ATG GCC AAT ATC GTT TTA TGC CGC ATC GAC AGC CGT TGT GTA GGC TGG AGC TGC TTC-3’	*dgaAB* knock out λ-Red
5’-CCT GTT GGG ATG TGT TAA GAA ACT GGC TGA TAA TGA CAT ATG AAT ATC CTC CTT A-3’	STM0576-STM0577 knock out λ-Red
5’-ATG ATC AAA TTA GTG CGC ATT GAT TAC CGC CTG CTG TGT GTA GGC TGG AGC TGC TTC-3’	STM0576-STM0577 knock out λ-Red
5’-CCG GTT GCG AGG CCG CAA ATT TCG GCA TAT TGA CCA CAT ATG AAT ATC CTC CTT A-3’	*gfrAB* knock out λ-Red
5’-ACG GGT GGA TCA TCG TTT ATT ACA TGG ACA GGT CGC TGT GTA GGC TGG AGC TGC TTC-3’	*gfrAB* knock out λ-Red
5’-TTG GCG TCT GGA TCT TCG-3’	STM0571 knock out overlapping PCR λ-Red
5’-CAG CCT ACA CAA TCG CTC AAG ATG GTG ACT AAT AAT GCT GCG-3’	STM0571 knock out overlapping PCR λ-Red
5’-CTG TGT CAC TGA AAA TTG CTT TGA GGT ATG ATT GAG CGA CTG GTC-3’	STM0571 knock out overlapping PCR λ-Red
5’-ATC AGG AGT CTA TCT GTA TTC TGC-3’	STM0571 knock out overlapping PCR λ-Red
5’-CAT CAT AAT TGC TGA TGT GAT AAT CGT GAG TGTGTA GGC TGG AGC TGC TTC-3’	*gfrR* knock out λ-Red
5’-TTA AAA CTG TTG ATC TTG CTC GAT AAG TTC CAT ATG AAT ATC CTC CTT A-3’	*gfrR* knock out λ-Red

Results

In order to address the importance of the PTS permeases in chicken colonization, two-day old broiler chicks were orally administered ~2 x 10^7 colony forming units (cfu) of wild-type *S.* Typhimurium SL1344 or a mutant strain in which the genes encoding all three mannose family PTS permeases (Δ*gfrAB*, Δ*dgaAB* and ΔSTM0577-0576; referred to as triple permease mutant) or all three of the RpoN-dependent activators of the PTS operons (Δ*gfrR*, Δ*dgaR* and ΔSTM0572; referred to as triple activator mutant) had been deleted. At this age the chicks had an established microflora which prevented morbidity and mortality by *Salmonella*, but the microflora was not fully developed as to completely inhibit *Salmonella* colonization.

Each treatment group contained 48 chicks. For each sampling, 6-9 chicks were sacrificed. To track colonization, chicks were sacrificed at different time points (days 3, 4, 5, 7, 14, and 21,

respectively). On days 3, 4, 5, and 7, nine birds from each treatment were sacrificed, and on days 14 and 21, six birds from each treatment were sacrificed. Chicks were euthanized and the jejuna, ilea, and ceca were removed to assess the number of viable CFUs of wild type and mutant *S.* Typhimurium in each location at different times following inoculation by serially diluting organ contents and then plating the dilutions on XLT4 agar.

Most strikingly, there were fewer chicks with detectable levels of the triple permease or triple activator mutants in the ileum as compared to wild type on days 3 and 4. For those that were detectable, the levels of the mutant strains were 10- to 1,000-fold lower compared to that of wild type. Using a Student's t-test, both the triple permease mutant and triple activator mutant were significantly different in load recovered on days 3 and 4 with P-values <0.05 (Fig. 3.3). Using a Wilcoxin Signed-Rank Test, which is often used to interpret scatter data, indicated that the cfu load recovered from either of the mutant strains compared to wild type were significantly different on days 3 and 4 with P-values <0.001 (Fig. 3.4). The colonization defect is especially surprising in the ileum and jejunum since this is an area that is rich in nutrients.

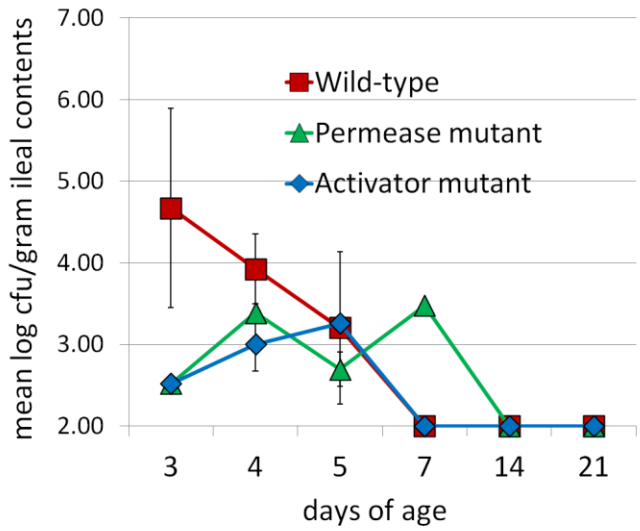

Figure 3.3. Average load *S.* Typhimurium recovered from the avian jejunum and ileum. Two-day old broiler chicks were orally administered ~2 x 107 colony forming units (cfu) of wild-type *S.* Typhimurium SL1344 or a mutant strain in which the genes encoding all three of the RpoN-dependent mannose family PTS permeases (permease mutant) or all three of the putative RpoN-dependent activators of the PTS operons (activator mutant) had been deleted. Each treatment group contained 48 chicks. For each sampling, 6-9 chicks were sacrificed, the small intestine and cecal contents were removed, serially diluted and plated on XLT4 agar. Mean cfu, after log transformation, is shown with error bars corresponding to sd. Ileum day 3 and 4 are significantly different ($p<0.05$) between wild-type and mutants.

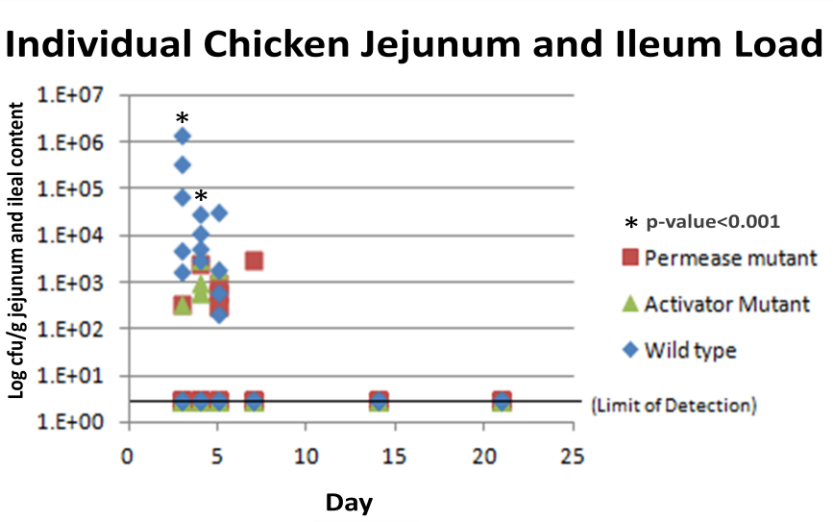

Figure 3.4. Recovery of mutant and wild-type *S*. Typhimurium from the avian jejunum and ileum. Using a Wilcoxin Signed-Rank Test, recovered samples from day 3 and day 4 in the jejunum and ileum are significantly different (P-value <0.001) between wild-type and the mutants.

On days 3, 7, and 14 the triple permease mutant and triple activator mutant loads were significantly less than that of wild type in the ceca suggesting that one or more of the permeases is important for colonization of the chick intestinal tract. Using Student's t-test, the triple activator mutant and triple permease mutant loads were significantly different from the load of wild type on days 3, 7 and 14 with P-values <0.05 (Figure 3.5). Using the Wilcoxin Signed-Rank Test to view individually colonized birds, both the triple activator mutant and the triple permease mutant colonized at a load significantly less than wild type on day 3 with a P-value <0.05 (Figure 3.6). The colonization defect in the ceca may be because there could be reasonable amounts of fructation and glycation products associated with proteins that are released by the primary degraders so that they are available to wild-type *Salmonella*. Both the triple permease mutant and the triple activator mutant persisted in the ceca longer than the wild

type. A potential explanation for this is that by deleting these PTS permeases or their activators, the carbohydrate hierarchy has changed so that the mutants are utilizing other carbohydrates and able to persist longer in the ceca.

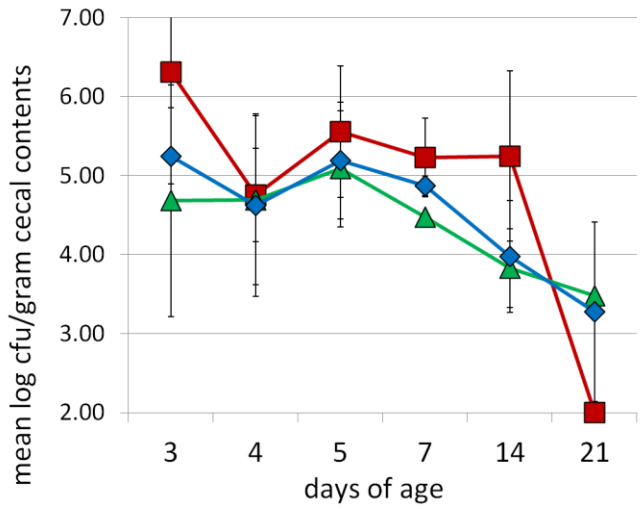

Figure 3.5. Average load *S.* Typhimurium recovered from the avian ceca. Wild-type in red, triple permease mutant in green, and triple activator mutant in blue. Mean cfu, after log transformation, is shown with error bars corresponding to one standard deviation. Ceca day 3, 7 and 14 are significantly different (P-value <0.05) between wild type strain and mutants.

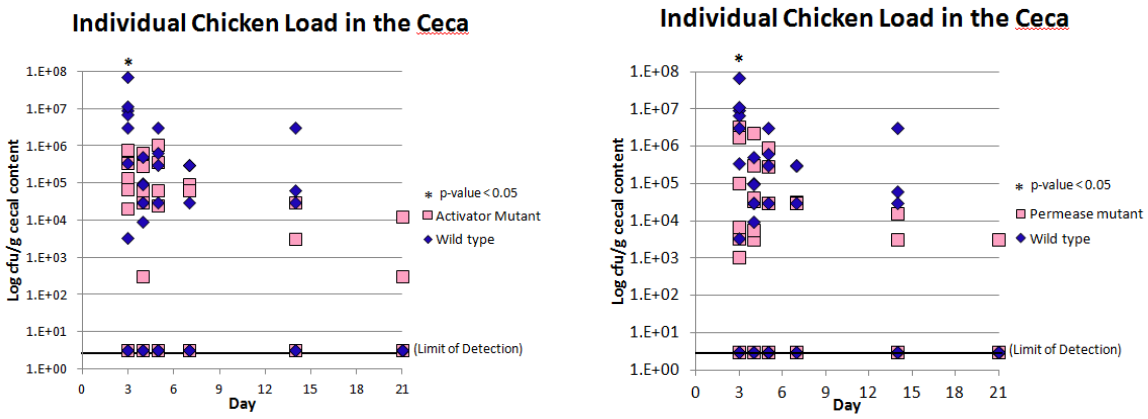

Figure 3.6. Recovery of mutant and wild-type *S*. Typhimurium from the avian small intestine and ceca. Using a Wilcoxin Signed-Rank Test, recovered samples from day 3 in the ceca are significantly different (P-value <0.05) between wild type and mutants.

Discussion

The triple permease and triple activator mutants displayed colonization defects in both the ileum/jejunum and ceca which may result from the inability of these mutants to utilize one or more carbohydrates as a nutrient source. The *dga* permease transports D-glucosaminic acid. D-glucosaminic acid can be formed by the oxidation of glucosamine by glucose oxidase (11), an enzyme that many microorganisms possess, including *E. coli* and *Salmonella*. Glucose oxidase is a periplasmic enzyme in *E. coli* and *Salmonella*, meaning that D-glucosaminate could be produced from the oxidation of glucosamine in the periplasmic space and then diffuse across the outer membrane into the surround medium where it could be used by other microorganisms. Glucose oxidase requires pyrroloquinoline quinine (PQQ) as a cofactor, but neither *E. coli* nor *Salmonella* are capable of making PQQ. It is an interesting conjecture that *Salmonella* may use glucose oxidase when it is growing in association with other microorganisms that produce PQQ to oxidize glucosamine to glucosaminate that it can use later but the neighboring microbiota

cannot. Glucosamine is a common carbohydrate and it is likely present at significant levels in the intestinal tract of the chick where bacteria there could oxidize some of it to glucosaminate. Glucosamine is a part of the structure of polysaccharides in chitosan and chitin and is found in the cell wall of fungi and higher organisms. Glucosamine is one of the most abundant monnosaccharides.

The *S*. Typhimurium *gfr* permease transports both fructoselysine and glucoselysine. Fructoselysine is formed by the spontaneous reaction of glucose with the ε-amine of lysine (either within a polypeptide or as a free amino acid)followed by a rearrangement known as an Amadori rearrangement. Glucoselysine is formed by the spontaneous reaction of fructose with the ε-amine of lysine followed by a rearrangement known as a Heyns rearrangement. Fructoselysine and glucoselysine are likely derived from modified proteins in the chick's feed, with such modification occurring during the growth and/or preparation of the feed or during digestion in the intestinal tract. Passage of fructoselysine and other fructosamines through the gut mucosa is very limited and may not be used by the host (12), and so these compounds may represent a significant nutrient source to *Salmonella* in the chick intestinal tract.

The STM0574-77 permease may transport fructosamines resulting from the reaction of glucose with the α-amino group of other amino acids. Preliminary studies suggest that one such α-glycated amino acid that is a substrate for the STM0574-0577 PTS permease is fructosemethionine (Chapter 6). We know that other fructosamines are important in host colonization by *Salmonella*. Ali and co-workers identified the *fra* locus in *S.* Typhimurium that is required for catabolism of fructose-asparagine, and showed that fructosamine utilization is essential for fitness of *Salmonella* in the inflamed mouse intestine model (13). Chaudhuri and co-workers reported results from a high throughput screen of random transposon insertion

mutants in *S.* Typhimurium that insertions within the *fra* locus resulted in colonization defects in chicken, pigs, and calves (14). Taken together, these data suggest that fructosamines are present in the gastrointestinal tract of the chicken and these compounds represent important sources of nutrients for *Salmonella*. Alternatively, one or more of the mannose family PTS permeases that were inactivated in this study may have a regulatory role that impacts the expression of other genes important for the colonization of the chick. In support of this hypothesis, *S.* Typhimurium GfrD was reported to modulate the activity of CadC (15), a positive regulator of *cadBA* which encode a lysine/cadaverine antiporter and lysine decarboxylase, respectively, and are involved in acid stress response in *S.* Typhimurium.

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CHAPTER 4: *SALMONELLA* UTILIZES D-GLUCOSAMINATE VIA A MANNOSE FAMILY PHOSPHOTRANSFERASE SYSTEM PERMEASE AND ASSOCIATED ENZYMES

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All of the genetic, molecular, and biochemical work was performed by Katherine Miller. All of the bioinformatic analysis was performed by Jan Mrázek.

Abstract

Salmonella enterica, a globally significant bacterial foodborne pathogen, utilizes a variety of carbon sources. We report here that *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) uses D-glucosaminate (2-amino-2-deoxy-D-gluconic acid) as a carbon and nitrogen source via a previously uncharacterized mannose family phosphotransferase system (PTS) permease, and designate the genes encoding the permease as *dgaABCD* (D-glucosaminate PTS permease components EIIA, EIIB, EIIC and EIID). Two other genes in the *dga* operon were required for wild-type growth of *S.* Typhimurium with D-glucosaminate, and we designate these genes as *dgaE* and *dgaF*. Transcription of *dgaABCDEF* was dependent on RpoN (σ^{54}) and an RpoN-dependent activator which we designate as *dgaR*. Introduction of a plasmid bearing *dgaABCDEF* under control of the *lac* promoter into *Escherichia coli* strains DH5α, BL21 and JM101 allowed these strains to grow on minimal medium containing D-glucosaminate as the sole carbon and nitrogen sources. Biochemical and genetic data support a catabolic pathway in which D-glucosaminate is phosphorylated at the C-6 position as it is transported across the cell membrane by DgaABCD. DgaE, a predicted pyridoxal phosphate-dependent enzyme, subsequently converts the resulting D-glucosaminate-6-phosphate to 2-keto-3-deoxygluconate 6-phosphate, which is then cleaved by the aldolase DgaF to form glyceraldehyde-3-phosphate and pyruvate. Examination of the Integrated Microbial Genomes database revealed that orthologs of the *dga* genes are largely restricted to certain enteric bacteria and a few species in the phylum Firmicutes.

Introduction

Salmonella is an enteropathogen that depending on the serovar causes gastroenteritis and/or fatal systemic disease in a variety of mammals, including humans, cattle and swine. Over 2,500 serovars of *Salmonella* have been identified to date (1). *Salmonella* serovars vary markedly with regard to their host specificity and the diseases they cause. While a considerable amount of work has focused on elucidating the mechanisms by which *Salmonella* pathogenicity genes promote infection (2-6), until recently little attention has been paid to understanding the nutritional and metabolic requirements of *Salmonella* during colonization (7, 8). Several recent papers have used -omics driven (i.e., genomics, transcriptomics, proteomics, metabolomics) systems biology approaches to investigate potential contributions of metabolic processes to *Salmonella* colonization and/or virulence (9-13). The utility of such approaches is hindered by gaps in our knowledge of metabolic pathways in *Salmonella* and dubious or uninformative gene annotations.

Salmonella is catabolically robust. Gutnick and co-workers tested roughly 600 compounds and found that approximately 90 of these compounds serve as carbon and/or nitrogen sources for *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) LT2 (14). Using a high-throughput phenotype microarray system from Biolog (Hayward, CA), AbuOun and co-workers (9) reported that the two *S.* Typhimurium strains (LT2 and DT104) examined were able to utilize to varying degrees 91 of the 195 carbon sources in the phenotype microarray plates and 34 of the 95 nitrogen sources, plus an additional 6 carbon sources and 6 nitrogen sources were utilized by one or the other of the two strains. The study by AbuOun and co-workers (9) identified three carbon sources (D-glucosaminate, D-psicose and D-tartarate) utilized by the *Salmonella* strains but not *Escherichia coli* MG1655 for which there was no information regarding their catabolism. Such results highlight the need for the continued investigation of metabolic pathways in *Salmonella*.

Phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) are responsible for the transport of many of the carbohydrates utilized by *Salmonella*. *S.* Typhimurium strain LT2 possesses over 20 different sugar PTS permeases, but not all of these PTS permeases are present in other *S.* Typhimurium strains. For example, *S.* Typhimurium 14028s (the strain used in the present study) lacks three of the PTS permeases found in *S.* Typhimurium LT2 – a galactitol-specific PTS, a D-tagatose-specific PTS, and a PTS of unknown function (15, 16). All *S.* Typhimurium strains whose genomes have been sequenced to date, however, possess a mannose PTS permease (ManXYZ) and three additional mannose family PTS permeases of unknown function. PTS-mediated sugar transport involves a cascade of phosphor-transfer reactions that culminates in the phosphorylation of the sugar as it is transported across the cell membrane (Fig. 4.1A). Enzyme I (EI) and HPr are cytoplasmic proteins that serve as common elements for transport of most PTS sugars, while Enzyme II (EII) complexes or PTS permeases are associated with the cell membrane and are specific for a particular sugar or group of structurally similar sugars (17). PTS permeases are organized into several families based on shared structural features. All PTS permeases contain three domains designated as EIIA, EIIB and EIIC, while mannose family PTS permeases contain a fourth domain designate as EIID (18, 19). These domains are arranged as either a single polypeptide or multiple polypeptides (20). In *Escherichia coli,* ManXYZ transports D-mannose and related hexoses with different substituents at the C-2 position (21).

Genes encoding the three uncharacterized mannose family PTS permeases in *S.* Typhimurium were predicted from bioinformatic analysis to be under control of the alternative sigma factor RpoN (σ^{54}) (22). Initiation of transcription by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) requires an activator which stimulates the isomerization of a closed complex

between σ^{54}-holoenzyme and the promoter to an open complex (23, 24). Genes encoding RpoN-dependent activators are often closely associated with the genes they regulate, and adjacent to each of the PTS permease operons is a gene encoding an RpoN-dependent activator.

We report here that D-glucosaminate (2-amino-2-deoxy-D-gluconic acid) is a substrate for the mannose family PTS permease encoded by the genes with locus numbers STM14_4548-STM14_4545 (shown in Fig. 4.1B) in *S.* Typhimurium 14028s, and designate these genes as *dgaABCD* (D-glucosaminate PTS permease components EIIA, EIIB, EIIC and EIID). Deleting the gene predicted to encode an RpoN-dependent activator located upstream of the *dgaABCDEF* operon (STM14_4550) abolished the ability of *S.* Typhimurium 14028s to utilize D-glucosaminate as a sole carbon source, and we designate this gene as *dgaR.* Two additional genes in the *dgaABCD* operon were found to be involved in the catabolism of D-glucosaminate and we designate these genes as *dgaE* and *dgaF*. Genetic and biochemical data were consistent with the hypothesis that D-glucosaminate is catabolized to pyruvate plus glyceraldehyde-3-phosphate by the products of the *dgaABCDEF* operon.

Materials and Methods

Bacterial strains, growth conditions and reagents. The *S.* Typhimurium strains and plasmids used in this study are listed in Tables 4.S1 and 4.S2, respectively. *E. coli* DH5α was used for routine cloning procedures. Strains were maintained in Luria-Bertani (LB) broth or agar supplemented as needed with 100 μg ml^{-1} ampicillin or 50 μg ml^{-1} kanamycin. Growth of *S.* Typhimurium on various carbon or nitrogen sources was tested using a version of a minimal medium developed by Neidhardt and co-workers (25) and modified by Maloy, Stewart and Taylor (26). The minimal medium was buffered with 40 mM 3-(N-morpholino)propanesulfonic

acid and 4 mM Tricine with the pH adjusted to 7.4 with NaOH. Additional components of the basic minimal medium were 20 mM $NaHCO_3$, 0.88 mM K_2HPO_4, 50 mM NaCl, 10 μM $FeSO_4 \cdot 7H_2O$, 0.55 mM $MgSO_4 \cdot 7H_2O$, 0.4 μM H_3BO_3, 0.5 μM $CaCl_2 \cdot 2H_2O$, 0.03 μM $CoCl_2 \cdot 6H_2O$, 0.01 μM $CuSO_4 \cdot 5H_2O$, 0.08 μM $MnCl_2 \cdot 4H_2O$ and 0.01 μM $ZnSO_4 \cdot 7H_2O$. Various carbon and nitrogen sources were added as indicated, generally to final concentrations of 20 mM and 13 or 5 mM, respectively.

Determination of growth rates and K_s. *S.* Typhimurium strains were grown aerobically at 37°C in minimal media and growth of cell cultures was monitored using a Klett colorimeter (model 900-3) using a green (520-580 nm) glass filter. Generation (g) times were calculated from the equation g = ln(2)/b, where b is the best fit constant from a plot of the log values of Klett units versus incubation time. The Michaelis or substrate concentration at half-maximal growth rate (K_μ) for D-glucosaminate was estimated by growing cells in minimal medium containing 20 mM L-arabinose as the primary carbon source and various concentrations of D-glucosaminate (ranging from 0 to 20 mM) as the sole nitrogen source. Cells were cultured in sterile 96-well plates (0.2 mL medium per well) at 37°C in a BioTek ELx808 Absorbance Microplate Reader (BioTek, Winooski, VT). Each culture condition was done with three biological replicates. Absorbance was measured every 30 min for 24 h. Generation times for each condition were calculated as described above after plotting log values of absorbance versus incubation time. The K_s value for D-glucosaminate was estimated from a double reciprocal plot (Lineweaver-Burk plot) of g values versus D-glucosaminate concentration using the SigmaPlot 12.5 software package.

A modification of the auxanographic technique described by Gutnick and co-workers (14) was used to test the ability of compounds to support the growth of *S.* Typhimurium. *S.* Typhimurium strains were grown overnight in nutrient broth, diluted 20-fold in minimal salts-soft agar containing 1.0 g K_2SO_4, 13.5 g K_2HPO_4, 4.7 g KH_2PO_4, 0.1 g $MgSO_4 \cdot 7H_2O$ and 6.0 g agar per liter, and the mixture was poured into sterile Petri dishes and allowed to solidify. For testing potential carbon sources, ammonium chloride was included in the soft agar at a final concentration of 13 mM. For testing potential nitrogen sources, L-arabinose was included in the soft agar at a final concentration of 20 mM. Compounds tested to support growth of *S.* Typhimurium were sterilized by irradiation with short wave (254 nm) UV light for 2 min using a hand-held UV lamp. Approximately 2 mg of each compound was placed on the surface of the solidified agar and bacterial growth in the agar was recorded after incubating the plates for 2 days at 37^{o}C.

Chemical syntheses. D-glucosaminic acid was purchased from Toronto Research Chemicals or synthesized by the oxidation of D-glucosamine hydrochloride (Sigma-Aldrich) with sodium chlorite (27). D-glucosamine hydrochloride (0.108 g, 0.5 mM) was dissolved in 4 ml water, after which 300 µl of 0.5 M NaH_2PO_4, 0.054 g $NaClO_2$ and 64 µl 30% H_2O_2 were added to the solution. The solution was stirred at room temperature overnight. The solution was decolorized by the addition of a small amount of $Na_2S_2O_5$ and the pH of the solution was adjusted to 7 by the drop-wise addition of 1 M NaOH (final volume of 1 M NaOH was ~1 ml). The solution was applied to a Dowex 50-X8 column (H^+) (10 ml bed volume), and the column was washed with water until the pH of the washings were neutral. The amino acid was then eluted with 1 M NH_3, and the total eluate (~30 ml) was evaporated *in vacuo* which resulted in a white solid. The residue was dissolved in a minimum volume of hot water. The resulting solution was diluted

with ethanol, cooled to room temperature and then allowed to stand at 4ºC overnight. The precipitate was filtered, washed with ethanol and dried. The final product was fluffy white crystals with a final yield of 0.036 g (37%). The ^{1}H NMR spectrum of the final material in D_2O was identical to a reference spectrum.

D-galactosaminic acid was synthesized from D-galactosamine hydrochloride (Sigma-Aldrich; 0.108 g, 0.5 mM) following the same procedure as described above for the synthesis of D-glucosaminic acid from D-glucosamine hydrochloride. The final yield of D-galactosaminic acid was 0.0457 g (48.7%). L-mannosaminic acid was synthesized from D-mannosamine hydrochloride (Sigma-Aldrich; 0.108 g, 0.5 mM) in the same manner except the product following evaporation of the aqueous NH_3 was suspended in 95% ethanol, filtered and dried *in vacuo* to give 0.039 g (41.6% yield) of a pale yellow solid.

D-glucosaminic acid 6-phosphate was synthesized from D-glucosamine-6-phosphate (50 mg, 0.193 mmol) dissolved in 1.5 mL water, to which 22.5 mg $NaClO_2$ was added, followed by 24.7 µL 30% H_2O_2 and 115.8 µL 0.5 M NaH_2PO_4. The reaction was left stirring at room temperature for 48 hours. The remaining oxidant was neutralized with a little $Na_2S_2O_5$, then the pH was brought up to ~7 with a few drops of 10% $NaHCO_3$ and the solution was stirred for two more hours. The solution was then loaded on a Dowex 50 column (5 mL, H^+ form), and washed with water until the pH of the washings was neutral. The amino acid product was eluted with 1 M NH_3, and the eluate was evaporated *in vacuo*. The white solid was dissolved in ~1 mL water, and 40 mg $Ba(OAc)_2$ was added, followed by 4 mL ethanol. A flocculent white precipitate formed immediately, and the solution was put at 4 ºC to complete precipitation. The white precipitate was collected by centrifugation, the supernatant was discarded, and the precipitate was resuspended in ~2 mL ethanol and collected by centrifugation. Drying the precipitate *in*

vacuo over P_2O_5 gave 14.5 mg (27.4% yield) of an amber solid. The product had the expected mass as analyzed by electrospray ionization mass spectrometry: m/z = 274 $(M-1)^-$.

Construction of mutant strains. *S.* Typhimurium mutants indicated in Table 4.S1 were constructed using the λ Red Recombineering method as described (28). Plasmid pKD4, which contains a kanamycin resistance (*kan)* cassette flanked by two Flp recognition targets (FRT sites), was used as a PCR template to create amplicons which contained sequences at their ends that were homologous to the genes targeted for deletion. Primer sets used to generate the amplicons for the targeted mutagenesis are listed in Table 4.S3. Amplified DNA was introduced by electroporation into *S.* Typhimurium 14028s bearing plasmid pKD46 which carries the λ phage recombinase genes under control of the *araBAD* promoter. Genomic DNA isolated from resulting kanamycin-resistant colonies was checked by PCR to confirm that the target gene had been knocked out using PCR primers that flanked the target deletion gene (primers are listed in Table 4.S3). Mutant alleles in which the target genes had been replaced with the *kan* cassette were moved into a clean genetic background by transduction using P22 HT *int*. The *kan* cassette was then removed by introducing plasmid pCP20 into the mutant strains. Plasmid pCP20 expresses the Flp recombinase which recognized the FRT sites and excised the *kan* cassette. Loss of the *kan* cassette was confirmed by susceptibility to kanamycin and PCR using the same flanking primers listed in Table 4.S3.

S. Typhimurium 14028s strains with disruptions in *ptsG*, *crr* or *crp* were constructed using P22 HT *int* to transduce antibiotic resistance markers in these genes from derivatives of *S.* Typhimurium LT2. These *S.* Typhimurium LT2 mutant strains were DM12321 (*met*$^+$ *ptsG4152*::Tn10d(Tc^r)), DM12310 (*met*$^+$ *crr307*::Tn10d(Tc^r)) and JE16465 (*met205 ara*-9

crp891::kan^{+}). Strains DM12321 and DM12310 were kindly provided by Diana Downs and strain JE16465 was kindly provided by Jorge Escalante-Semerena.

Construction of plasmids. The *dgaABCDEF* operon and *dgaF* were amplified by PCR from *S.* Typhimurium 14028s genomic DNA with Phusion High-Fidelity DNA polymerase (New England Biolabs) using the primer sets indicated in Table 4.S3. The PCR primer sets introduced BglII and HindIII sites for subsequent cloning into the vector pLAC22 (29). In the resulting plasmids, designated as pLAC22+*dgaABCDEF* and pLAC22+*dgaF*, *dgaABCDEF* and *dgaF* were placed under control of the *lac* promoter/operator. Plasmids for overexpression of DgaE and DgaF were constructed by amplifying *dgaE* and *dgaF* by PCR from *S*. Typhimurium 14028s genomic DNA with Phusion High-Fidelity DNA polymerase using the primer sets indicated in Table 4.S2. A's were added to the 3'ends of the amplicons using Taq DNA polymerase (Thermo Fisher Scientific). The amplicons were cloned into pCR2.1-TOPO (Invitrogen) and the sequences of the cloned fragments were confirmed by DNA sequencing (Genewiz). NdeI and HindIII sites introduced by the primer sets used to clone *dgaE* and *dgaF* into the expression vector pET21a (Novagen). The resulting plasmids, designated as pET21a+*dgaE* and pET21a+*dgaF*, expressed the native DgaE and DgaF that lacked the hexahistidine tag.

For all transformations involving *S.* Typhimurium 14028s, DNA was introduced into the bacterium by electroporation using the parameters 2.4 kV, 25 μF and 400Ω. Following electroporation, cells were allowed to recover in SOC broth (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose) for 1 h at 37^{o}C. Chemically competent cells which were prepared using the calcium chloride method were used for transformation of all *E. coli* strains, and cells were allowed to recover in LB broth for 1 h at 37^{o}C following the heat shock step.

Biolog Phenotype MicroArray plate assays. *S.* Typhimurium strains were grown on Biolog Universal Growth (BUG) agar supplemented with 5% sheep blood at 37^{o}C for 24 h. Cells were resuspended in IF-O+dye solution (Biolog) to a final cell density of 85% transmittance, which was determined by a spectrophotometer. The cell suspension was used to inoculate Biolog PM1 and PM2 plates. Before inoculating the Biolog PM3 plates, sodium succinate and ferric citrate were added to the cell suspension to give in final concentrations of 20 mM and 2 µM, respectively. Plates were incubated in an OmniLog PM System at 37°C for 48 h and scanned every 15 min. Collected data were analyzed with OmniLog software.

Purification of DgaE. Plasmid pET21a+*dgaE* was transformed into *E. coli* B21 DE3λ containing plasmid pLysE (30). *E. coli* B21 DE3l expresses T7 RNA polymerase which was required for transcription of *dgaE* from pET21a+*dgaE*. The cell extract from 1 liter of cells cultured in rich auto-induction medium (31) was prepared by sonication for 4 minutes at 1 minute intervals, followed by centrifugation for 90 minutes at 2,500 x g. The supernatant was applied to a Ni affinity column (10 mL), and washed with 0.05 M potassium phosphate, pH 7.0, 0.1 mM pyridoxyl-5'-phosphate (PLP), 0.3 M NaCl until, the absorbance at 280 nm was back to baseline. The column was then eluted with 100 mL of buffer containing 0.2 M imidazole. Peak protein fractions were pooled, concentrated in a centrifugal concentrator (YM-30 membrane), then dialyzed overnight against 500 mL 0.05 M potassium phosphate, pH 7.0, 0.1 mM PLP.

Identification of DgaE reaction product. Solutions were prepared containing 0.1 M NH_4HCO_3 and 1 mM D-glucosaminic acid-6-phosphate in a total of 200 µL. To one solution was added 5 µL purified DgaE (24.4 mg/mL), and the solutions were allowed to stand at room temperature for 48 hours. The solutions were then analyzed by electrospray ionization mass spectrometry. MS(ESI): m/z = 257 $(M-1)^{-}$.

Coupled assay for DgaE and DgaF. The reaction mixtures contained 0.1 M potassium phosphate, pH 7.0, 1 mM D-glucosamine-6-phosphate, 40 μM PLP, 0.2 mM NADH, 10 μg lactate dehydrogenase (Sigma-Aldrich), and 10-20 ug of crude cell extract containing DgaF. Cell extracts containing DgaF were prepared from *E. coli* B21 DE3λ containing plasmids pET21a+*dgaF* and pLysE. Cells were grown overnight in rich auto-induction medium, harvested by centrifugation, lysed by sonication for 4 minutes at 1 minute intervals, and the resulting crude cell extract was clarified by centrifugation for 90 minutes at 2,500 x g. The coupled enzyme reactions were performed at 37 ºC, and the absorbance decrease at 340 nm ($\Delta\varepsilon = 6220\ M^{-1}\ cm^{-1}$) was recorded.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) assays. Transcript levels of *dgaAB* in wild-type *S.* Typhimurium 14028s cultured in minimal medium that contained D-glucose, D-glucosaminate, D-gluconate or L-arabinose (20 mM final concentration for each sugar) as the sole carbon source were assessed by quantitative reverse-transcription PCR (qRT-PCR). One ml of cell culture grown to mid-log phase was used for RNA extraction. RNA was isolated using Aurum Total RNA Mini Kit (BioRad) and treated with an additional DNase treatment using TURBO DNA-free Kit (Ambion). cDNA was synthesized using iScript cDNA Synthesis Kit (BioRad), after which qRT-PCR was carried out using an iCycler iQ system (BioRad). Primer specificity was confirmed by PCR using genomic DNA as a template. Three biological samples and three technical replicates were assessed for expression of *dgaAB* and were normalized to levels of *rpoD,* an internal control for *S.* Typhimurium gene expression. All reactions totaled 20 μL and included 10 μL of iQ SYBR Green Supermix (BioRad), 5 μL of 100-fold diluted cDNA from each reaction and 200 nM of primers specific for either *dgaAB* or *rpoD*. Gene expression levels were determined by using the $2^{-\Delta\Delta ct}$ equation (32).

Computational analysis. Complete genomes of *Salmonella enterica* serovar Typhimurium 14028s, *Escherichia coli* IAI1, *Enterobacter aerogenes* KCTC 2190 and *Enterococcus faecalis* 62 were downloaded from the NCBI FTP server ftp://ftp.ncbi.nih.gov/genomes/Bacteria/. Sequences of the *dga* loci were extracted from the complete genomes. The *dga* loci span the genes STM14_4550 through STM14_4543, ECIAI1_3068 through ECIAI1_3062, EAE_10150 through EAE_10120 and EF62_0067 through EF62_0061, respectively. The *dga* loci and their host genomes were compared in terms of their overall G+C content, G+C content at codon site 3 (S3) and relative abundance of the tetranucleotide CTAG (τ^*_{CTAG}). CTAG is strongly suppressed in γ-Proteobacteria but generally not in Firmicutes, and any laterally transferred DNA segment between γ-Proteobacteria and Firmicutes could therefore exhibit anomalous CTAG representation with respect to the bulk of the host genome (33, 34). The relative abundance measures an excess or deficit of a given oligonucleotide relative to the expected occurrence based on known frequencies of all embedded shorter oligonucleotides. For a tetranucleotide XYZW, its relative abundance τ^*_{XYZW} is assessed as

$$\tau^*_{XYZW} = \frac{f^*_{XYZW} f^*_{XY} f^*_{XNZ} f^*_{XNNW} f^*_{YZ} f^*_{YNW} f^*_{ZW} f^*_{XYZW} f^*_{XYZW} f^*_{XYZW} f^*_{XYZW} f^*_{XYZW}}{f^*_{XYZ} f^*_{XYNW} f^*_{XNZW} f^*_{YZW} f^*_{X} f^*_{Y} f^*_{Z} f^*_{W}},$$

where f^*_{XYZW} denotes the symmetrized frequency of the tetranucleotide XYZW, f^*_{XY} denotes the symmetrized frequency of the dinucleotide XY, etc. N stands for any nucleotide. For double-stranded DNA, symmetrized frequencies are calculated from the nucleotide sequence at hand concatenated with its inverted complement. Relative abundance values ~1 signify that the tetranucleotide occurs approximately as expected, values <1 indicate that it occurs less frequently than expected and values >1 indicate that it occurs more than expected. Values between 0.78 and 1.23 are considered "normal range" (33, 35).

δ-differences.* The relative abundance of a dinucleotide XY is defined as

$$\rho^*_{XY} = \frac{f^*_{XY}}{f^*_X f^*_Y}.$$

The vector of the 16 dinucleotide relative abundances constitutes a genome signature (36, 37). A difference between two nucleotide sequences *A* and *B* can subsequently be defined as

$$\delta^*(A,B) = \frac{1}{16}\sum_{XY}|\rho^*_{XY}(A) - \rho^*_{XY}(B)|,$$

Where $\rho^*_{XY}(A)$ signifies the relative abundance of the dinucleotide XY in the sequence *A* and $\rho^*_{XY}(B)$ signifies the relative abundance of the dinucleotide XY in the sequence *B*. To avoid statistical artifacts when comparing sequences of vastly different sizes it is reasonable to divide the compared sequences into disjoint samples of approximately equal sizes and use the mean distance among all pairwise comparisons between different samples as a measure of dissimilarity of the analyzed sequences. In this work, we use the sample size 7,000 bp, which is similar to the size of the *dga* locus. The software for genome signature comparisons and assessments of oligonucleotide relative abundances is available at http://www.cmbl.uga.edu/software.html.

Results

A mannose family PTS permease in *S*. Typhimurium is required for utilization of D-glucosaminate. Functions of three previously uncharacterized mannose family PTS permeases in *S*. Typhimurium 14028s were examined by deleting the genes encoding the EIIA and EIIB components of each of the PTS permeases and analyzing the phenotypes of the resulting mutants. Each of the PTS operons has a predicted RpoN-dependent promoter and a gene encoding a putative RpoN-dependent activator in the vicinity (either immediately downstream or upstream of the operon), suggesting transcription of the PTS genes is dependent on σ^{54}-

holoenzyme (22). Genes encoding the activators were similarly deleted and the phenotypes of the resulting mutants analyzed. Given the broad substrate specificity of *E. coli* ManXYZ (21), we thought there may be overlap in the substrates of the PTS permeases. Therefore, strains were constructed in which genes encoding all three PTS permeases or activators were deleted.

Phenotypes of the single and triple mutants were analyzed using Biolog Phenotype Microarrays. The two carbon source plates (PM-1 and PM-2) and the nitrogen source plate (PM-3) were used for the analysis, which allowed us to assess the ability of the mutant strains to metabolize 190 different carbon sources and 95 different nitrogen sources (compounds included on these plates are listed on http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf). Mutant strains that lacked all three PTS permeases, all three activators, *dgaAB* or *dgaR* were deficient in metabolizing D-glucosaminate compared to the parental strain. This was the only phenotypic difference observed consistently between the parental strain and any of the mutant strains.

The Δ*dgaAB* and Δ*dgaR* mutants were examined for the ability to grow aerobically in a minimal medium with D-glucosaminate as the sole carbon source. Wild-type *S.* Typhimurium 14028s grew in the minimal medium with a doubling time of 91 ± 3 min. Cells grew faster when ammonium chloride was omitted from the minimal medium and D-glucosaminate was the sole carbon and nitrogen source (80 ± 2 min). The Δ*dgaAB* and Δ*dgaR* mutants mutants failed to grow with D-glucosaminate as the sole carbon source indicating that the *dga* locus was responsible for D-glucosaminate utilization.

Consistent with the prediction that transcription of *dgaABCD* is dependent on RpoN, a Δ*rpoN* mutant failed to grow in a D-glucosaminate minimal medium supplemented with 5 mM L-glutamine. L-glutamine was included in the medium as deletion of *rpoN* in *S.* Typhimurium

results in glutamine auxotrophy (38). A *S*. Typhimurium Δ*ptsH* (encodes HPr) mutant also failed to grow in the D-glucosaminate minimal medium indicating that the general PTS component HPr is required for D-glucosaminate utilization.

To see if *S*. Typhimurium 14028s employs DgaABCD to utilize sugars which are structurally similar to D-glucosaminate, we used an auxanographic method to assess the ability of *S.* Typhimurium 14028s strains to utilize various compounds as carbon or nitrogen sources. The Δ*dgaAB* mutant was able to grow with D-gluconate, D-glucosamine or N-acetylglucosamine as a carbon source, which was expected as there are known transporters of these compounds in *S*. Typhimurium. Wild-type *S*. Typhimurium 14028s failed to grow with D-galactosaminate or L-mannosaminate as a carbon or nitrogen source indicating that *S*. Typhimurium is unable to transport or metabolize these compounds.

D-glucosaminate supports robust growth of *S*. Typhimurium as a nitrogen source. To determine the efficacy of D-glucosaminate as a nitrogen source for *S*. Typhimurium, we compared the growth of *S*. Typhimurium 14028s in minimal media that contained D-glucosaminate as the sole nitrogen source and various primary carbon sources. For these experiments we compared the ability of *S*. Typhimurium to utilize D-glucosaminate versus a good nitrogen source (ammonium chloride) and a poor nitrogen source (L-arginine). Primary carbon sources that were tested included three PTS sugars (D-glucose, D-mannose and D-fructose) and a non-PTS sugar (L-arabinose). Transport of D-glucose and D-mannose involves EI and HPr, while the D-fructose transport system relies on its own HPr-like and EI-like activities (39, 40). Cultures of *S*. Typhimurium 14028s were grown overnight in the basic MOPS minimal medium with various combinations of nitrogen sources and sugars. The

overnight cultures were diluted into fresh medium and doubling times during exponential growth were measured for each culture condition.

S. Typhimurium failed to grow in a minimal medium containing glucose and D-glucosaminate as the sole nitrogen source. *S.* Typhimurium was able to grow in a minimal medium that contained glucose as the primary carbon source and both D-glucosaminate and ammonium as nitrogen sources. The doubling time of *S.* Typhimurium in the glucose minimal medium containing both D-glucosaminate and ammonium was similar to that of *S.* Typhimurium grown in glucose minimal medium containing ammonium as the sole nitrogen source (44 ± 4 min versus 45 ± 3 min, respectively). These findings indicate that the failure of *S.* Typhimurium to grow in the glucose minimal medium containing D-glucosaminate as the nitrogen source results from glucose inhibiting utilization of D-glucosaminate.

In minimal media that contained L-arabinose or D-fructose as the primary carbon source, D-glucosaminate as the nitrogen source supported growth rates of *S.* Typhimurium that were comparable to those with ammonium chloride (Table 4.1). Compared to minimal medium containing D-mannose and ammonium chloride, *S.* Typhimurium grew significantly slower in medium containing D-mannose and D-glucosaminate. The slower growth rate of *S.* Typhimurium in the minimal medium with D-glucosaminate plus D-mannose compared to the minimal medium with D-glucosaminate plus L-arabinose or D-fructose may be due to competition between the mannose PTS and D-glucosaminate PTS for P~HPr. For all of the sugars tested, D-glucosaminate served as a significantly better nitrogen source than L-arginine, and was comparable to ammonium chloride in its efficacy of promoting rapid growth of *S.* Typhimurium.

To determine the affinity of the Dga PTS for D-glucosaminate, the half-saturation constant, K_s, for D-glucosaminate was estimated. The K_s for D-glucosaminate was estimated by measuring the growth rates for *S*. Typhimurium grown in minimal medium with L-arabinose as the primary carbon source and varying amounts of D-glucosaminate as the sole nitrogen source. The estimated K_s was 0.098 ± 0.02 mM D-glucosaminate, suggesting that D-glucosaminate is a physiologically relevant substrate for the Dga PTS.

Two genes downstream of *dgaABCD* function in catabolism of D-glucosaminate. The two genes located immediately downstream of *dgaABCD* (STM14_4544 and STM14_4543) were deleted and the phenotypes of the resulting mutants were analyzed. The STM14_4544 deletion mutant failed to grow in the D-glucosaminate minimal medium indicating that this gene is essential for catabolism of D-glucosaminate, and so we refer to it as *dgaE*. DgaE belongs to the fold type I or aspartate aminotransferase (AAT) superfamily of PLP-dependent enzymes (41). In reactions catalyzed by AAT superfamily members, PLP combines with an α-amino acid to form a Schiff base intermediate. Depending on the enzyme, the Schiff base serves as a substrate in a transamination, racemization, decarboxylation or one of a variety of side-chain reactions. As expected, the *S*. Typhimurium 14028s strain in which both *dgaE* and STM14_4543 were deleted failed to grow in the D-glucosaminate minimal medium.

We postulated that D-glucosaminate is phosphorylated at the C-6 position as it is transported across the cell membrane and that the resulting D-glucosaminate 6-phosphate is converted to 2-keto-3-deoxygluconate 6-phosphate (KDGP) by DgaE (Fig. 4.2). Consistent with this hypothesis, incubation of purified DgaE with D-glucosaminic acid 6-phosphate resulted in a new peak with m/z = 257, demonstrating the net loss of 17 amu (NH_3) from the molecule. Incubation in the absence of DgaE did not show the peak at 257, but only the D-glucosaminic acid 6-

phosphate peak at 274. The mass of the product is consistent with the formation of KDGP from D-glucosaminic acid 6-phosphate indicating that DgaE functions as a glucosaminic acid 6-phosphate dehydratase.

In contrast to the Δ*dgaE* mutant, the STM14_4543 deletion mutant grew in the D-glucosaminate minimal medium, although the growth rate of the mutant was significantly slower (100 ± 4 min doubling time for the mutant versus 80 ± 2 min doubling time for wild type). These data indicated that STM14_4543 is not essential for D-glucosaminate catabolism but is required for wild-type growth on D-glucosaminate and so we designate the gene as *dgaF*.

DgaF belongs to the DUF1341 superfamily. Some members of this family are annotated as KDGP aldolase (Entner-Doudoroff aldolase; Eda) or 4-hydroxy-2-oxoglutarate aldolase, although it is not clear if there is experimental evidence to support these annotations. Based on the above observations indicating that DgaE converts glucosaminic acid 6-phosphate to KDGP, we postulated that DgaF is an aldolase and that Eda can substitute for DgaF in D-glucosaminate catabolism. Deletion of *eda* had no effect on growth of *S.* Typhimurium 14028s in the D-glucosaminate minimal medium as the *Δeda* mutant had a doubling time of 74 ± 1 min compared to 80 ± 2 min for wild type. A strain lacking both *dgaF* and *eda*, however, failed to grow in the D-glucosaminate minimal medium indicating that Eda does indeed substitute for DgaF in the catabolism of D-glucosaminate. Conversely, DgaF was able to substitute for Eda. Eda is required for growth of *S*. Typhimurium with D-glucuronate or D-gluconate as a carbon source. Introduction of a plasmid-borne copy of *dgaF* under control of the *lac* promoter into the Δ*eda* mutant restored the ability of the mutant to grow on a minimal medium with D-glucuronate or D-gluconate as the carbon source (data not shown).

These combined genetic and biochemical data indicated that DgaE and DgaF function together to convert D-glucosaminic acid 6-phosphate to pyruvate plus glyeraldehyde-3-phosphate (Fig. 4.2). Consistent with this hypothesis, addition of DgaE to reaction mixtures containing D-glucosaminic acid 6-phosphate, a cellular extract containing DgaF, lactate dehydrogenase and NADH resulted in the DgaE-dependent consumption of NADH, demonstrating that pyruvate is formed from D-glucosaminic acid 6-phosphate by the combined action of DgaE and DgaF. The specific activity of DgaE in the couple assay was 6.2 μmol/min-mg.

The *dgaABCDEF* operon is sufficient for D-glucosaminate utilization in *E. coli*. The *S.* Typhimurium *dgaABCDEF* operon was cloned into an expression vector and placed under control of an inducible *lac* promoter (plasmid pLAC22+*dgaABCDEF*). The *dgaABCDEF* expression vector restored the ability of the *S.* Typhimurium Δ*dgaAB* and Δ*dgaR* mutants to grow on the D-glucosaminate minimal medium if IPTG was included in the medium to induce expression of the *dga* genes. *E. coli* strains BL21, DH5α and JM101 are typical of most *E. coli* strains whose genomes have been sequenced to date in that they lack the *dga* locus. These three *E. coli* strains failed to utilize D-glucosaminate as a carbon or nitrogen source. Introduction of the *dgaABCDEF* expression vector into the strains, however, allowed them to grow in the D-glucosaminate minimal medium if IPTG was included in the medium to induce expression of the *dga* genes. *E. coli* IAI1 is a strain that possesses the *dga* locus and we found that it utilizes D-glucosaminate as a carbon and nitrogen source. There does not appear to be any obvious trait (e.g., commensal versus pathogen) that distinguishes the *E. coli* strains which possess the *dga* locus from those that lack the locus.

D-Glucosaminate induces expression of the *dgaABCDEF* operon. DgaR contains a PTS regulation domain (PRD) which is phosphorylation target of certain PTS proteins (17, 42). *Bacillus subtilis* LevR, the best characterized PRD-containing RpoN-dependent activator, stimulates transcription of the levanase operon which encodes a fructose-specific PTS permease (43). Phosphorylation of a specific PRD histidine residue by P~HPr stimulates LevR activity, while phosphorylation of a different PRD histidine residue by the phosphorylated EIIB component (P~LevE) of the levanase PTS inhibits LevR activity (44). The negative regulation of LevR is the basis for induction of the levanase operon by fructose as in the absence of fructose in the medium, P~LevE predominates and phosphorylates LevR to inhibit transcription of the levanase operon.

Given that fructose induces expression of the *B. subtilis lev* operon, we postulated that D-glucosaminate similarly induces expression of the *dga* operon. To test this hypothesis, qRT-PCR was used to measure *dgaAB* transcript levels in *S.* Typhimurium 14028s grown in minimal medium that contained D-glucosaminate, D-glucose, D-gluconate or L-arabinose as the sole carbon source. For these assays, *dgaAB* transcript levels were normalized to *rpoD* transcript levels. Levels of *dgaAB* transcript were ~1,000-fold higher in cultures grown with D-glucosaminate compared to cultures grown with the other carbohydrates tested (Fig. 4.3), indicating that D-glucosaminate induces expression of the *dga* operon.

Glucose inhibition of D-glucosaminate utilization is mediated by the glucose PTS. We wished to determine the basis for the glucose inhibition of D-glucosaminate utilization. One possible explanation is that transcription of *dgaR* or *dgaABCDEF* is dependent on the cAMP-receptor protein (CRP) and glucose inhibits *dga* expression by depressing cAMP levels inside the cell. To address this hypothesis we constructed a Δ*crp* mutant in *S.* Typhimurium 14028s

and tested its ability to utilize D-glucosaminate. The Δ*crp* mutant grew as well as the parental strain in minimal medium with D-glucosaminate as the sole carbon and nitrogen source (doubling times of the Δ*crp* mutant and wild type were 76 ± 4 min and 80 ± 2 min, respectively), indicating that CRP is not required for expression of the *dga* operon.

We next introduced a *ptsG*::Tn10d(Tc^r) mutation into *S*. Typhimurium 14028s to determine if the glucose-specific PTS was required for the glucose inhibition of D-glucosaminate utilization (*ptsG* encodes the EIIBC component of the glucose-specific PTS). ManXYZ transports glucose efficiently and in the absence of the glucose-specific PTS is able to support rapid growth on glucose (45, 46). The *ptsG* mutant grew with a doubling time of 46 ± 4 min in a minimal medium containing 20 mM glucose as the primary carbon source and 5 mM D-glucosaminate as the sole nitrogen source. These data indicate that glucose does not directly inhibit transport or utilization of D-glucosaminate. We postulate that glucose inhibition of D-glucosaminate utilization in wild-type *S*. Typhimurium results from the glucose-specific PTS inhibiting transcription of the *dgaABCDEF* operon by diverting P~HPr from phosphorylating DgaR and thereby preventing activation of DgaR.

Phylogenetic distribution of the *dga* locus. Bacterial genomes in the Integrated Microbial Genomes (IMG; http://img.jgi.doe) were searched for potential orthologs of the *dga* genes using the synteny of *dga* genes to facilitate the identification of orthologs (data for the analysis were collected on February 20, 2013). The IMG Conserved Neighborhood tool, which searches for orthologs in user-selected genomes and displays neighborhoods of similar sized orthologs (47) was used for the analysis. Each *dga* gene from *S*. Typhimurium 14028s was compared with the genomes in the IMG database, and the resulting gene ortholog neighborhoods were examined for the co-localization of the *dga* genes. Bacterial genomes that possessed the *dga* locus using these

criteria were limited to seven genera (*Salmonella*, *Escherichia*, *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella* and *Serratia*) within the family Enterobacteriaceae in the subphylum γ-Proteobacteria, one member of the subphylum β-Proteobacteria (*Chitiniphilus shinanonensis* DSM 23277) and some members of the genera *Enterococcus* and *Lactobacillus* in the phylum Firmicutes (Table 4.S4). *Collinsella tanakaei* YIT 12063, a member of the phylum Actinobacteria, possesses orthologs of the *dgaABCDEF* genes, but lacks homologs of *dgaR* and *rpoN*, indicating that expression of the *dga* orthologs in this bacterium is regulated in a manner that differs from that of *S*. Typhimurium.

For most of the *Salmonella* genome sequences in the IMG database we were able to unambiguously identify complete *dga* loci (68 of 86). Most of the *S*. Typhi genomes in the database are draft sequences that consist of numerous and short contigs; and while these genones possess at least some of the *dga* orthologs, we were unable to ascertain if they contained complete sets of *dga* genes. The three finished *S*. Typhi genomes (*S*. Typhi CT18, *S*. Typhi Ty2 and *S*. Typhi P-stx-12) and one draft genome sequence (*S*. Typhi E98-3139) in the database, however, allowed us to unambiguously assess the content of *dga* genes in these strains. All four of these *S*. Typhi strains possess *dgaABCDEF* orthologs but contain the same apparent frameshift mutation (G inserted between nucleotides 207 and 208) in *dgaR*, suggesting that they lack a functional DgaR. Sequences which matched the RpoN-dependent promoter consensus sequence generated by Barrios and co-workers (48) were found upstream of *dgaA* for all of the *Salmonella* strains (Table 4.S4).

All of the *Enterococcus faecalis* genomes in the IMG database contain at least some of the *dga* orthologs. Many of the *E. faecalis* strains lack full-length versions of either or both *dgaE* or *dgaF* within the *dga* operon but possess *dgaEF* orthologs at a separate locus. We postulate that

this altered gene arrangement resulted from a gene duplication and subsequent deletion of the *dgaEF* genes at the *dga* locus since some *E. faecalis* strains (e.g., AR01/DG) possess two full-length copies of *dgaEF*. In the case of *E. faecalis* AR01/DG, the DgaF paralogs share 100% amino acid identity over their entire length, while the DgaE paralogs share 98% amino acid identity over their entire length.

Two of the eight *Lactobacillus rhamnosus* genome contain the *dga* locus (strains LRHMDP2 and LRHMDP3). In both of these strains *rpoN* is situated between the *dgaA* and *dgaR* homologs, while the six *L. rhamnnosus* strains which lack the *dga* locus also do not possess *rpoN* elsewhere in their genomes. Thus, *L. rhamnosus* strains LRHMDP2 and LRHMDP3 appear to have acquired the *dga* locus together with the gene encoding the σ factor required for transcription of the *dga* operon through lateral gene transfer.

RpoN-dependent PTS operons are more prevalent in Firmicutes than Proteobacteria, and so we wished to test the hypothesis that enterobacteria acquired the *dga* genes from *E. faecalis.* To test this hypothesis, *dga* orthologs of four bacterial species for which complete genome sequences are available were compared in terms of G+C content, G+C at codon site 3 and suppression of the CTAG tetranucleotide. These comparisons showed that the *dga* loci match the properties of their respective host genomes, arguing against recent lateral transfer of the *dga* locus between *E. faecalis* and the enterobacteria (Table 4.S5). Genome signature comparisons of the genomes and *dga* loci revealed that *E. faecalis* is an outlier among the four genomes and the *dga* loci feature similar signatures to those of their host genomes (Table 4.S6); and BLAST comparisons among orthologous genes in the four *dga* loci show that for each of the six genes, the three enterobacterial orthologs are invariably more similar to each other than any is to the *E.*

faecalis ortholog (Table 4.S7). Taken together, these data fail to support the hypothesis that the *dga* genes were transferred from *E. faecalis* to the enteric bacteria or *vice versa*.

Discussion

We show here that *S.* Typhimurium uses a mannose family PTS permease encoded by *dgaABCD* together with a novel D-glucosaminic acid 6-phosphate dehydratase (DgaE) and a redundant KDGP aldolase (DgaF) to transport and catabolize D-glucosaminate (Fig. 4.2). The proposed DgaE activity is similar to that of D-glucosaminate dehydratase, a PLP-dependent enzyme that has been purified from *Agrobacterium radiobacter* and *Pseudomonas fluorescens* which converts D-glucosaminate to 2-keto-3-deoxygluconate (49-52). We could not compare the sequence of DgaE with sequences of the *A. radiobacter* and *P. fluorescens* enzymes since sequences for these enzymes have not been reported. DgaE does share 40% amino acid identity (59% similarity) over its entire length with a predicted protein present in most of the *Agrobacterium* strains whose genomes have been sequenced and it is possible that the *Agrobacterium* DgaE homolog is D-glucosaminate dehydratase. The only *P. fluroescens* protein that shares significant homology with DgaE is selenocysteine synthase, but this sequence homology is considerably less than that of the *Agrobacterium* DgaE homolog (24% identity and 42% similarity over 287 amino acid residues).

It is not obvious where *Salmonella* might encounter D-glucosaminate in nature. Given that many of the bacteria that possess the D-glucosaminate PTS can colonize animal intestinal tracts it is possible that *Salmonella* encounters D-glucosaminate in such environments. We are unaware though of any reports of D-glucosaminate in the intestinal tract contents of any animal. There have been numerous studies in which the *Salmonella* transcriptome has been analyzed

from bacteria grown *in vivo* (e.g., during infection of macrophage-like cells or epithelial cells; or isolated from the intestinal tract), cultured under different conditions, or exposed to various chemical stimuli (53-61). In addition, there are several reports on how the loss of known global regulators affects the *Salmonella* transcriptome (62-64). A recent study used proteomic, mutant phenotyping and computational approaches to investigate the *Salmonella* nutrition in a mouse typhoid fever model (65). None of these studies, however, identified conditions that resulted in up-regulation of the *dga* operon or offered clues as to where *Salmonella* might encounter D-glucosaminate.

It is possible that *S*. Typhimurium obtains D-glucosaminate from other microorganisms. D-glucosaminate is a component of *Rhizobium leguminosarum* lipid A (66), and *S*. Typhimurium could obtain D-glucosaminate from bacteria that produce it for the biosynthesis of lipid A or other macromolecules. Another possible source of D-glucosaminate is from the oxidation of D-glucosamine by glucose oxidase. *E. coli* glucose oxidase converts D-glucosamine to D-glucosaminate effectively, having a catalytic efficiency for D-glucosamine that is about half that for D-glucose (67). *S*. Typhimurium possesses a glucose oxidase and could use the enzyme to convert D-glucosamine to D-glucosaminate. The benefit of such a scheme is not obvious since D-glucosamine can be transported directly by the mannose and glucose PTS (19, 68). Moreover, glucose oxidase requires the cofactor pyrroloquinoline quinone (PQQ), but *S*. Typhimurium is unable to synthesize PQQ (69). In nature, *S*. Typhimurium presumably obtains PQQ from the environment to activate glucose oxidase. It is possible that *S*. Typhimurium uses glucose oxidase as a strategy to compete for limiting nutrients. For example, if *S*. Typhimurium obtained PQQ from associated bacteria in the environment it could use its glucose oxidase to convert sugars, such as D-glucosamine, to compounds that it could use but its neighbors could not.

Alternatively, *S.* Typhimurium may obtain D-glucosaminate generated from D-glucosamine as an unwanted side reaction of the glucose oxidases of other bacteria. Since glucose oxidase is located on the periplasmic side of the cell membrane in Gram-negative type bacteria, D-glucosaminate formed from the oxidation of D-glucosamine could diffuse into the surrounding area and be available for *S.* Typhimurium (14). Regardless of the source of D-glucosaminate, the D-glucosaminate PTS is likely a scavenging system that allows *S.* Typhimurium to compete for limiting nutrients in one or more of its native environments.

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Table 4.1. Bacterial strains used for this study.

species	strain	relevant genotype	source
S. Typhimurium	14028s	wild type	ATCC
S. Typhimurium	KAM30	Δ*dgaR::kan*	this study
S. Typhimurium	KAM68	Δ*dgaR*	this study
S. Typhimurium	KAM44	Δ*dgaAB::kan*	this study
S. Typhimurium	KAM71	Δ*dgaAB*	this study
S. Typhimurium	KAM117	Δ*dgaE::kan*	this study
S. Typhimurium	KAM123	Δ*dgaE*	this study
S. Typhimurium	KAM118	Δ*dgaF::kan*	this study
S. Typhimurium	KAM124	Δ*dgaF*	this study
S. Typhimurium	KAM126	Δ*dgaFΔeda::kan*	this study
S. Typhimurium	KAM1262	Δ*dgaFΔeda*	this study
S. Typhimurium	KAM111	Δ*eda::kan*	this study
S. Typhimurium	KAM106	Δ*eda*	this study
S. Typhimurium	KAM121	Δ*dgaEF::kan*	this study
S. Typhimurium	KAM122	Δ*dgaEF*	this study
S. Typhimurium	KAM27	ΔSTM14_0667::*kan*	this study
S. Typhimurium	KAM69	ΔSTM14_0667	this study
S. Typhimurium	KAM31	ΔSTM14_5448::*kan*	this study
S. Typhimurium	KAM67	ΔSTM14_5448	this study
S. Typhimurium	KAM75	ΔSTM14_0673 ΔSTM14_0672 Δ*dgaAB* ΔSTM14_5449 ΔSTM14_5450::*kan*	this study
S.	KAM81	ΔSTM14_0673 ΔSTM14_0672	this study

Typhimurium		Δ*dgaAB* ΔSTM14_5449 ΔSTM14_5450	
S. Typhimurium	KAM96	ΔSTM14_0667 Δ*dgaR* ΔSTM14_5448::*kan*	this study
S. Typhimurium	KAM100	ΔSTM14_0667 Δ*dgaR* ΔSTM14_5448	this study
S. Typhimurium	ACB01	Δ*rpoN*	A. Bono
S. Typhimurium	KAM91	Δ*ptsH::kan*	this study
S. Typhimurium	KAM95	Δ*ptsH*	this study
S. Typhimurium	KAM131	Δ*dgaAB* Δ*eda*	this study
S. Typhimurium	KAM139	Δ*eda/* pLAC22+*dgaF*	this study
S. Typhimurium	KAM143	Δ*eda/* pLAC22	this study
S. Typhimurium	KAM140	Δ*dgaAB /* pLAC22+*dgaABCDEF*	this study
S. Typhimurium	KAM145	Δ*dgaAB* / pLAC22	this study
S. Typhimurium	KAM141	Δ*dgaR* / pLAC22+*dgaABCDEF*	this study
S. Typhimurium	KAM146	Δ*dgaR* / pLAC22	this study
E. coli	DH5α	*fhuA2 lac(del)U169 phoA glnV44 Φ80' lacZ(del)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	S. Kustu
E. coli	KAM134	DH5α / pLAC22+*dgaABCDEF*	this study
E. coli	DH5α	DH5α / pLAC22	E. Altman
E. coli	JM101	*glnV44 thi*-1 Δ(*lac-proAB*) F'[$lacI^{q}$ZΔM15 *traD36 proAB*$^{+}$]	S. Kustu
E. coli	KAM142	JM101 / pLAC22+*dgaABCDEF*	this study
E. coli	KAM150	JM101 / pLAC22	this study
E. coli	BL21	*E. coli* B F- *dcm ompT hsdS*(r_B- m_B-) *gal* [$malB^{+}$]$_{K-12}$(λ^{S})	S. Kustu
E. coli	KAM143	BL21 / pLAC22+*dgaABCDEF*	this study
E. coli	KAM149	BL21 / pLAC22	this study

E. coli	IAI1	Wild type commensal	A. Maurelli and E. Denamur

Table 4.2. Plasmids used in this study.

plasmid	relevant characteristics	Ref.
pKD46	expresses phage λ recombinase genes *γ*, *β* and *exo* from arabinose-inducible P_{araB} promoter; temperature-sensitive replicon	(28)
pKD4	template plasmid used to generate amplicons for inactivation of target genes in the λ Red system	(28)
pCP20	expresses FLP enzyme for removal of *kan* gene flanked by FRT sites; temperature-sensitive replicon	(70)
pLAC22	expression vector derived from pBR322 that carries the *E. coli lac* promoter/operator and $lacI^q$	(29)
pLAC22+ *dgaABCDEF*	carries *S.* Typhimurium *dgaABCDEF* under control of *lac* promoter/operator in pLAC22	this study
pLAC22+ *dgaF*	carries *S.* Typhimurium *dgaF* under control of *lac* promoter/operator in pLAC22	this study

Table 4.3. Growth rates of *S.* Typhimurium 14028s grown in minimal medium with various combinations of carbon and nitrogen sources.

	L-arabinose	**D-mannose**	**D-fructose**
nitrogen source	**doubling time (min)**		
NH_4Cl	44 ± 2	39 ± 4	43 ± 2
D-glucosaminate	43 ± 4	50 ± 1	45 ± 2
L-arginine	100 ± 5	130 ± 9	110 ± 5

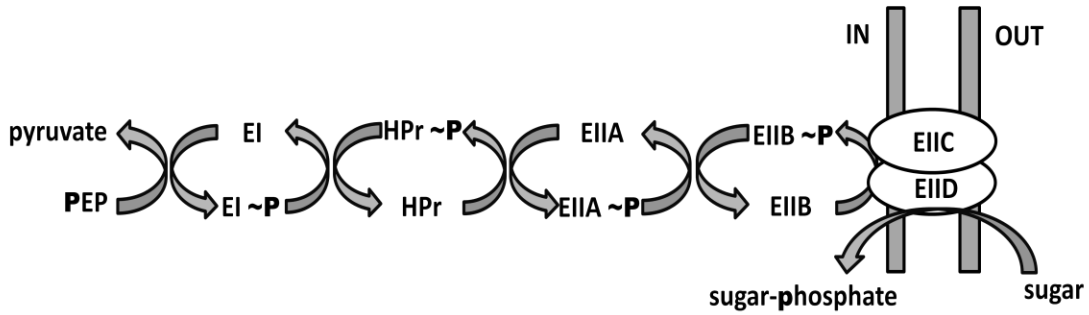

Figure 4.1. Mannose family PTS-mediated carbohydrate transport. Phosphoenolpyruvate (PEP) serves as the phosphor-donor for a phosphorylation cascade that is formed by the general PTS proteins EI and HPr, and an EII complex (PTS permease) which is specific for a given sugar(s). Mannose family PTS permeases consist of four domains (A, B, C and D) arranged on one to four polypeptides. EIIC and EIID are integral membrane proteins, while the EIIA and EIIB are soluble cytoplasmic proteins if not part of a polypeptide that includes EIIC or EIID. As the carbohydrate is transported across the membrane it is phosphorylated by the EII complex.

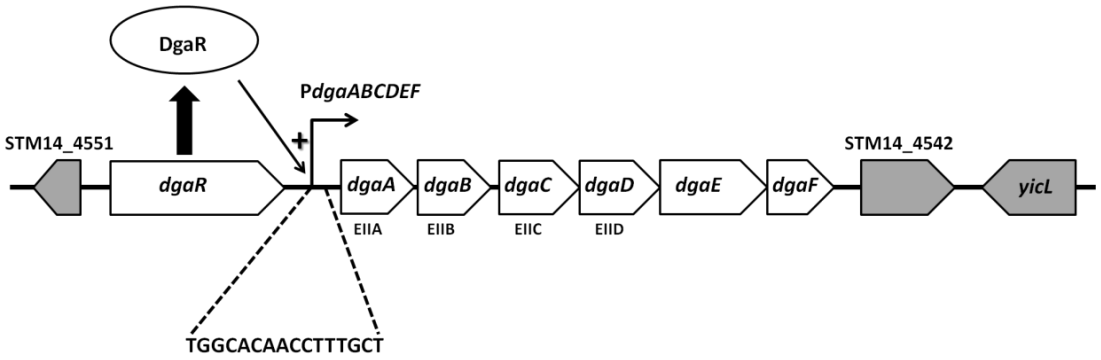

Figure 4.2. The *S*. Typhimurium *dga* locus. The EIIA, EIIB, EIIC and EIID components of the D-glucosaminate PTS permease are encoded by *dgaA*, *dgaB*, *dgaC* and *dgaD*, respectively. The other genes in the operon are *dgaE*, which we predict encodes a dehydratase that converts D-glucosaminate-6-phosphate to 2-keto-3-deoxy-6-phosphogluconate (KDGP), and *dgaF*, which we predict encodes a KDGP aldolase. DgaR is an RpoN-dependent activator required for expression of *dgaABCDEF*. Located 138 bp upstream of the predicted translational start site of *dgaA* is a sequence (5'-TGGCACAACCTTTGCT-3') that matches the RpoN-dependent promoter consensus sequence. Genes that flank the *dga* locus in *S*. Typhimurium 14028s are indicated in gray.

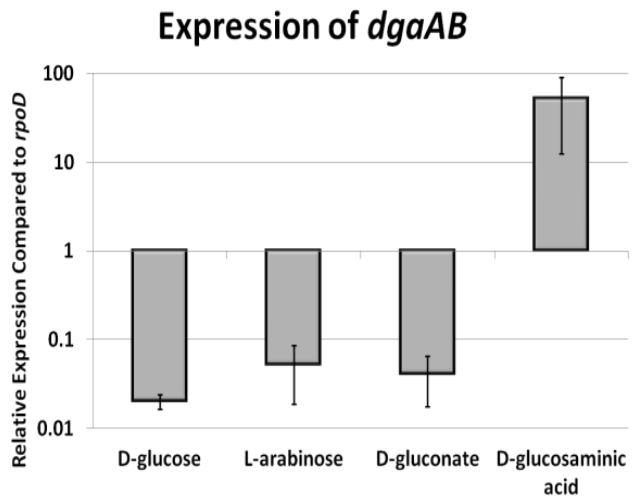

Figure 4.4. Relative levels of *dgaAB* transcript in *S*. Typhimurium grown on minimal media containing different carbon sources. Cultures were grown to mid-log phase in minimal medium containing 20 mM D-glucose, L-arabinose, D-gluconate or D-glucosaminate as the carbon source. cDNA was prepared from RNA isolated from the cultures and quantified by qRT-PCR. Levels of *dgaAB* transcripts were compared to *rpoD* transcript levels. Average values for three sample replicates of three biological replicates are shown. Error bars indicate standard deviations. Levels of *dgaAB* transcript from the culture grown with D-glucosaminate differed significantly from those of the other cultures as assessed using the Student's t-test ($P<0.03$).

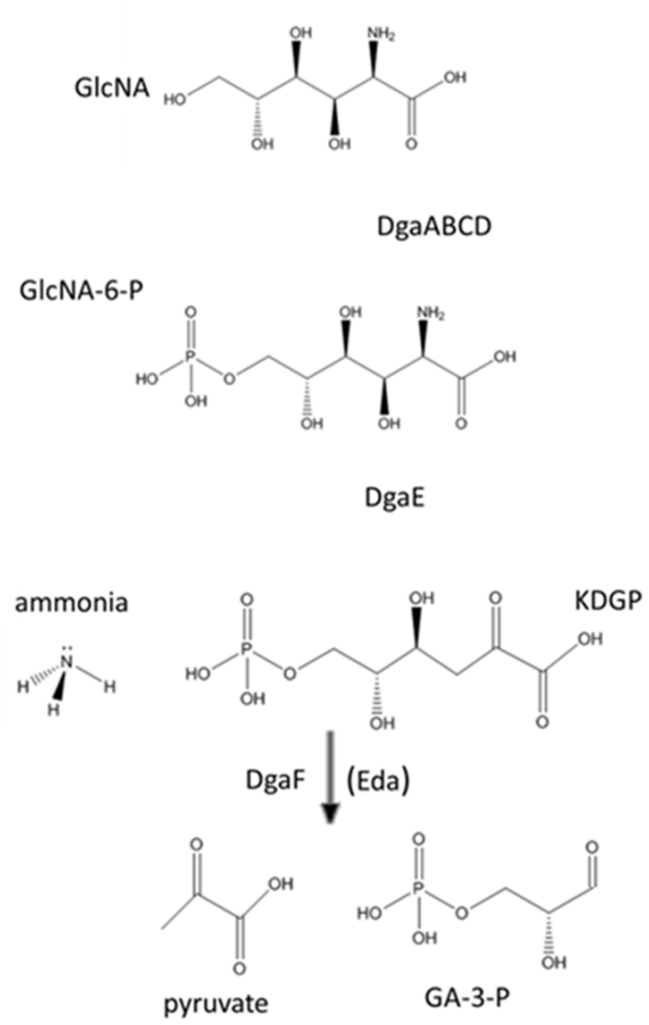

Figure 4.5. Proposed pathway for D-glucosaminate catabolism in *S*. Typhimurium. D-glucosaminate (GlcNA) is phosphorylated at the C-6 position as it is transported across the cell membrane by the DgaABCD permease. DgaE is a predicted dehydratase that converts D-glucosaminate-6-phosphate (GlcNA-6-P) to KDGP. KDGP is subsequently cleaved by the aldolase DgaF to form glyceraldehyde-3-phosphate (GA-3-P) and pyruvate. KDGP can also be cleaved by the Entner-Doudoroff aldolase, Eda.

Table 4.S1. Primers used in this study.

Sequence	Experiment
5’-ATG AGA CGT ATT GAG ATC GTA CTG GGA GAG TGT GTA GGC TGG AGC TGC TTC-3’	*dgaR* knock out λ-Red
5’-TTA ACT ATA GAG CAG TTC GTA GAT ATA AAC ATA TGA ATA TCC TCC TTA-3’	*dgaR* knock out λ-Red
5’-CAT TTT TCG CCA GGC CTT TAA TCA GGA AGG CGA CAC CAT ATG AAT ATC CTC CTT A-3’	*dgaAB* knock out λ-Red
5’-ATG GCC AAT ATC GTT TTA TGC CGC ATC GAC AGC CGT TGT GTA GGC TGG AGC TGC TTC-3’	*dgaAB* knock out λ-Red
5’-ATG ACG CCG AAT ATC TAT CAA CAA CTG GGC ATA TGA ATA TCC TCC TTA-3’	*dgaE* knock out λ-Red
5’-TTA ATC TGC TGC ATG TTC TGC AAT CTC CCT TGT AGG CTG GAG CTG CTT C-3’	*dgaE* knock out λ-Red
5’-GGC GAT CCG TCA CCT GAG GAA TAA GAT GAC TGT GTA GGC TGG AGC TGC TTC-3’	*dgaF* knock out λ-Red
5’-AGG GAG ATT GCA GAA CAT GCA GAT TAA CAT ATG AAT ATC CTT A-3’	*dgaF* knock out λ-Red
5’- CACTAG TAA TCA GGC GAG AGA AGA ATT CCG TGT GTA GGC TGG AGC TGC TTC-3’	*eda* knock out λ-Red
5’-ATA AGC AAG CGC CAT CGG GCA TTT AAC GGC CAT ATG AAT ATC CTC CTT A-3’	*eda* knock out λ-Red
5’-CCG TAA AGC AAA CTG TTG AAG TCA CCA ATA AGC TGG TGT GTA GGC TGG AGC TGC TTC-3’	*ptsO* knock out λ-Red
5’-GGT GAC TGT CAT CAA ATA CCG GGA CGG CGT TGG CAT ATG AAT ATC CTC CTT A-3’	*ptsO* knock out λ-Red
5’-CCT GTT GGG ATG TGT TAA GAA ACT GGC TGA TAA TGA CAT ATG AAT ATC CTC CTT A-3’	STM14_0673/STM14_0672 knock out λ-Red
5’-ATG ATC AAA TTA GTG CGC ATT GAT TAC CGC CTG CTG TGT GTA GGC TGG AGC TGC TTC-3’	STM14_0673/STM14_0672 knock out λ-Red
5’-CCG GTT GCG AGG CCG CAA ATT TCG GCA TAT TGA CCA CAT ATG AAT ATC CTC CTT A-3’	STM14_5449/STM14_5450 knock out λ-Red
5’-ACG GGT GGA TCA TCG TTT ATT ACA TGG ACA GGT CGC TGT GTA GGC TGG AGC TGC TTC-3’	STM14_5449/STM14_5450 knock out λ-Red
5’-TTG GCG TCT GGA TCT TCG-3’	STM14_0667 knock out overlapping PCR λ-Red
5’-CAG CCT ACA CAA TCG CTC AAG ATG GTG ACT AAT AAT GCT GCG-3’	STM14_0667 knock out overlapping PCR λ-Red
5’-CTG TGT CAC TGA AAA TTG CTT TGA GGT ATG ATT GAG CGA CTG GTC-3’	STM14_0667 knock out overlapping PCR λ-Red
5’-ATC AGG AGT CTA TCT GTA TTC TGC-3’	STM14_0667 knock out overlapping PCR λ-Red
5’-CAT CAT AAT TGC TGA TGT GAT AAT CGT GAG TGTGTA GGC TGG AGC TGC TTC-3’	STM14_5448 knock out λ-Red
5’-TTA AAA CTG TTG ATC TTG CTC GAT AAG TTC	STM14_5448 knock out λ-Red

CAT ATG AAT ATC CTC CTT A-3’	
5’-GGA AGG CTC GCT GAT CCT GA-3’	*dgaAB* qRT-PCR
5’-CAG GCG GTT AGC ATC CCT TG-3’	*dgaAB* qRT-PCR
5’-TAT CGA AGA CGG GAT CAA CC-3’	*rpoD* qRT-PCR
5’-GGA CAA ACG AGC CTC TTC AG-3’	*rpoD* qRT-PCR
5’- CAT ATG ACG CCG AAT ATC TAT CAA-3’	DgaE purification
5’-AAG CTT ATC TGC TGC ATG TTC TGC-3’	DgaE purification
5’-CAT ATG CAG CAG ATT AAT TTT TAT CG-3’	DgaF purification
5’-AAG CTT CAC CAG CGC TTT GAC TAT C-3’	DgaF purification

Table 4.S2. BLAST comparisons among proteins encoded by the *dga* locus.

DgaR[a]

Gene	STM14_4550	ECIAI1_3068	EAE_10150	EF62_0067
STM14_4550	1907	1674	1580	582
ECIAI1_3068		1941	1550	579
EAE_10150			1938	588
EF62_0067				1910

DgaA[a]

Gene	STM14_4548	ECIAI1_3067	EAE_10145	EF62_0066
STM14_4548	285	243	226	91
ECIAI1_3067		289	226	95
EAE_10145			286	97
EF62_0066				269

DgaB[a]

Gene	STM14_4547	ECIAI1_3066	EAE_10140	EF62_0065
STM14_4547	326	304	301	172
ECIAI1_3066		326	295	169

EAE_10140			327	170
EF62_0065				326

DgaC[a]

Gene	STM14_4546	ECIAI1_3065	EAE_10135	EF62_0064
STM14_4546	496	483	484	333
ECIAI1_3065		496	476	329
EAE_10135			495	338
EF62_0064				487

DgaD[a]

Gene	STM14_4545	ECIAI1_3064	EAE_10130	EF62_0063
STM14_4545	571	560	538	350
ECIAI1_3064		572	529	352
EAE_10130			568	350
EF62_0063				570

DgaE[a]

Gene	STM14_4544	ECIAI1_3063	EAE_10125	EF62_0062
STM14_4544	752	636	570	287
ECIAI1_3063		750	558	286
EAE_10125			751	266
EF62_0062				748

DgaF[a]				
Gene	STM14_4543	ECIAI1_3062	EAE_10120	EF62_0061
STM14_4543	504	415	396	207
ECIAI1_3062		515	392	200
EAE_10120			507	202
EF62_0061				507

The table shows pairwise BLAST bit scores between protein products of orthologous genes. The data were obtained running the blastp at the NCBI server (http://blast.ncbi.nlm.nih.gov/) with default parameters.

[a] Protein product assignments based on the results of this study with *S.* Typhimurium 14028s.

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CHAPTER 5: FRUCTOSELYSINE AND GLUCOSELYSINE UTILIZATION IN *SALMONELLA* TYPHIMURIUM

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Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the leading causes of food borne illness. *Salmonella* causes 11% of all infections caused by bacterial foodborne pathogens in the United States and is responsible for an estimated 1 million cases of salmonellosis in the United States each year including 19,000 hospitalizations and approximately 400 deaths (1). *S.* Typhimurium causes gastroenteritis in humans, but is capable of colonizing a wide range of animals with little or no disease symptoms. *S.* Typhimurium utilizes a broad array of carbon and nitrogen sources (2), which may contribute to the wide host range of this bacterium. Chadhuri and coworkers found that genes encoding enzymes in a variety of catabolic processes are important for *S.* Typhimurium colonization of the chicken, pig, and calf (3). Among those catabolic genes, some encoded phosphoenolpyruvate-dependent sugar phosphotransferase systems (PTS) permeases of unknown function.

PTS permeases, which are major routes for carbohydrate transport in bacteria, couple the phosphorylation of sugar substrates with their translocation across the cell membrane. The general mechanism of PTS-mediated transport is as follows. A phosphoryl group from phosphoenolpyruvate is transferred to enzyme I (EI) which in turn transfers it to a phosphoryl carrier, the histidine protein (HPr). From HPr, the phosphoryl group is transferred to a sugar-specific permease, a membrane-bound complex known as enzyme 2 (EII), which phosphorylates the sugar as it transports the sugar across the cell membrane. EII complexes (PTS permeases) are associated with the cell membrane and are specific for a single substrate or set of substrates with similar structures. EII complexes consists of at least three distinct domains (IIA, IIB and IIC) which are fused into a single polypeptide or exist as multiple, interacting polypeptides. Mannose family PTS permeases have an addition domain, EIID (4, 5). The best described mannose family

PTS permease is ManXYZ which transports mannose and other hexoses (6). Enzyme I and HPr are cytoplasmic proteins used in conjunction with most PTS permeases, although some permeases (e.g., FruA and FrwBCD fructose-specific PTS permeases) have dedicated Enzyme I and/or HPr homologs (7).

One of the PTS permeases identified by Chadhuri and co-workers as important in colonization of the chicken, pig, and calf was homologous to a PTS permease encoded in the *Enterococcus faecium gfrABCDEF* operon. Wiame and co-workers showed the *gfrE* and *gfrF* encode deglycases involved in fructoselysine and glucoselysine catabolism, respectively (8).

Fructoselysine is formed by the reaction of glucose with the ε-amine of lysine followed by a spontaneous isomerization of the sugar referred to as an Amadori rearrangement (9). Glucoselysine is formed from the reaction of fructose with the ε-amine of lysine which is also followed by a rearrangement of the sugar although it differs from an Amadori rearrangement. After reacting with a primary amine, fructose can undergo various rearrangements (Heyns rearrangements) which lead to the formation of 2-amino-aldose derivative or 2-amino-3-hexulose derivatives, each of which can be in either a glucosamine or mannosamine configuration (10). The spontaneous reactions of primary amines with glucose and fructose are referred to as glycation and fructation, respectively. Fructosamines are common in nature and can result from the modification of free amino acids as well as proteins. Fructoselysine occurs in dehydrated fruits, grains and vegetables, including raisins, prunes, dates, figs, apricots, onion, cereal, and carrots (11). Other fructosamines are found in rotting fruits and vegetables, accounting for as much as 7% of the fresh mass (12).

The putative *S.* Typhimurium *gfrABCDEF* operon was shown to be under control of the alternative sigma factor RpoN (σ^{54}) (13, 14). Transcription from RpoN-dependent promoters requires an activator to stimulate isomerization of a closed complex between RNA polymerase and the promoter to an open complex that is competent to initiate transcription (15). Genes encoding RpoN-dependent activators are often closely associated with the genes they regulate, and a gene encoding a predicted RpoN-dependent activator is located immediately upstream of the *gfrABCDEF* operon (Fig 5.1).

We show here that the predicted *gfrABCDEF* operon is responsible for the transport and utilization of both fructoselysine and glucoselysine in *S.* Typhimurium. Fructoselysine and glucoselysine can serve as both the sole carbon and sole nitrogen source for S. Typhimurium. Fructoselysine is a good nitrogen source for *S.* Typhimurium, with the growth rate of *S.* Typhimurium cultures grown in a minimal medium containing fructoselysine as the sole nitrogen source comparable to that of cultures grown in medium containing ammonium as the nitrogen source. Interestingly, although lysine derived from fructoselysine and glucoselysine can be used as a nitrogen source for *S.* Typhimurium, exogenous lysine does not support the growth of *S.* Typhimurium.

Methods and Materials

Bacterial strains, growth conditions, and reagents.

Strains were maintained in Luria-Bertani (LB) broth or agar supplemented with 100 µg/ml of ampicillin or 50 µg/ml kanamycin as needed. Growth of *S.* Typhimurium on different carbon and nitrogen sources was carried out using the basal MOPS minimal medium (16) with the modifications described by Maloy *et al*. (17). Various carbon and nitrogen sources were

added to the minimal medium at the concentrations indicated. Strains used in this study are listed in Table 5.S1.

Construction of plasmids.

Plasmids used for this study are listed in Table 5.S2. Both *gfrE* and *gfrF* were amplified by PCR from *S.* Typhimurium 14028s genomic DNA using Phusion High-Fidelity DNA polymerase (New England BioLabs) using primers sets listed in Table 5.S3. Adenine residues were added to the 3'-ends of the amplicons using *Taq* DNA polymerase (Thermo Fisher Scientific). The amplicons were cloned into pCR2.1-TOPO (Invitrogen), and the sequences of the cloned fragments were confirmed using DNA sequencing (Genewiz). NdeI and HindIII sites were introduced by the primer sets and were used to clone *gfrE* and *gfrF* into the expression vector pET21a (Novagen). The resulting plasmids, designated pET21a+*gfrE* and pET21a+*gfrF*, expressed the proteins with a C-terminal hexahistidine tag.

For all transformations involving *S.* Typhimurium 14028s, DNA was introduced by electroporation at 2.4 kV, 25 μF, and 400 Ω. Following electroporation, cells were allowed to recover in SOC broth (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) for 1 hour at 37°C. Chemically competent cells were prepared using the calcium chloride method and were used for all transformations involving *E. coli*. Chemically competent cells were allowed to recover in LB broth for 1 hour at 37°C following the heat shock step.

Expression and purification of proteins.

Plasmids pET21a+*gfrE* and pET21a+*gfrF* were transformed into *E. coli* BL21 (DE3λ) containing the plasmid pLysE (18). *E. coli* BL21 (DE3λ) expresses T7 RNA polymerase, which

is needed for transcription of *gfrE* and *gfrF* from pET21a+*gfrE* and pET21a+*gfrF*. Overnight cultures were subcultured into 1 liter of LB broth with appropriate antibiotics and grown at 30°C until OD_{650} = 0.4-0.6. Cells were then moved to ice for 30 minutes, after which isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to the medium to induce expression of the desired proteins. Cells were then allowed to grow at 18°C for 48 hours. Cells were harvested by centrifugation at 8,000 x g for 5 min then resuspended in 30 ml 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (pH 7) containing 100 mM potassium thiocyanate (buffer A). Cells were then lysed by sonication using a Branson Sonifer 450 with 100% output and 4 1-minute intervals, maintaining the cell extract on ice to prevent heating. The cell lysate was clarified by centrifugation at 2,500 x g for 90 minutes. The resulting supernatant was applied to a nickel–nitrilotriacetic acid agarose column (Sigma;10 ml) and washed with buffer A until the absorbance at 280 nm was baseline. The his-tagged proteins were eluted from the column with 100 ml of buffer A containing 0.15 M imidazole. Peak protein fractions were pooled, concentrated using a centrifugal concentrator (YM-30 membrane), and then dialyzed against 50 mM MOPS buffer (pH 7).

Determination of growth rates and K_s.

S. Typhimurium was grown aerobically at 37°C in MOPS minimal medium containing various carbon and nitrogen sources. Cell growth was measured using a Klett colorimeter (model 900-3) with a green (520 to 580 nm) glass filter. Doubling times (DT) were calculated using the equation DT = ln(2)/b, where b is the best-fit constant from a plot of log values of Klett units versus incubation time. The substrate concentration at half-maximal growth rate (K_s) for fructoselysine was estimated by growing cells in minimal media containing 20 mM L-arabinose as the primary carbon source and fructoselysine at concentrations ranging from 0 to 4 mM as the

sole nitrogen source. Cells were grown in 96-well plates at 37°C in a BioTek ELx808 absorbance microplate reader (BioTek, Winooski, VT). Each culture condition was performed with three biological replicates. Absorbance was measured every 30 min for 24 h. Generation times for each condition were calculated as described above, after plotting log values of absorbance versus incubation times. The K_s value for fructoselysine was estimated from a double-reciprocal plot (Lineweaver-Burk plot) of 1/μ (μ=ln2/g) values versus 1/[fructoselysine].

Sugar-amine synthesis

Sugar-amines were synthesized as described in (19, 20). For the synthesis of fructoselysine, D-glucose (3.3 gm, 18.3 mmol) and N_α-(*tert*-butoxycarbonyl)-L-lysine (Boc-Lys-OH, Sigma Aldrich) (1 gm, 4 mmol) were refluxed in 50 mL of methanol for 5 hours under argon gas. The reaction was freeze dried and the resulting solids were resuspended in 16.7 mL of 1 M HCl to remove the *tert*-butoxycarbonyl protecting group. The HCl was removed under vacuum and the product was resuspended in 20 mL distilled water. The sample was incubated with 5 g of BioRad (Ag 50W X-8) cation exchange resin for 1 hour to separate unreacted sugar from the desired product. After the separation, the resin was incubated in 30 mL NH_4OH to remove the product from the resin. The sample was decolorized with charcoal, filtrated and then freeze dried. The yield for the synthesis ranged from 32 to 53%. Synthesis of glucoselysine was essentially the same as that described for fructoselysine, except fructose (3.3 gm, 18.3 mmol) was used in place of glucose. The Heyns rearrangement result in several products following the reaction of fructose with Boc-Lys-OH and we did not determine what proportion of the final product was glucoselysine.

Ribuloselysine was formed by reacting D-ribose (0.6 gm, 4 mmols) with Boc-Lys-OH 0.22 gm, 0.9 mmol) for 5 hours at 50^{o}C in 50 mL methanol as described (Fortpied, 2005). The Boc protecting group was removed using HCl and ribuloselysine was separated from the unreacted D-ribose using the cation exchange resin as described for the synthesis of fructoselysine. Erythrulose was synthesized by reacting D-erythrose (0.12 gm, 1 mmol) with Doc-Lys-OH (0.22 gm, 0.9 mmol) in 50 mL methanol for 1 h at 40^{o}C as described (Fortpied, 2005), the Boc protecting group was removed, and erythruloselysine was separated using the cation exchange resin as described above. The yields for ribuloselysine and erythruloselysine were both ~56%. Other sugar-amines were synthesized and purified following the same general procedure used for fructoselysine synthesis. Tagatoselysine was synthesized by reacting D-galactose (3.3 gm, 18.3 mmol) with Boc-Lys-OH (1 gm, 4 mmols), and the final yield was ~25%. Fructose-D-lysine was formed by reacting glucose (3.3 gm, 18.3 mmol) with Boc-D-Lys-OH (1 gm, 4 mmol), and the final yield was ~27%. Fructose-ornithine was formed by reacting D-glucose (3.3 gm, 18.3 mmol) with N_{α}-*tert*-butoxycarbonyl-L-ornithine (0.94 gm, 4 mmol; Sigma Aldrich), and the final yield was ~44%. All products were verified using ^{1}H nuclear magnetic resonance (NMR) and mass spectrometry (Supplemental Data).

Construction of mutant strains.

The *S.* Typhimurium mutants indicated in Table 5.S1 were constructed using the λ Red recombineering method (21). Plasmid pKD4, which contains a kanamycin resistance (*kan*) cassette flanked by two Flp recognition sights (FRT sites), was used to PCR amplify linear DNA which included sequences at the ends that were homologous to the genes targeted for deletion. Primer sets used to generate the amplicons for targeted mutagenesis are listed in Table 5.S3. The amplicons were introduced into *S.* Typhimurium 14028s bearing the plasmid pKD46, which

carries the λ phage recombinase genes under the control of the *araBAD* promoter, by electroporation. DNA isolated from the resulting kanamycin-resistant colonies was checked by PCR, using primers that flanked the target gene deletion (primers are listed in Table 5.S3), to confirm that the target gene had been deleted. Mutant alleles in which the target genes had been replaced with the kanamycin-resistance (*kan*) cassette were moved into a clean genetic background by transduction using P22 HT *int*. The *kan* cassette was then removed by introducing plasmid pCP20 into the mutant strains by electroporation. Plasmid pCP20 expresses the Flp recombinase, which recognized the FRT sites and excised the *kan* cassette. Loss of the *kan* cassette was confirmed by susceptibility to kanamycin and by PCR using the same flanking primers (listed in Table 5.S3).

Fructoselysine-6-phosphate deglycase assay.

Deglycase assays were carried out as previously described (8). Fructoselysine-6-phosphate was generated by incubating 5 mM fructoselysine, 10 mM ATP, 10 mM $MgCl_2$, 50 mM HEPES, pH 7, and 5 units/ml of purified *E. coli* fructoselysine kinase (YhfQ) for 30 min at 30°C. Fructoselysine kinase was purified as described previously (22). Deglycase activity was measured spectrophotometrically at 340 nm in a coupled assay through the formation of glucose-6-phosphate. The mixture contained 50 mM HEPES, pH 7.1, 50 mM KCl, 5 mM $MgCl_2$, 0.25 mM NADP, 0.1 mM fructoselysine-6-phosphate, and 5μg glucose-6-phosphate dehydrogenase (Sigma), and various concentrations of purified GfrF to obtain linear slopes.

Testing fructoselysine analogs for growth inhibition.

The fructoselysine analogs fructose-D-lysine and fructose-L-ornithine were examined for their ability to inhibit growth of S. Typhimurium 14028s in MOPS minimal medium containing

20 mM L-arabinose and either 10 mM ammonium chloride or 5 mM fructoselysine. *S.* Typhimurium cultures grown aerobically overnight in either medium were used to inoculate fresh medium containing various concentrations of the fructoselysine analogs (0 to 20 mM) in 96 well plates (0.2 ml growth medium per well) at 37°C in a BioTek ELx808 absorbance microplate reader (BioTek, Winooski, VT). Each culture condition was performed with three biological replicates. Absorbance was measured every 30 min for 24 h. Absorbance vs time was plotted using Prism and resulting growth trends were analyzed.

qRT-PCR assays.

Transcript levels of *gfrAB* in wild-type *S.* Typhimurium 14028s cultured in minimal medium that contained 20 mM L-arabinose and either 5 mM fructoselysine or 10 mM ammonium were assessed by quantitative reverse transcription-PCR (qRT-PCR). Ten milliliters of cell culture grown to mid-log phase was used for RNA extraction. RNA was isolated using a boil method (23). RNA was then treated with RQ1 DNase (Promega). cDNA was synthesized using RT Superscript III synthesis kit (Invitrogen), after which qRT-PCR was carried out using an iCycler iQ system (Bio-Rad). Primer specificity was confirmed by PCR using a genomic DNA template. Three biological replicates and three technical replicates were assessed for expression of *gfrAB.* All expression of *gfrAB* was normalized to levels of *rpoD*, an internal control for *S.* Typhimurium gene expression. All reactions total 20 µL and included 10µL of iQ SYBR green Supermix (Bio-Rad), 5µL off 100-fold diluted cDNA, and 200nM of each primer specific for either *gfrAB* or *rpoD.* Gene expression levels were determined using the equation $2^{-\Delta\Delta CT}$ (24). The fold change of *gfr* expression of cells grown with fructoselysine compared to ammonium for all three biological replicates were averaged and standard deviation was calculated. A Student T-test was preformed to calculate P-value.

Mass spectrometry analysis

To examine intracellular metabolites in S. Typhimurium grown with fructoselysine, *S.* Typhimurium cultures were grown overnight in MOPS minimal media containing either 20 mM L-arabinose and 13 mM ammonium or 20 mM fructoselysine at 37°C. 1 mL of overnight culture was subjected to 10 cycles of freezing and thawing using a dry ice /ethanol bath. After the last thaw, cell debris was removed by centrifugation at 6,000 x g for 1 min and the resulting supernatant was removed for analysis by electrospray mass spectrometry using a Burker Esquire 3000 Plus mass spectrometer.

Results

A mannose family PTS permease in *S.* Typhimurium is required for utilization of fructoselysine and glucoselysine. Wiame and co-workers identified deglycases in *E. faecium* that use fructoselysine-6-P and glucoselysine-6-P as substrates (8). The genes encoding these enzymes appeared to be part of an operon with genes encoding a mannose family PTS permease. They proposed that the genes in the operon were involved in the transport and catabolism of fructoselysine and glucoselysine. Additionally, they noted that *S.* Typhimurium possesses homologs to the PTS and deglycase genes and postulated that the *S.* Typhimurium homologs were similarly involved in fructoselysine and glucoselysine transport and catabolism (8). We wished to test this hypothesis by seeing if *S.* Typhimurium could use fructoselysine and glucoselysine as carbon or nitrogen sources, and if so, whether the PTS and deglycase genes were required for the utilization of these compounds.

At 37^{o}C under aerobic conditions, wild-type *S.* Typhimurium grew in a minimal medium with fructoselysine as the sole carbon source with a doubling time of 88 ± 5 min (Table 5.1).

The minimal medium contained 13 mM ammonium chloride as a nitrogen source, and *S.* Typhimurium grew somewhat better when ammonium chloride was withheld from the medium (80 ± 3 min doubling time). Including L-arabinose in the minimal medium with fructoselysine further improved the growth rate of *S.* Typhimurium (55 ± 2 min doubling time). By comparison, the doubling time of *S.* Typhimurium in minimal medium containing L-arabinose as a carbon source and ammonium chloride as a nitrogen source was 44 ± 2 min (Table 5.1). Using minimal medium that contained L-arabinose and fructoselysine, we estimated the *Ks* (concentration that gives half maximal growth rate) for fructoselysine to be 0.44 mM. Taken together, these data indicate that fructoselysine is a good nitrogen source for *S.* Typhimurium and is a physiologically relevant substrate.

Glucoselysine was similarly used by *S.* Typhimurium as a carbon and nitrogen source, although it did not support the same rapid growth observed with fructoselysine (Table 5.1). In minimal medium in which glucoselysine was the sole carbon source (glucoselysine plus ammonium), sole carbon and nitrogen source (glucoselysine only), or sole nitrogen source (L-arabinose plus glucoselysine), doubling times for *S.* Typhimurium cultures were 131 ± 13 min, 140 ± 8 min, and 124 ± 12 min, respectively. The reason for the slower growth rate of *S.* Typhimurium with glucoselysine compared to fructoselysine could be due to a couple of reasons. First, fructoselysine may be transported or catabolized by *S.* Typhimurium more efficiently than glucoselysine. Alternatively, the synthesis of glucoselysine likely resulted in a mixture of Heyns products and glucoselysine may not have been the major end product or some of the other Heyns products may have inhibited growth of *S.* Typhimurium.

Genes encoding the EIIAB components (*gfrAB* homologs) or the deglycases (*gfrE* and *grfF* homologs) were deleted to assess their requirement for growth on fructoselysine and

glucoselysine. The *gfrABCDEF* operon has a predicted RpoN-dependent promoter and a gene encoding a putative RpoN-dependent activator immediately upstream, and we also deleted this gene (*gfrR*). The Δ*gfrAB*, Δ*gfrR*, Δ*gfrE,* and Δ*gfrF* mutants were examined for their ability to grow in minimal media with fructoselysine or glucoselysine as the sole carbon source. The Δ*gfrAB* and the Δ*gfrR* mutants failed to grow in minimal media with either fructoselysine or glucoselysine as the sole carbon source, indicating that the *gfr* locus is responsible for both fructoselysine and glucoselysine utilization. The Δ*gfrE* failed to grow in minimal medium in which glucoselysine was the sole carbon or nitrogen source, but grew normally in minimal medium that contained fructoselysine as the sole carbon and nitrogen source (doubling time was 83 ± 4 min). Conversely, the Δ*gfrF* mutant failed to grow in minimal medium that contained fructoselysine as the sole carbon or nitrogen, but grew normally in minimal medium containing glucoselysine as the sole carbon and nitrogen source (doubling time of 113 ± 13 min). These results were consistent with the prediction that GrfE and GrfF are deglycases for glucoselysine-6-phosphate and fructoselysine-6-phosphate, respectively (8) .

Consistent with the prediction that transcription of the *gfrABCDEF* operon is dependent on RpoN, a Δ*rpoN* mutant failed to grow in minimal media containing fructoselysine or glucoselysine as the sole carbon source. For these phenotypic assays the medium was supplemented with 5 mM L-glutamine as deletion of *rpoN* results in glutamine auxotrophy (25). L-glutamine is a poor nitrogen source and does not support growth of *S.* Typhimurium at the concentration used for the experiment. A Δ*ptsH* mutant (*ptsH* encodes HPr) likewise failed to grow in minimal medium containing either fructoselysine or glucoselysine. *S.* Typhimurium 14028s possesses a paralog of HPr (STM14_4558) and two additional HPr homologs fused to other PTS components (FruF and PtsA). The inability of the Δ*ptsH* mutant to utilize

fructoselysine or glucoselysine indicates that these other HPr homologs are either unable to substitute for HPr in the transport of fructoselysine and glucoselysine or are not expressed at sufficient levels under the assay conditions.

Regulated expression of the *gfr* locus

GfrR possesses a C-terminal PTS Regulation Domain (PRD), a regulatory domain found in RpoN-dependent activators of PTS operons such as *Bacillus subtilis* LevR (26). LevR regulates expression of the *levDEFGsacC* operon, which is required for fructose transport and catabolism (26). In the absence of fructose in the medium, the LevR PRD is phosphorylated at conserved histidine by the EIIBC component of the fructose-specific PTS permease (i.e., LevEF) which inhibits the activity of LevR (26). To determine if fructoselysine induces expression of the *gfrABCDEF* operon, *gfrAB* transcript levels in *S.* Typhimurium grown in minimal medium containing 20 mM L-arabinose and either 5 mM fructoselysine or 10 mM ammonium chloride were measured by qRT-PCR. For these assays, *gfrAB* transcripts were normalized to *rpoD* transcript levels. Levels of *gfrAB* transcripts were 13-fold higher (13 ± 4.6) (P-value <0.01) in cultures grown with fructoselysine as a nitrogen source compared to cultures in which ammonium was the nitrogen source, indicating that fructoselysine induces expression of the *gfrABCDEF* operon.

The *S.* Typhimurium D-glucosaminate PTS permease, which is encoded with the *dgaABCDEF* operon, is another member of the mannose family PTS permease. Transcription of *dgaABCDEF* is controlled by the RpoN-dependent activator DgaR which, like GfrR, has a PRD at its carboxy-terminus. The presence of glucose in the medium prevents *S.* Typhimurium from utilizing D-glucosaminate suggesting that expression of the *dga* locus is subject to catabolite repression (27). *S.* Typhimurium was able to grow in minimal medium containing glucose and

fructoselysine as the sole nitrogen source indicating that, in contrast to the *dga* locus, expression of the *gfr* locus is not subject to catabolite repression by glucose.

GfrF has a fructoselysine-6-phosphate deglycase activity.

Wiame and co-workers showed that *E. faecium* GfrE and GfrF have glucoselysine-6-phosphate deglycase and fructoselysine-6-phosphate deglycase activity, respectively, and proposed that the *S.* Typhimurium counterparts have the same activities (8) . The phenotypes of the *S.* Typhimurium *gfrE* and *gfrF* mutants were consistent with the activities for the products of these genes proposed by Wiame and co-workers. To verify the proposed activities of *S.* Typhimurium GfrE and GfrF, we expressed his-tagged versions of the proteins in *E. coli* and attempted to purify the proteins by immobilized nickel-affinity chromatography. Unfortunately, we were unable to purify GfrE as it was insoluble under all of the conditions we tested for inducing its expression, including induction at temperatures as low as 4°C. We were able to purify GfrF, however, and assay the purified protein for fructoselysine-6-phosphate deglycase activity using a coupled enzyme assay with glucose-6-dehydrogenase in which formation of glucose-6-phosphate from fructoselysine-6-phosphate was monitored spectrophotometrically at 340 nm. The specific activity of the purified *S.* Typhimurium GfrF was 0.78 µmol/min/mg, which was similar to the specific activity reported for *Enterococcus faecium* GfrF (0.4 µmol/min/mg) (8).

Examining the substrate specificity of the fructoselysine/glucoselysine PTS permease and GfrF.

To examine the substrate specificities of the fructoselysine/glucoselysine PTS permease and GfrF, we synthesized analogs of fructoselysine and other sugar-amines. In addition, we

determined if the fructoselysine analogs inhibited growth of *S.* Typhimurium when fructoselysine was present in the medium as a nitrogen source. Fructoselysine analogs were synthesized using either D-lysine or L-ornithine, an amino acid with a side chain that is one carbon shorter than the side chain of lysine. *S.* Typhimurium was able to use fructose-D-lysine as a carbon source but not as a nitrogen source. These data indicate that fructose-D-lysine is transported and phosphorylated by the fructoselysine/glucoselysine PTS permease and the resulting fructose-D-lysine-6-phosphate is recognized by GfrF, but that D-lysine is not utilized as a nitrogen source. S. Typhimurium was unable to use fructose-ornithine as a carbon or nitrogen source, suggesting that it is not recognized as a substrate by the fructoselysine/glucoselysine PTS permease and/or GfrF.

We tested the ability of the fructoselysine analogs to inhibit growth of S. Typhimurium in minimal medium that contained L-arabinose as the primary carbon source and either ammonium chloride (10 mM) or fructoselysine (5 mM) as the nitrogen source. Cells grown with fructoselysine as the nitrogen source were not inhibited by fructose-D-lysine or fructose-L-ornithine at concentrations ranging from 2 to 20 mM of the analog.

Ribuloselysine, erythuloselysine and tagatoselysine were synthesized and tested for their ability to support growth of *S.* Typhimurium. These compounds were chosen since they are derived from the reaction of three relatively common sugars (ribose, erythrose and galactose) with the ε-amine of lysine. None of these compounds supported the growth of *S.* Typhimurium when included in the medium as either the sole carbon or nitrogen source indicating that the fructoselysine/glucoselysine PTS permease and/or GfrF (or GfrE) does not recognize these compounds as substrates.

The reason *S*. Typhimurium is able to use fructoselysine as a nitrogen source, but not lysine, remains a mystery.

Gutnick and Ames reported that *S*. Typhimurium LT2 is unable to use L-lysine as a nitrogen source (2). We confirmed that *S*. Typhimurium 14028s is unable to grow in minimal medium with L-lysine as the sole nitrogen source. We postulated that when *S*. Typhimurium is grown on fructoselysine, lysine accumulates to high levels inside the cell which allows the bacterium to use lysine as a nitrogen source, and such intracellular levels of lysine cannot be achieved by the lysine transporters. We reasoned that one or more transaminases in the cell may be able to use lysine as a substrate when intracellular levels of lysine are high which would allow the bacterium to use lysine derived from fructoselysine as a nitrogen source. Deamination of L-lysine results in (*S*)-2-amino-6-oxohexanoate, which undergoes a spontaneous intramolecular dehydration to form Δ^1-piperideine-6-carboxylate. To test our hypothesis, we used mass spectrometry to examine *S*. Typhimurium grown with fructoselysine for the accumulation of extracellular or intracellular Δ^1-piperideine-6-carboxylate.

S. Typhimurium cultures were grown to stationary phase in a minimal medium that contained 20 mM fructoselysine as the sole carbon and nitrogen source at which point the cells were removed from the medium by centrifugation and the resulting supernatant liquid was analyzed. Alternatively, part of the culture was subjected to multiple freeze-thaw cycles to lyse the cells which were then removed by centrifugation and the resulting supernatant liquid was analyzed. We did not observe an obvious peak for the expected mass of Δ^1-piperideine-6-carboxylate (m/z 128.1, $(M+1)^+$) in the mass spectra of either the extracellular-only fraction or the combined extracellular and intracellular fractions. From the mass spectra, however, we noted that the fructoselysine had been depleted from the spent medium and observed a prominent peak

with the mass expected for lysine (m/z 147.2, $(M+1)^+$) in the mass spectrum of the sample of the combined extracellular and intracellular fractions (Fig. 5.S1). We also observed a prominent peak with the mass expected for histidine (m/z 156.1, $(M+1)^+$) in the mass spectrum of the sample of the combined extracellular and intracellular fractions (Fig. 5.S1). Neither peak was evident in the mass spectrum of extracts of *S.* Typhimurium grown in minimal medium with arabinose as the carbon source and ammonium as the nitrogen source (data not shown).

In the histidine biosynthesis pathway, histidinol-phosphate aminotransferase (HisC) catalyzes the conversion of imidazole acetol-phosphate to histidinol-phosphate using L-glutamate as an amino donor. We hypothesized that HisC uses lysine as an amino donor when intracellular concentrations of lysine are high and this reaction allows *S.* Typhimurium to use fructoselysine as a nitrogen source. HisC is known to be promiscuous, reacting with phenylalanine and tyrosine as an amino donor, as well as glutamate (28). To test the hypothesis that HisC is needed for *S.* Typhimurium to use fructoselysine as a nitrogen source, we deleted *hisC* in *S.* Typhimurium 14028s and examined the growth of the resulting mutant in medium that contained fructoselysine (5 mM) as the primary nitrogen source. Since the Δ*hisC* mutant is a histidine auxotroph, the medium was supplemented with 140 μM L-histidine, an amount sufficient to allow growth of the mutant but insufficient to support a significant growth yield. Compared to the wild-type strain the Δ*hisC* mutant grew somewhat slower on medium containing fructoselysine as the primary nitrogen source (116 ± 13 min doubling time for the mutant versus 80 ± 3 min for wild type), but growth yields for the two strains on this medium were the same indicating that HisC is not essential for catabolism of lysine derived from fructoselysine.

S. Typhimurium GfrD was reported to modulate the activity of CadC (29), a positive regulator of the *cadBA* which encode a lysine/cadaverine antiporter and lysine decarboxylase, respectively, and are involved in acid stress response in *S.* Typhimurium. We postulated that growth of *S.* Typhimurium on fructoselysine may up-regulate expression of *cadA* and allow the bacterium to use lysine derived from fructoselysine as a nitrogen source. To test this hypothesis we disrupted *cadA,* as well as *cadC,* in *S.* Typhimurium 14028s, but found that both mutant strains were able to use fructoselysine as a nitrogen source. *S.* Typhimurium possesses a second lysine decarboxylase, LdcC, and so we constructed a mutant strain that lacked both *cadA* and *ldcC.* Growth of the *cadA*/*ldcC* double mutant with fructoselysine as a nitrogen source was slightly impaired but not blocked (doubling time of 99 ± 14 min for the mutant versus 80 ± 3 min for wild type), indicating that decarboxylation of lysine derived from fructoselysine is not essential for *S.* Typhimurium to utilize it. Consistent with this observation, deletion of genes involved in cadaverine degradation (*patA*, *patD*and *gabT*) did not significantly impair *S.* Typhimurium in its ability to use fructoselysine as a nitrogen source (data not shown).

Discussion

We verified that the *S.* Typhimurium *gfr* locus encodes a mannose family PTS permease and associated deglycases required for the transport and catabolism of fructoselysine and glucoselysine (Fig. 5.2). The *gfr* locus allows *S.* Typhimurium to use fructoselysine and glucoselysine as both carbon and nitrogen sources, although these compounds support more rapid growth of *S.* Typhimurium as nitrogen sources than as carbon sources. In the initial steps of the Maillard reaction, Amadori products (fructosamines when glucose is the reacting sugar) and Heyns products (e.g., glucoselysine) are formed by the spontaneous reaction of the carbonyl groups of reducing sugars with the free amino groups of proteins or amino acids.

Microorganisms likely encounter Amadori products frequently in their environments since a variety of microorganisms are able to utilize these compounds. Enteric bacteria, such as *Salmonella*, may encounter fructoselysine from glycated proteins in its hosts' diets as the passage of fructosamines through the intestinal mucosa is very limited (12). Other Amadori products encountered by enteric bacteria could result from the glycation of peptides or amino acids during digestion. In soil environments, the Maillard reaction takes place during the formation of humic substances (12). Significant levels of Amadori products are also present in rotting fruits and vegetables (11), as well as dried fruits, nuts and grains (12).

A couple of different types of pathways for the catabolism of fructosamines have been described to date. Fructosyl amino acid oxidases (also known as Amadoriases) catalyze the oxidative degradation of fructosamines to generate the corresponding amino acid, glucosone and hydrogen peroxide (30). Amadoriases have been found in fungi such as *Aspergillus* and *Penicillium*, as well as bacteria such as *Arthrobacter*, *Pseudomonas* and *Corynebacterium* (30). A second type of pathway is found in *E. coli* and *B. subtilis* and involves phosphorylation of the fructosamine by a fructosamine 6-kinase (FlrD) and a subsequent cleavage by a deglycase (FrlB) to generate the corresponding amino acid and glucose-6-phosphate (22). The *E. coli* and *B. subtilis* FrlB/FrlD systems share about 30% amino acid identity but have different substrate specificities. The *E. coli* FrlB/FrlD system is specific for ε-glycated lysine, whereas the *B. subtilis* enzymes catalyze reactions with α-glycated amino acids and have a broader substrate specificity being able to recognize a number of different α-glycated amino acids (31). The *E. coli frl* locus includes a gene that encodes a fructoselysine 3-epimerase (*frlC*) which catalyzes the reversible interconversion of fructoselysine with its C-3 epimer, psicoselysine, and allows the bacterium to utilize this unusual Amadori product (32).

Ali and co-workers recently showed that *S.* Typhimurium possesses an FrlB/FrlD-like system that is required for utilization of α-glycated asparagine (i.e., fructose-asparagine) (33). The *S.* Typhimurium operon that contains the genes encoding the FrlB/FrlD-like system (*fraB* and *fraD*) also contains genes predicted to encode a transporter (*fraA*) and an asparaginase (*fraE*). FraE is thought to be localized to the periplasm where it converts fructose-asparagine to fructose-aspartate which is subsequently transported across the cell membrane by FlaA. Once inside the cell, fructose-aspartate is converted to aspartate and glucose-6-phosphate by FraB and FraD (33). The *fra* locus was found to be essential for *Salmonella* fitness in an inflamed mouse intestine model, suggesting that fructose-asparagine is a significant nutrient for *Salmonella* in the inflamed intestine (33).

The enzymes encoded within the *S.* Typhimurium and *E. faecium gfr* loci represent a variation on the FrlB/FrlD-like system used for utilization of Amadori products. In *E. coli*, fructoselysine and psicoselysine are transported across the cell membrane by the permease FrlA, while in *B. subtilis* the α-glycated amino acid substrates are transported across the membrane by an ABC transport system (FrlONM) (31). These Amadori products are subsequently phosphorylated by the FrlB kinase once inside the cell. In *S.* Typhimurium and *E. faecium*, fructoselysine and glucoselysine are transported by a mannose-type PTS permease which phosphorylates the sugar moieties of these compounds at the C-6 position as they are translocated across the cell membrane. As with the FrlD enzymes, the GfrE and GfrF deglycases in *S.* Typhimurium and *E. faecium* then cleave the phosphorylated products to generate lysine and either glucose-6-phosphate or fructose-6-phosphate.

Paradoxically, *S.* Typhimurium grows well with fructoselysine as a nitrogen source but not with lysine. We postulate that *S.* Typhimurium must accumulate lysine to high intracellular

levels to use it as a nitrogen source and such concentrations cannot be achieved through the lysine permeases. *S.* Typhimurium has at least two transport systems for lysine, the lysine/arginine/ornithine periplasmic transport protein (ArgT) which functions in concert with the HisQMP ABC transporter and the lysine-specific permease LysP (34). Consistent with the hypothesis that high intracellular levels of lysine are needed for S. Typhimurium to utilizes this amino acid as a nitrogen source, intracellular levels of lysine appeared to be high in *S.* Typhimurium grown in minimal medium containing fructoselysine as the sole carbon and nitrogen source (Fig. 5.S1). While we were unable to ascertain a pathway that was essential for catabolism of lysine derived from fructoselysine, it is possible that multiple pathways are involved in catabolism of endogenously generated lysine. The observation that both the *hisC* mutant and *cadA*/*ldcC* double mutant were slightly impaired in their ability to utilize fructoselysine as a nitrogen source is consistent with such a hypothesis.

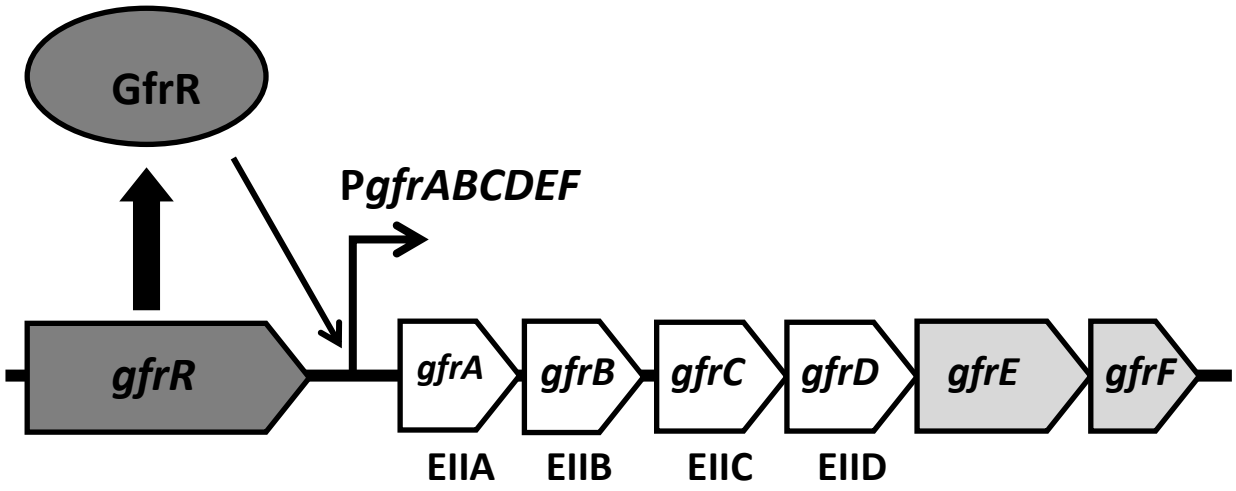

Figure 5.1. The *S.* Typhimurium *gfr* locus. The EIIA, EIIB, EIIC and EIID components of the fructoselysine/glucoselysine PTS permease are encoded by *gfrA*, *gfrB*, *gfrC* and *gfrD*, respectively. The other genes in the operon, *gfrE* and *gfrF*, are predicted to encode glucoselysine-6-phosphate deglycase and fructoselysine-6-phosphate deglycase, respectively. GfrR is an RpoN-dependent activator predicted to be required for transcriptional activation of *gfrABCDEF*.

	carbon source		
	L-arabinose	fructoselysine	glucoselysine
nitrogen source	doubling time (min)		
NH_4Cl	44 ± 2	88 ± 5	131 ± 13
fructoselysine	55 ± 2	80 ± 3	ND
glucoselysine	124 ± 12	ND	140 ± 8

Table 5.1. Growth rate of *S.* Typhimurium on various carbon and nitrogen sources.

Doubling times for glucoselysine and fructoselysine together were not determined (ND).

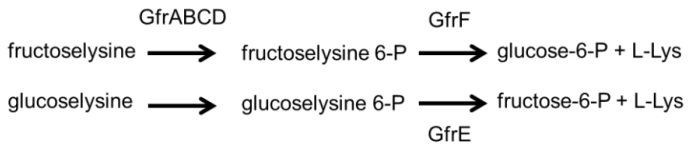

Figure 5.2. Proposed pathway for utilization of fructoselysine and glucoselysine. GfrABCD is a PTS permease that transports both fructoselysine and glucoselysine. Inside the cell, GfrF and GfrE work as deglycases and cleave fructoselysine 6-P and glucoselysine 6-P, respectively, resulting in the corresponding hexose-6-phosphates and lysine.

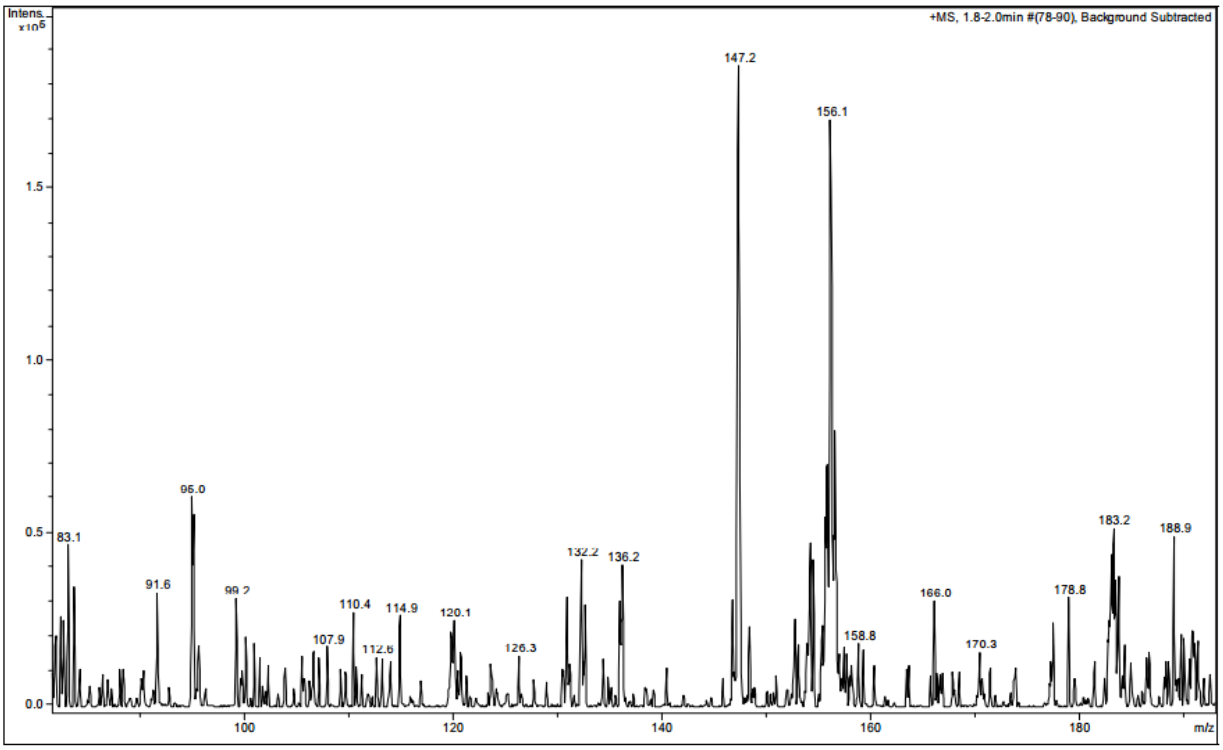

Figure 5.S1. Apparent accumulation of lysine and histidine in cells grown with fructoselysine. The mass spectrum of cell extracts prepared from *S.* Typhimurium grown on minimal medium containing fructoselysine as the sole carbon and nitrogen source revealed prominent peaks with the mass expected for lysine (m/z 147.2, $(M+1)^+$) and histidine (m/z 156.1, $(M+1)^+$). Both of these peaks were absent in the mass spectrum of extracts prepared from cells grown with arabinose and ammonium.

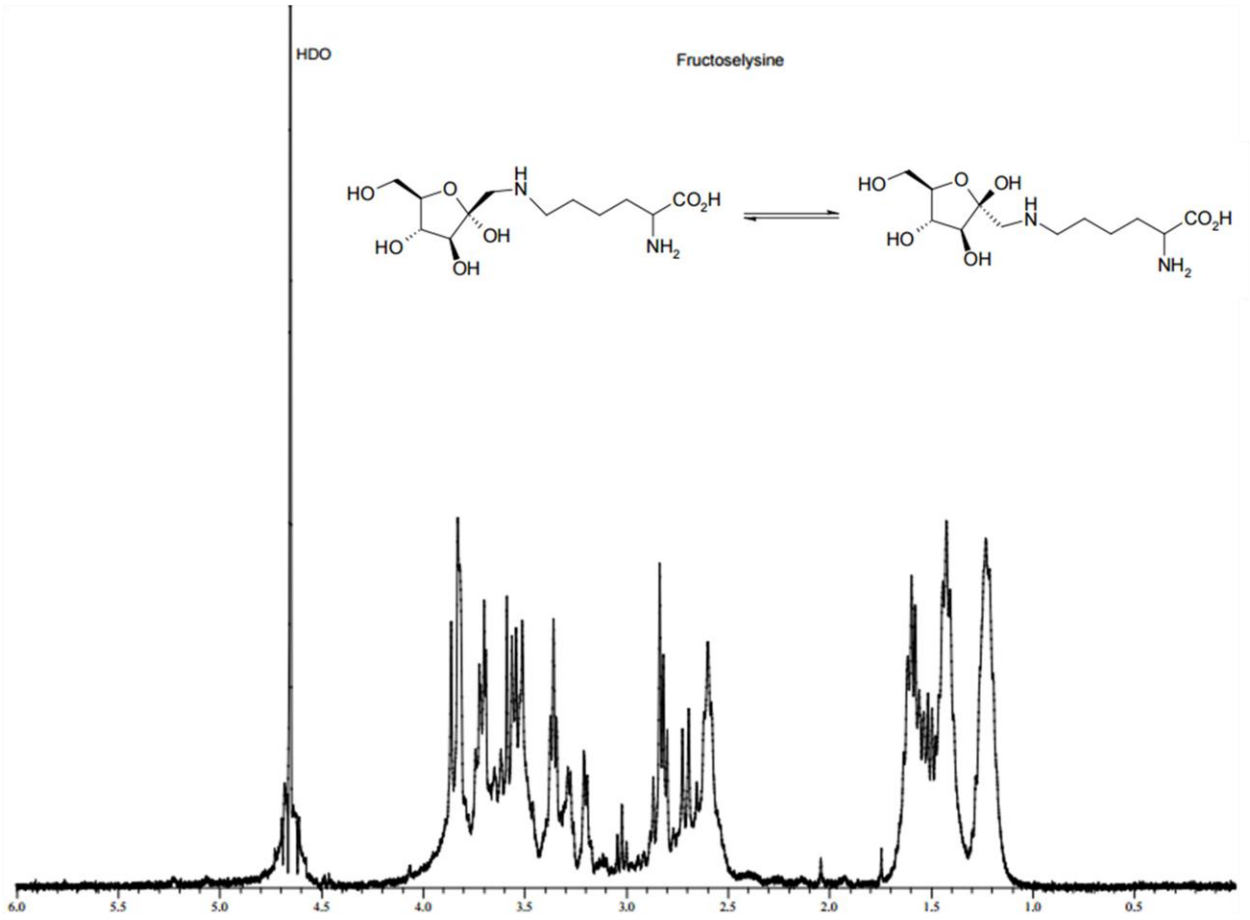

Figure 5.S2. H^1-NMR of fructoselysine. The H^1-NMR spectrum was similar to that reported by Wiame *et al*. (8). The structures of fructoselysine with the sugar in the alpha (right) and beta (left) forms are shown.

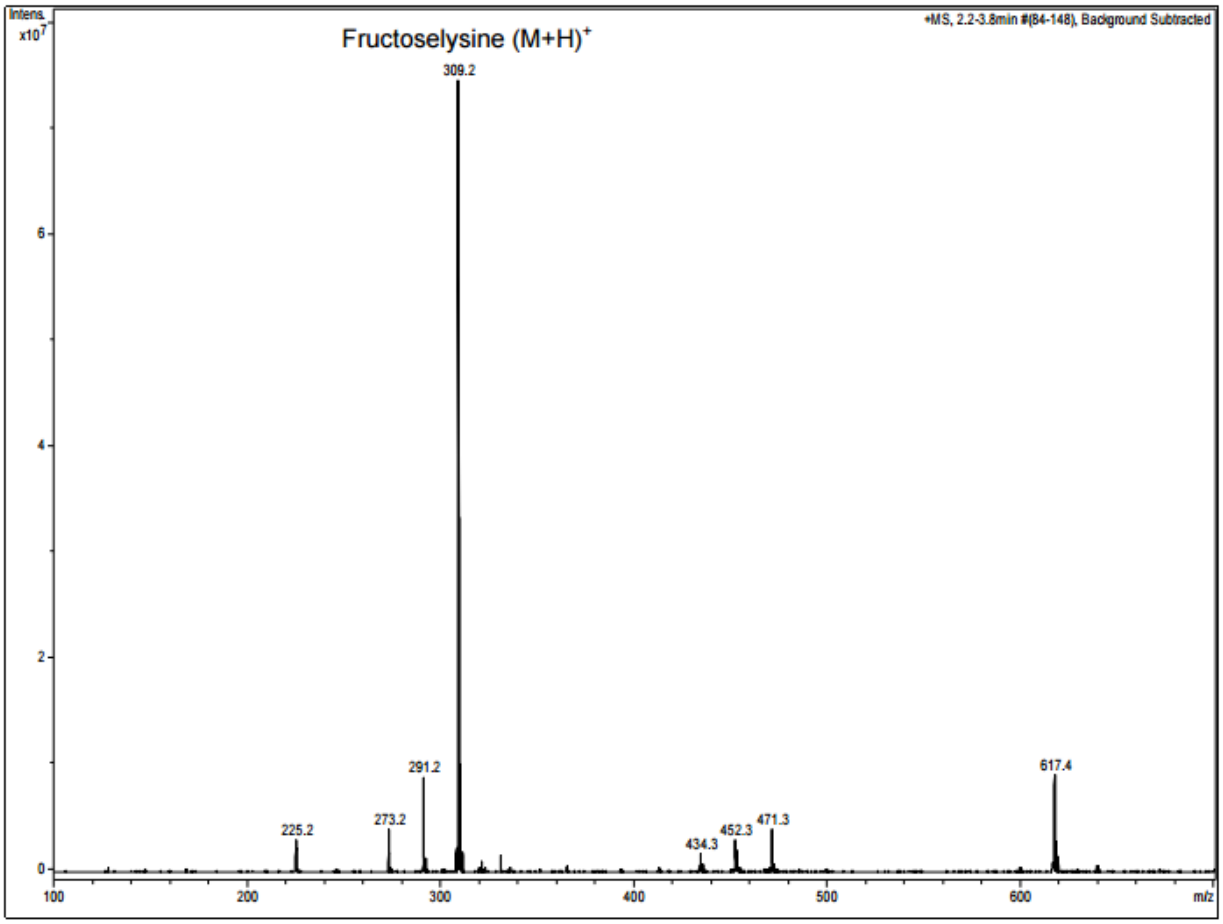

Figure 5.S3. Mass spectrum of fructoselysine. A prominent peak with the expected mass for fructoselysine (m/z 309, $(M+1)^+$) was observed in the mass spectrum of a sample of the final product. Note that there are not significant amounts of the reacting materials in the preparation: Boc-lysine (m/z 247.3, $(M+1)^+$); glucose (m/z 181.2, $(M+1)^+$); lysine (m/z 147.2, $(M+1)^+$).

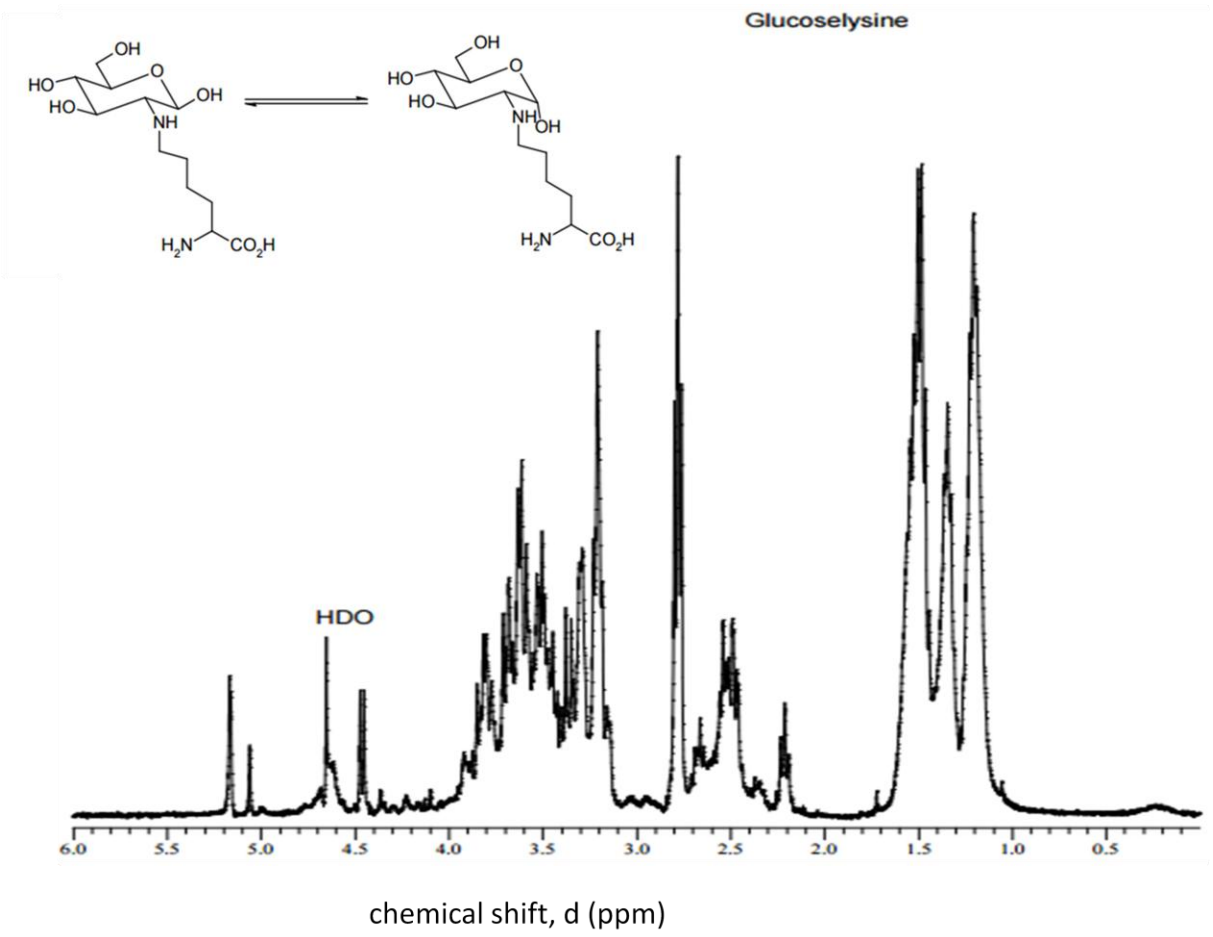

Figure 5.S4. H^1-NMR of glucoselysine. The H^1-NMR spectrum was as expected and included the two signals corresponding to the anomeric proton of glucose (δ = 5.2 and 5.1). The structures of glucoselysine with the sugar in the alpha (right) and beta (left) forms are shown.

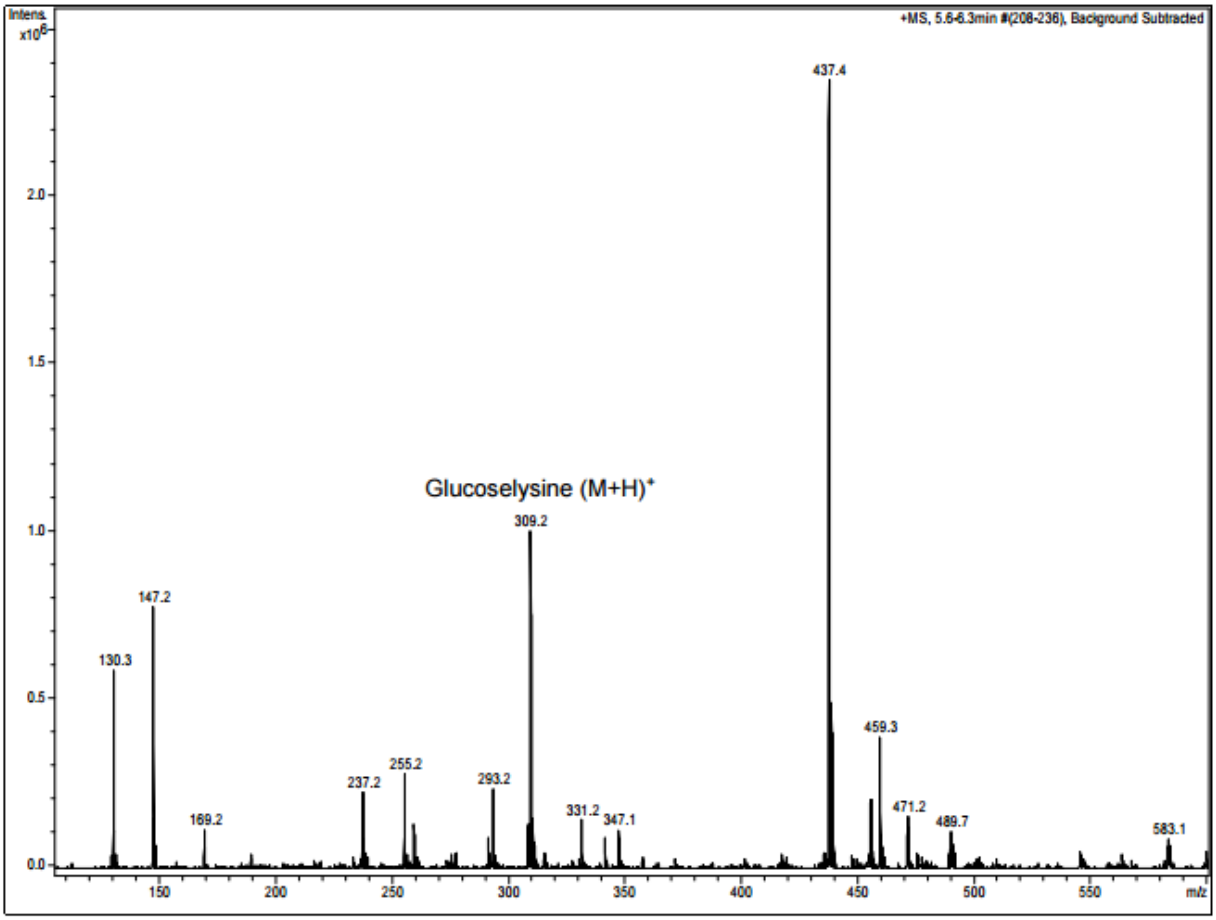

Figure 5.S5. Mass spectrum of glucoselysine. A peak with the expected mass for glucoselysine (m/z 309, $(M+1)^+$) was observed in a sample of the final product. Although there are not significant amounts of the reacting materials: Boc-lysine (m/z 247.3, $(M+1)^+$) or fructose (m/z 181.2, $(M+1)^+$), there does appear to be some lysine (m/z 147.2, $(M+1)^+$) in the final product.

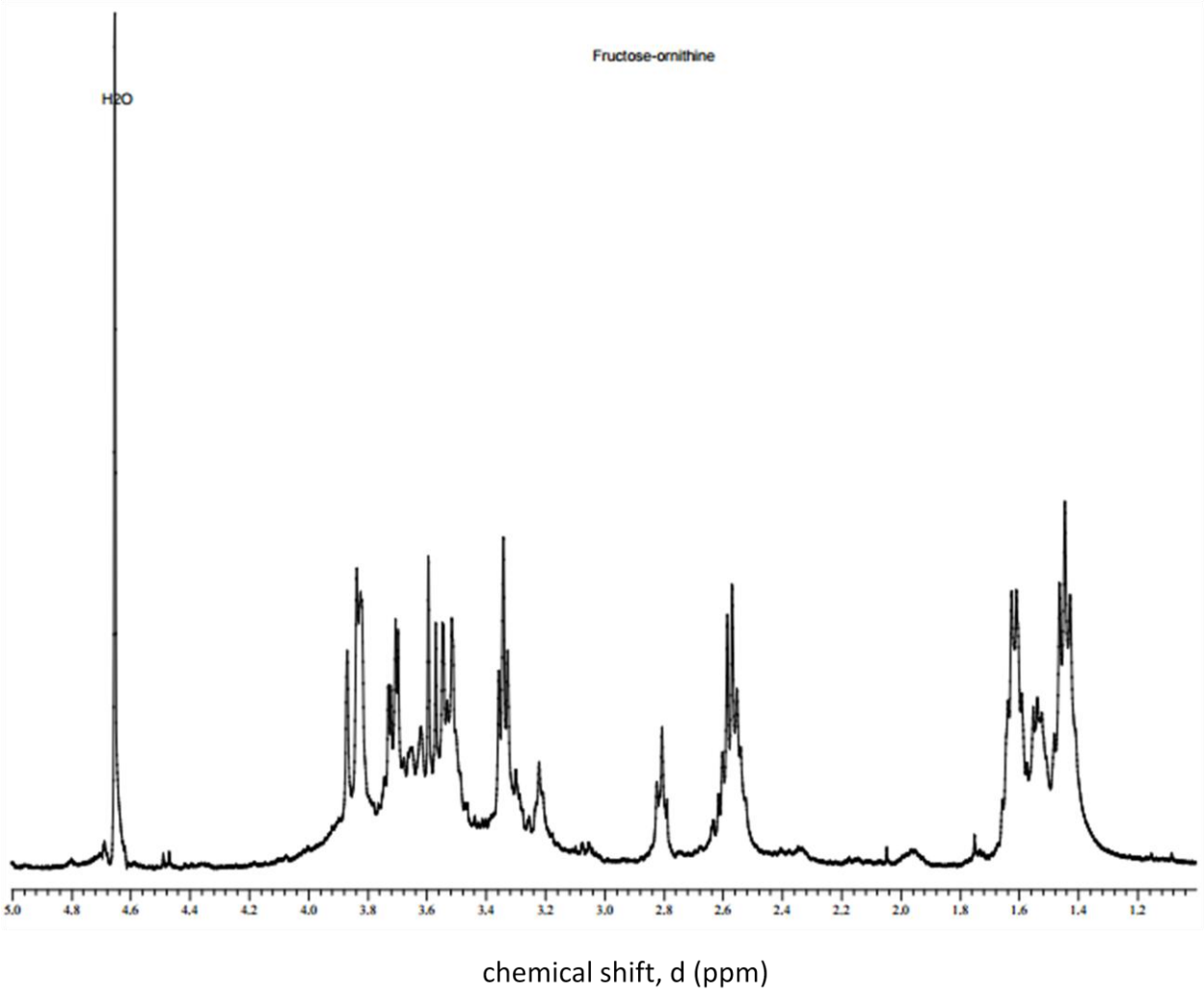

Figure 5.S6. H^1-NMR of fructose-ornithine. The H^1-NMR of the fructose-ornithine preparation was as expected.

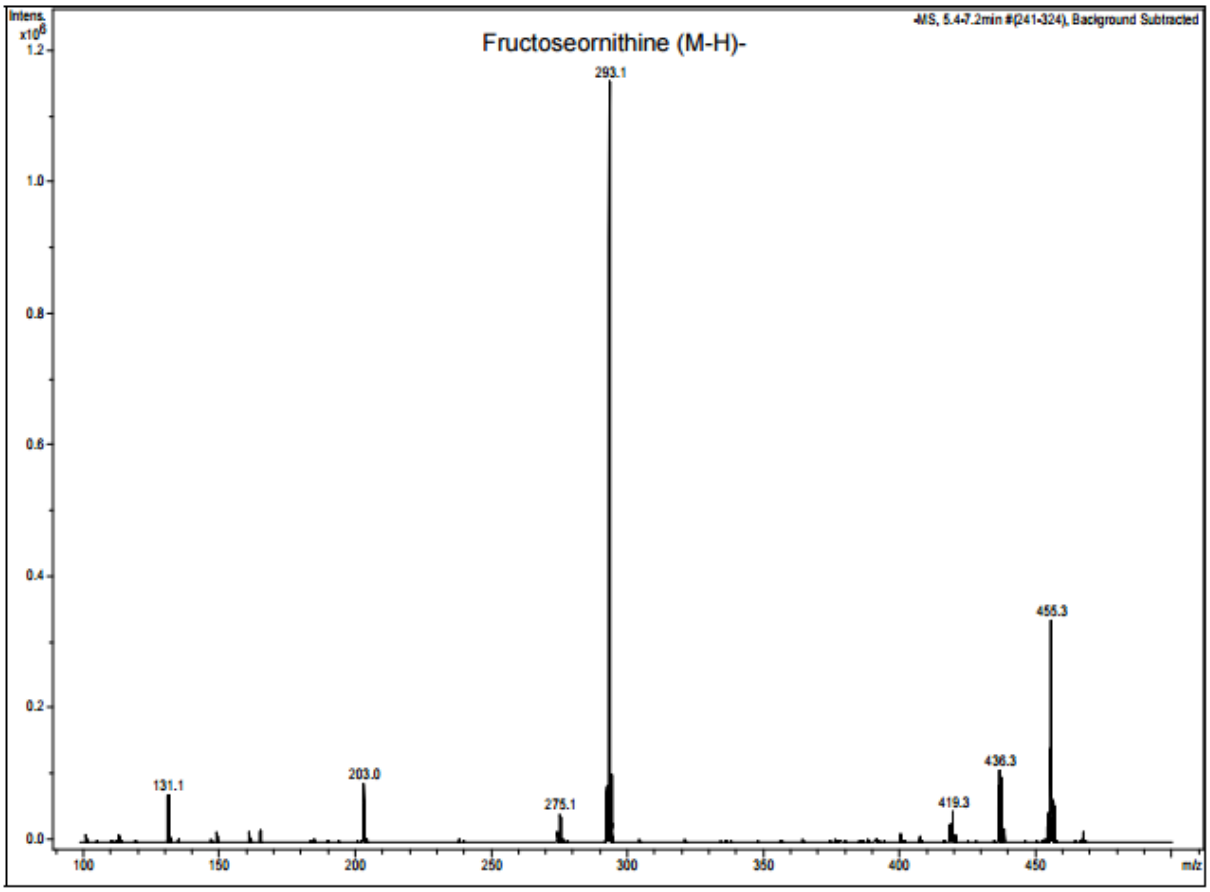

Figure 5.S7. Mass spectrum of fructose-ornithine. A prominent peak with the expected mass for fructose-ornithine (m/z 291, $(M-1)^-$) was observed in the final product. There appeared to be some ornithine (m/z 131.1, $(M-1)^-$) in the final product.

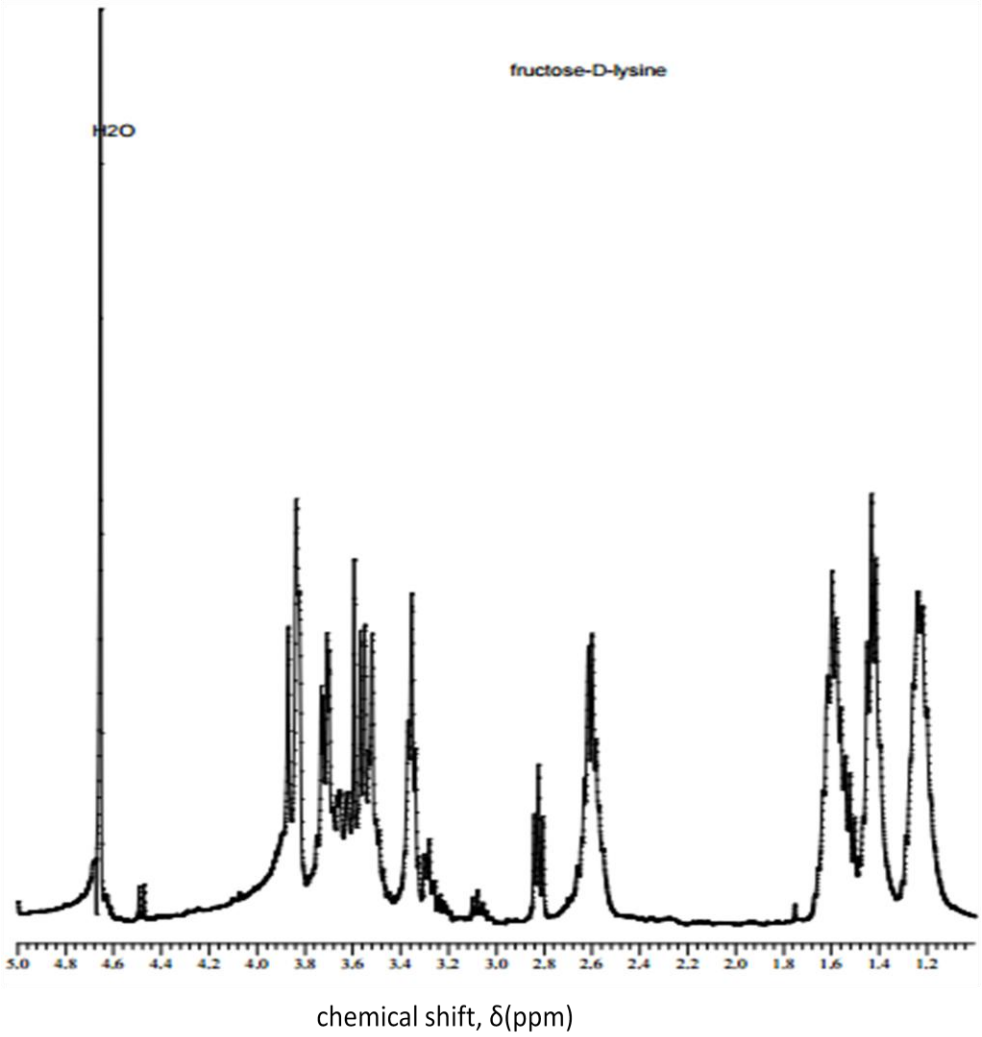

Figure 5.S8. H^1-NMR of fructose-D-lysine. The H^1-NMR spectrum was similar to that for fructose-L-lysine reported by Wiame *et al*. (8).

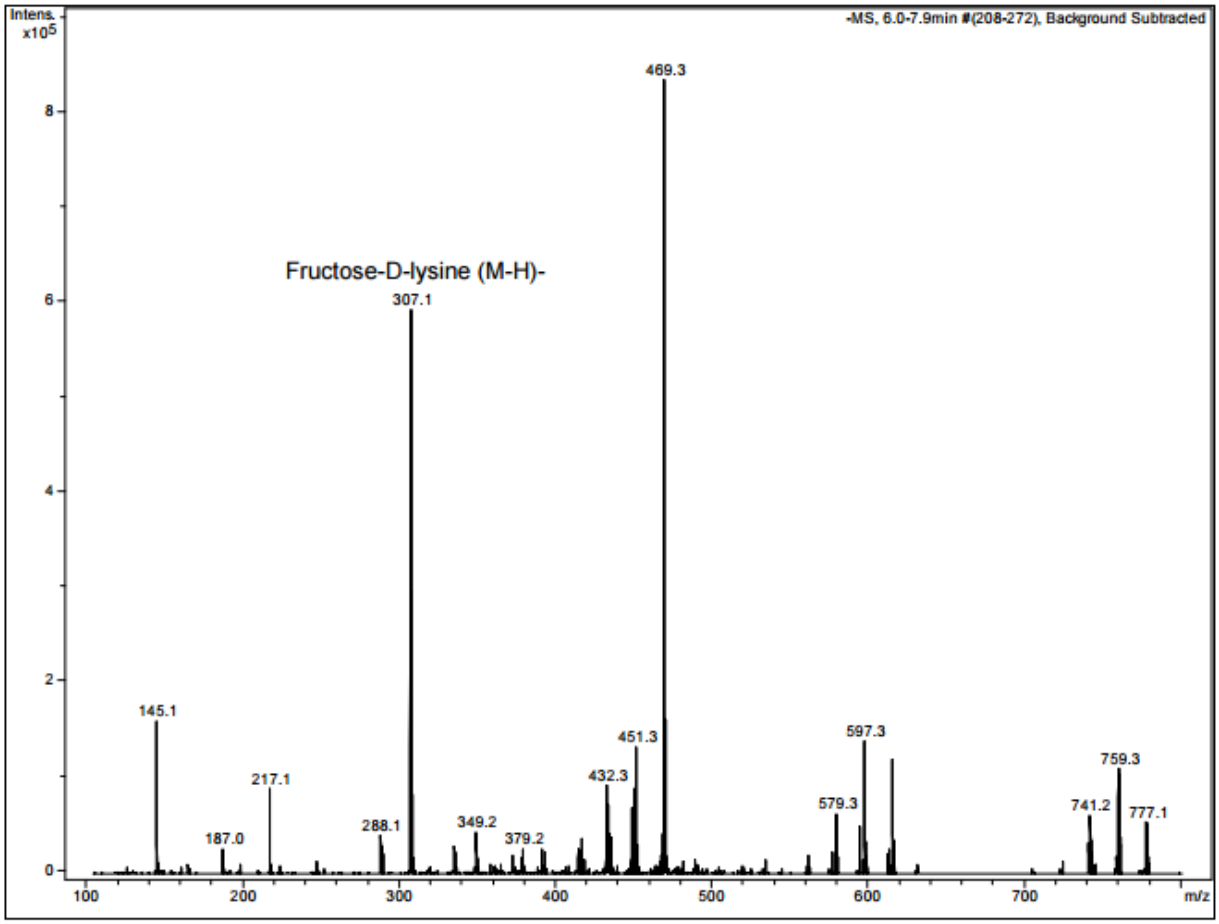

Figure 5.S9. Mass spectrum of fructose-D-lysine. A prominent peak with the expected mass for fructose-D-lysine (m/z 307.1, $(M-1)^-$) was observed in the final product. The final product appeared to have some unreacted lysine (m/z 145.1, $(M-1)^-$).

Table 5.S1. Bacterial strains used in this study.

species	strain	relevant genotype	source
S. Typhimurium	14028s	Wild-type	ATCC
S. Typhimurium	KAM31	Δ*gfrR::kan*	(27)
S. Typhimurium	KAM67	Δ*gfrR*	(27)
S. Typhimurium	KAM32	Δ*gfrAB::kan*	(27)
S. Typhimurium	KAM42	Δ*gfrAB*	(27)
S. Typhimurium	GLS01	Δ*gfrE::kan*	this study
S. Typhimurium	GLS03	Δ*gfrE*	this study
S. Typhimurium	GLS02	Δ*gfrF::kan*	this study

S. Typhimurium	GLS04	Δ*gfrF*	this study
S. Typhimurium	KAM91	Δ*ptsH::kan*	(27)
S. Typhimurium	KAM95	Δ*ptsH*	(27)
S. Typhimurium	GLS05	Δ*ldcC::kan*	this study
S. Typhimurium	KAM129	Δ*ldcC*	this study
S. Typhimurium	DM2591	Δ*cadA::mudJ*	D. Downs
S. Typhimurium	KAM136	Δ*cadA::mudJ* Δ*ldcC*	this study
S. Typhimurium	GLS06	Δ*patA::kan*	this study
S. Typhimurium	KAM130	Δ*patA*	this study
S. Typhimurium	GLS07	Δ*patD::kan*	this study
S. Typhimurium	KAM131	Δ*patD*	this study
S. Typhimurium	GLS08	Δ*gabT::kan*	this study
S. Typhimurium	KAM133	Δ*gabT*	this study
S. Typhimurium	PBK01	Δ*hisC::kan*	this study
S. Typhimurium	PBK02	Δ*hisC*	this study
S. Typhimurium	ACB01	Δ*rpoN*	A. Bono
E. coli	BL21 (DE3)	F– *ompT gal dcm lon hsdSB(rB-mB-)* λ(DE3 [*lacI lac*UV5-T7 gene 1 *ind1 sam7 nin5*])	(18)
E. coli	KAM216	BL21/DE3λ/pACYC184+*plyE*/pET21a+*gfrE*	this study
E. coli	KAM217	BL21/DE3λ/pACYC184+*plyE*/pET21a+*gfrF*	this study

Table 5.S2. Plasmids used in this study.

Plasmid	Relevant characteristics	Ref.
pKD46	expresses λ phage recombinase genes γ, β and exo from arabinose-inducible P*araB* promoter; temperature-sensitive replicon	(21)
pKD4	template plasmid used to generate amplicons for inactivation of target genes in the λ Red system	(21)
pCP20	expresses FLP enzyme for removal of kan gene flanked by FRT sites; temperature-sensitive replicon	(35)
pET21a	vectors carries an N-terminal T7 Tag sequence and T7 promoter	Novagen
pET21a+*gfrE*	carries *S.* Typhimurium *gfrE* under control of T7 promoter in pET21a	this study
pET21a+*gfrF*	carries *S.* Typhimurium *gfrF* under control of T7 promoter in pET21a	this study

Table 5.S3. Primers used in this study.

Sequence	Experiment
5'-ATTTTCGGTTCCTGGATAGGGTTATTTTATGTGTAGGCTGGAGCTGCTTC-3'	*gfrE* knock out λ red
5'-CAGGTACTCGTCCTGATTAAAACCCAACATCATATGAATATCCTCCTTA-3'	*gfrE* knock out λ red
5'-TTAAAAGAGAATGTCATATATGCCGGGTCATATGAATATCCTCCTTA-3'	*gfrF* knock out λ red
5'-CGATTTCGATCGCGTTTTAAAAAGTAAAATTGTGTAGGCTGGAGCTGCTTC-3'	*gfrF* knock out λ red
5'-GCCTGGGCCGATGCATGGAAGGCATTGTAATGTGTAGGCTGGAGCTGCTTC-3'	*ldcC* knock out λ red
5'-	*ldcC* knock out

GATGTGATGAACCTGTTTTAATCCCAGCATCATATGAATATCCTCC TTA-3	λ red
5’-GCCTGCCTACTATGAGCC-3’	*ldcC* flanking
5’-GATGTGATGAACCTGTTTTAATC-3’	*ldcC* flanking
5’-TTTCGGTTCCTGGATAGG-3’	*gfrEF* flanking
5’-TGGAGATCAAAGTGGTGAATG-3’	*gfrEF* flanking
5’- TGAAAACTACGCGCAATATGCTGGCGTAAATGTGTAGGCTGGAGC TGCTTC-3’	*hisC* knock out λ red
5’- GTTCTACGCAGGCGGTCTGTTGCAGGGCATCATATGAATATCCTCC TTA-3’	*hisC* knock out λ red
5’-CGCGTAAACGCCCTCAAG-3’	*hisC* flanking
5’-CCGGTCGATGAAAAGATACTTC-3’	*hisC* flanking
5’- ATCGAGAAGCGAACGCTAAACCATGAGGAATGTGTAGGCTGGAGC TGCTTC-3’	*patA* knock out λ red
5’- GGATAAGCACAGCGCCATCCGGCATCGTTTCATATGAATATCCTCC TTA-3’	*patA* knock out λ red
5’-ACGCACTGAATCTCATCG-3’	*patA* flanking
5’-TGGTGATTGTGATGGGATTC-3’	*patA* flanking
5’- AGCGGCGGCCCGTTCGCCGGAAATCGATTATGTGTAGGCTGGAGC TGCTTC-3’	*patD* knock out λ red
5’- GGATCATTTACTGTTCAGAAAAATCGTGAACATATGAATATCCTCC TTA-3’	*patD* knock out λ red
5’-GTCAGCTCACAACCTGAC-3’	*patD* flanking
5’-CGCAGCAAGTACTCAAAGG-3’	*patD* flanking
5’- GAAATCAAATATATGTGCATCGGCCTTTAATGTGTAGGCTGGAGCT GCTTC-3’	*gabT* knock out λ red
5’- ACGTCGCCACCCGACGCTGCTTATTGGACACATATGAATATCCTCC TTA-3’	*gabT* knock out λ red
5’-GTTCCAAATACGGCATCGAAG-3’	*gabT* flanking
5’-CCGGATAAGACAGTTACGTCG-3’	*gabT* flanking
5’-CATATGATGTCACCAACCATGCTG-3’	GfrE purification
5’-AAGCTTGATTTTACTTTTTAAAACGCGATCG-3’	GfrE purification
5’-CATATGATGTTGGGTTTTAATCAGGACG-3’	GfrF purification
5’-AAGCTTATAATCGAACTGACGGTAGTAACG-3’	GfrF purify

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CHAPTER 6: CHARACTERIZATION OF ADDITIONAL *SALMONELLA* RPON-DEPENDENT GENES OF UNKOWN FUNCTION

Introduction

A goal of my research has been to identify functions for products of genes within the *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) RpoN regulon. RpoN is an alternative sigma factor required for transcription of genes involved in a range of metabolic pathways including nitrogen metabolism, H_2 evolution and uptake, carbohydrate transport and degradation of aromatic compounds (1-3). The RpoN regulon in *Salmonella* is not fully characterized. Many RpoN-dependent genes have unknown function, all of which appear to be involved in metabolism. Here, we describe the work that we have done on three RpoN-dependent operons in *S.* Typhimurium that contain genes of unknown function, some of which may have important roles in host colonization.

Materials and Methods

Bacterial strains, growth conditions, and reagents.

Strains were maintained in Luria-Bertani (LB) broth or agar supplemented with 100 µg/ml of ampicillin or 50 µg/ml kanamycin as needed. Growth of *S.* Typhimurium on different carbon and nitrogen sources was carried out in a MOPS minimal media described by Neidhardt (4) and modified by Maloy (5). The MOPS media was buffered with 40 mM 3-(*N*-morpholino)

propanesulfonic acid and 4 mM Tricine, with the pH adjusted to 7.4 with NaOH. Additional components of the basic minimal medium were 20 mM $NaHCO_3$, 0.88 mM K_2HPO_4, 50 mM NaCl, 10 μM $FeSO_4 \cdot 7H_2O$, 0.55 mM $MgSO_4 \cdot 7H_2O$, 0.4 μM H_3BO_3, 0.5 μM $CaCl_2 \cdot 2H_2O$, 0.03 μM $CoCl_2 \cdot 6H_2O$, 0.01 μM $CuSO_4 \cdot 5H_2O$, 0.08 μM $MnCl_2 \cdot 4H_2O$, and 0.01 μM $ZnSO_4 \cdot 7H_2O$.

Construction of mutant strains.

The *S.* Typhimurium mutants indicated in Table 6.1 were constructed using the λ Red recombineering method (6). Plasmid pKD4, which contains a kanamycin resistance (*kan*) cassette flanked by two Flp recognition sights (FRT sites), was used to PCR amplify linear DNA which included sequences at the ends that were homologous to the genes targeted for deletion. Primer sets used to generate the amplicons for targeted mutagenesis are listed in Table 6.2. The amplicons were introduced into *S.* Typhimurium 14028s bearing the plasmid pKD46, which carries the λ phage recombinase genes under the control of the *araBAD* promoter, by electroporation. DNA isolated from the resulting kanamycin-resistant colonies was checked by PCR, using primers that flanked the target gene deletion (primers are listed in Table 6.S3), to confirm that the target gene had been knocked out. Mutant alleles in which the target genes had been replaced with the kanamycin-resistance (*kan*) cassette were moved into a clean genetic background by transduction using P22 HT *int*. The *kan* cassette was then removed by introducing plasmid pCP20 into the mutant strains by electroporation. Plasmid pCP20 expresses the Flp recombinase, which recognized the FRT sites and excised the *kan* cassette. Loss of the *kan* cassette was confirmed by susceptibility to kanamycin and by PCR using the same flanking primers (listed in Table 6.2).

Construction of plasmids.

Plasmids used for this study are listed in Table 6.1. All genes were amplified by PCR from *S.* Typhimurium 14028s genomic DNA using Phusion High-Fidelity DNA polymerase (New England BioLabs) using primers sets listed in Table 6.2. Adenine overhangs were added to the 3' ends of the amplicons using *Taq* DNA polymerase (Thermo Fisher Scientific). The amplicons were cloned into pCR2.1-TOPO (Invitrogen), and the sequences of the cloned fragments were confirmed using DNA sequencing (Genewiz). For overexpression of STM0649-50, BglII and HindIII sites were introduced by the primer sets and were used to clone the fragments into pLAC22 (7). The resulting plasmid, designated pLAC22+STM0649-50 is under control of a *lac* promoter. For purification of STM2358 and STM2360, NdeI and HindIII sites were introduced by the primer sets and were used to clone STM2358 and STM2360 into the expression vector pET21a (Novagen). The resulting plasmids, designated pET21a+STM2358 and pET21a+STM2360, expressed proteins with C-terminal His tags.

For all transformations involving *S.* Typhimurium 14028s, DNA was introduced by electroporation at 2.4 kV, 25 μF, and 400 Ω. Following electroporation, cells were recovered in SOC broth (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) for 1 hour at 37°C. Chemically competent cells were prepared using the calcium chloride method and were used for all transformations involving *E. coli*. Chemically competent cells were recovered in LB broth for 1 hour at 37°C following the heat shock step.

Expression and purification of proteins.

Plasmids pET21a+STM2358 and pET21a+STM2360 were transformed into *E. coli* BL21 (DE3λ) containing the plasmid pLysE (8). *E. coli* BL21 (DE3λ) expresses T7 RNA polymerase, which is needed for transcription of STM2358 and STM2360 from pET21a+STM2358 and pET21a+STM2360. Overnight cultures were subcultured into 1 liter of LB broth with appropriate antibiotics and grown at 30°C until OD_{650} = 0.4 to 0.6. Cells were then moved to ice for 30 min, after which 50 mM IPTG was used to induce the desired proteins. Cells were then allowed to grow at 10°C for 96 hours. The cellular extract from the liter of cells was prepared by sonication for 4 minutes for 1-minute intervals in 50 mM MOPS, pH 7, with 100 mM potassium thiocyanate (buffer A) and 1µg/ml pyridoxine, followed by centrifugation for 90 min at 2,500 x *g*. The supernatant was applied to a Ni-NTA agarose affinity column (Sigma) (10 ml) and washed with 50 mM MOPS, pH 7, with buffer A until the absorbance at 280 nm was back to baseline. The column was then eluted with 100 ml of buffer A containing 0.2 M imidazole. Peak protein fractions were pooled, concentrated in a centrifugal concentrator (YM-30 membrane), and then dialyzed against 50 mM MOPS, pH 7.

L-alanine racemase assay

The reaction mix for L-alanine racemase consisting of 50 mM phosphate buffer, pH 8, 50 µM PLP, 0.2 mM NAD, 10 mM D-alanine and 10 µL L-alanine dehydrogenase. For a positive control, L-alanine was used place of D-alanine. 5 µg, 10 µg, or 20 µg of purified STM2358 were added to the reaction mix and the reaction was followed for 10 minutes at 340 nm.

Transamination reactions

Transamination reaction mixes included 50 mM potassium phosphate buffer, pH 7, 10 mM of the tested amino acid (D- or L- stereoisomer), and 10 µg of purified STM2358 or STM2360 protein. Reactions containing STM2360 and STM2358 proteins were monitored spectrophotometrically for 8 h and 30 min, respectively.

Decarboxylase assays

Decarboxylase assays included 60 µL of 1 mg/ml *Zea mays* phosphoenolpyruvate (PEP) carboxylase (Fisher Calbiochem) in 0.05M Tris, pH 7.8, 10 µg malate dehydrogenase (0.1U) (Fisher Calbiochem) in 0.05 M potassium phosphate buffer, pH 7, 60 µL of 10 mM PEP (trisodium salt; Sigma), 60 µL 2 mM NADH (USB) in 10 mM Tris, pH 7.8, 30 µL 1M potassium phosphate buffer, 12 µL 2 mM PLP, and 6 µL 1% Triton X-100. All substrates tested for decarboxylase activity contained 10 mM of the amino acid.

Determining the keto acid of D-arginine transamination

D-arginine transaminase reactions contained either 10 mM D-arginine in 50 mM potassium phosphate buffer, pH 7, along with 60 µg of purified STM2360 protein. Reactions were incubated for 1 h at 37°C then put through a PD-10 desalting column. Assays were carried out on the resulting enzyme and PMP, since incubation with D-arginine transforms the PLP attached to the enzyme to PMP, along with 50 mM potassium phosphate buffer (pH 7) and 10 mM of the keto acid. Transamination reactions were confirmed spectrophotemetrically.

Polarimeter assays

A polarimeter was used to monitor the change in the optical rotation of L-arginine as it was converted to D-arginine at various times. For these reactions, purified enzyme was incubated with L-arginine and aliquots were removed at various times and then spun down before the supernatant was measured on an Atago Polax-2L polarimeter.

Table 6.1. Plasmids used in this study.

Plasmid	**Relevant characteristics**	**Ref.**
pET21a	vectors carries an N-terminal T7 Tag sequence and T7 promoter	Novagen
pET21a+STM2358	carries *S.* Typhimurium STM2358 under control of T7 promoter in pET21a	this study
pET21a+STM2360	carries *S.* Typhimurium STM2360 under control of T7 promoter in pET21a	this study
pLAC22	expression vector derived from pBR322 that carries the E. coli *lac* promoter/operator and $lacI^q$	(7)
pLAC22+STM0649-50	carries *S.* Typhimurium STM0649-50 under control of *lac* promoter/operator in pLAC22	this study
pKD46	expresses λ phage recombinase genes γ, β and exo from arabinose-inducible P*araB* promoter; temperature-sensitive replicon	(6)
pKD4	template plasmid used to generate amplicons for inactivation of target genes in the λ Red system	(6)
pCP20	expresses FLP enzyme for removal of kan gene flanked	(9)

	by FRT sites; temperature-sensitive replicon	

Table 6.2. Primers used in this study.

Sequence	**Experiment**
5’-GCTAGCATGACTGACTCGATTATGCAG-3’	STM2360 purification
5’-AAGCTTATAAATGTCGTACTTGACCATATC-3’	STM2360 purification
5’-CATATGTATATGCCAGTTCTGG-3’	STM2358 purification
5’-AAGCTTACGCGTAAACCTCCAGG-3’	STM2358 purification
5’-AGATCTAGCAAAGGAGTTAATCGTG-3’	STM0649-50 overexpression
5’-AAGCTTTTATTCCCAGGAGATAACC-3’	STM0649-50 overexpression
5'-GAAGACAGGACTAAAGCAAAGGAGTTAATCTGTGTAGGCTGGAGCTGCTTC-3'	STM0649-50 knock out λ red
5'-TCAGAACGTCGGCGCGATCTTATAAATAGACATATGAATATCCTCCTTA-3'	STM0649-50 knock out λ red
5'- CGCAGTTCCTGTCACCG-3'	STM0649-50 flanking
5'- CGGTACAACCATCATACCACC-3'	STM0649-50 flanking
5’-GTCGGCGATAAAATACGGTTTACCGTATGATGTGTAGGCTGGAGCTGCTGC -3’	*fraB* knock out λ red
5’-GTCGCCAATACCCAATACGCTGATGCTCATCATATGAATATCCTCCTTA-3’	*fraB* knock out λ red
5’-TTGTATGAATGCTTACGCGAG-3’	*fraB* flanking
5’-CTGCTAATTTCGCATAGACAGC-3’	*fraB* flanking
5’-GGCGGTTAAAACCAGTGTTAATGTACTCACTGTGTAGGCTGGAGCTGCTTC-3’	*gntK* knock out λ red
5’-TTCACCTTGTCACCGGGCGGATCTATTTAGCATATGAATATCCTCC	*gntK* knock out λ red

TTA-3’	
5’-ACAGCAGCAGTAAGACGG-3’	*gntK* flanking
5’-TAGGTTTCACCTTGTCACC-3’	*gntK* flanking
5’-TGTTACACTACAATGTTACGCATAACGTGATGTGTAGGCTGGAGCTGCTTC-3’	*idnK* knock out λ red
5’-TATCGTGTAGTGCTAAAAGCTGACTTCTGACATATGAATATCCTCCTTA-3’	*idnK* knock out λ red
5’-CTCACTGGTCAGGTAGTTAC-3’	*idnK* flanking
5’-TGTCACAAATCAACGAGGC-3’	*idnK* flanking

Results and Discussion

Characterization of STM0572-STM0577 operon

The organization of the genes within the STM0572-0577 operon (Fig. 6.1) is very comparable to that of the *gfr* locus suggesting the involvement of the STM0572-0577 operon in transport and catabolism of fructosamines or other Amadori products. In fact, analysis of the predicted amino acid sequences of STM0572, STM0573, GfrE and GfrF using the SEED annotation/analysis tool (http://theseed.uchicago.edu/FIG/index.cgi) revealed that the STM0572 is more closely related to fructoselysine 6-phosphate deglycases (FrlB) from *E. coli* and *Shigella* than is GrfF. Moreover, both the STM0572 protein and GfrF belong to the same COG (Cluster of Orthologous Genes) family, COG2222 (fructoselysine-6-P-deglycase FrlB and related proteins with duplicated sugar isomerase (SIS) domain).

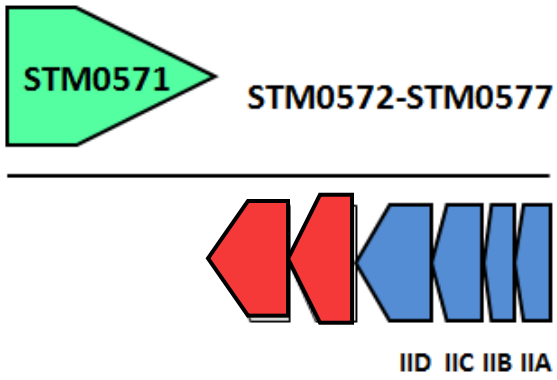

Figure 6.1. Operon organization of STM0572-0577. STM0577 to STM0574 encode the EIIABCD components of a mannose family PTS permease. STM0573 and STM0572 encode putative deglycases. Adjacent to the STM0572-0577 operon is STM0571, which is presumed to encode an RpoN-dependent activator of the STM0572-0577 operon.

To attempt to identify substrates of the STM0572-0577 operon, we synthesized several Amadori products. We started by synthesizing compounds in which various sugars were joined to the ε-amine of lysine. These Amadori products included ribuloselysine, erythruloselysine, tagatoselysine, and psicoselysine. Details for the synthesis of these compounds are found in Chapter 5). *S.* Typhimurium was unable to utilize any of these Amadori products as a sole carbon or nitrogen source suggesting that they are not substrates for the products encoded by the STM0572-0577 operon.

Bacillus subtilis FrlB shares about 30% amino acid identity with *E. coli* FrlB but unlike the *E. coli* enzyme, *B. subtilis* FrlB catalyzes reactions with α-glycated amino acids (10). Therefore, we examined the ability of *S.* Typhimurium to utilize α-glycated amino acids, starting with fructosemethionine which was synthesized by reacting glucose and methionine as described in Chapter 5. The fructosemethionine preparation served as the sole carbon and nitrogen source

for *S.* Typhimurium although it was contaminated with unreacted methionine. Exogenous methionine did not support growth of *S.* Typhimurium as a nitrogen or carbon source (data not shown;(11)), indicating that *S.* Typhimurium is able to utilize fructosemethionine. Fructosemethionine served as a good nitrogen source for *S.* Typhimurium. In MOPS minimal media with L-arabinose as the primary carbon source and fructosemethionine as the sole nitrogen source, *S.* Typhimurium cultures had doubling times of 48 ± 1.4 min when grown aerobically at 37^{o}C. Fructosemethionine did not support as rapid growth as a carbon source for *S.* Typhimurium as cultures had doubling times of 100.1 ± 3.9 min when grown in minimal medium with fructosemethionine as the sole carbon and nitrogen source, and 114 ± 11.6 min when grown with fructosemethionine plus ammonium.

A mutant strain in which STM0572 and STM0573 had been deleted was able to utilize fructosemethionine, suggesting that the putative deglycases encoded by these genes are not required for catabolism of fructosemethionine. We reasoned that it was possible, however, that the STM0572-0577 operon does have a role in fructosemethionine utilization, but that another deglycase in *S.* Typhimurium may have overlapping substrate specificity with one of the putative deglycases encoded by STM0572 and STM0573. *S.* Typhimurium *fraB* encodes a putative fructose-aspartate 6-phosphate deglycase (12). Since FraB appears to use a α-glycated amino acid as a substrate it seemed like a likely candidate as a fructosemethionine 6-phosphate deglycase. A *S.* Typhimurium Δ*fraB* mutant was able to use fructosemethionine as the sole carbon and nitrogen source, as did a Δ*gfrEF* mutant. A mutant strain in which all five of the putative deglycases were deleted (ΔSTM0572/0573, Δ*gfrEF* and Δ*fraB*), however, failed to grow on fructosemethionine as the sole carbon and nitrogen source, suggesting that there is overlap in the substrate specificities for at least two of these deglycases.

To follow up on these preliminary findings, we plan to examine various combinations of the deglycase mutants to determine which ones are required for fructosemethionine utilization. In addition, we plan to determine if the mannose-type PTS permease encoded by STM0574-0577 is required for transport of fructosemethionine or if this Amadori product is transported into the cell by another permease (other likely candidates are FraA, which is a putative transporter for fructose-aspartate (12), and the GfrABCD PTS permease). In addition, we plan to use RNA-seq to examine how growth on fructosemethionine affects global gene expression in *S.* Typhimurium to determine the pathway for utilization of methionine derived from fructosemethionine since *S.* Typhimurium is unable to use methionine as a nitrogen source. It should be noted that cells grown on fructosemethionine do not appear to accumulate methionine intercellularly (Figure 6.2) which suggests methionine derived from fructosemethionine is catabolized relatively efficiently inside the cell. Finally, we plan to determine if *S.* Typhimurium utilizes other α-glycated amino acids, and if so, if any of the known or putative deglycases are required for their utilization.

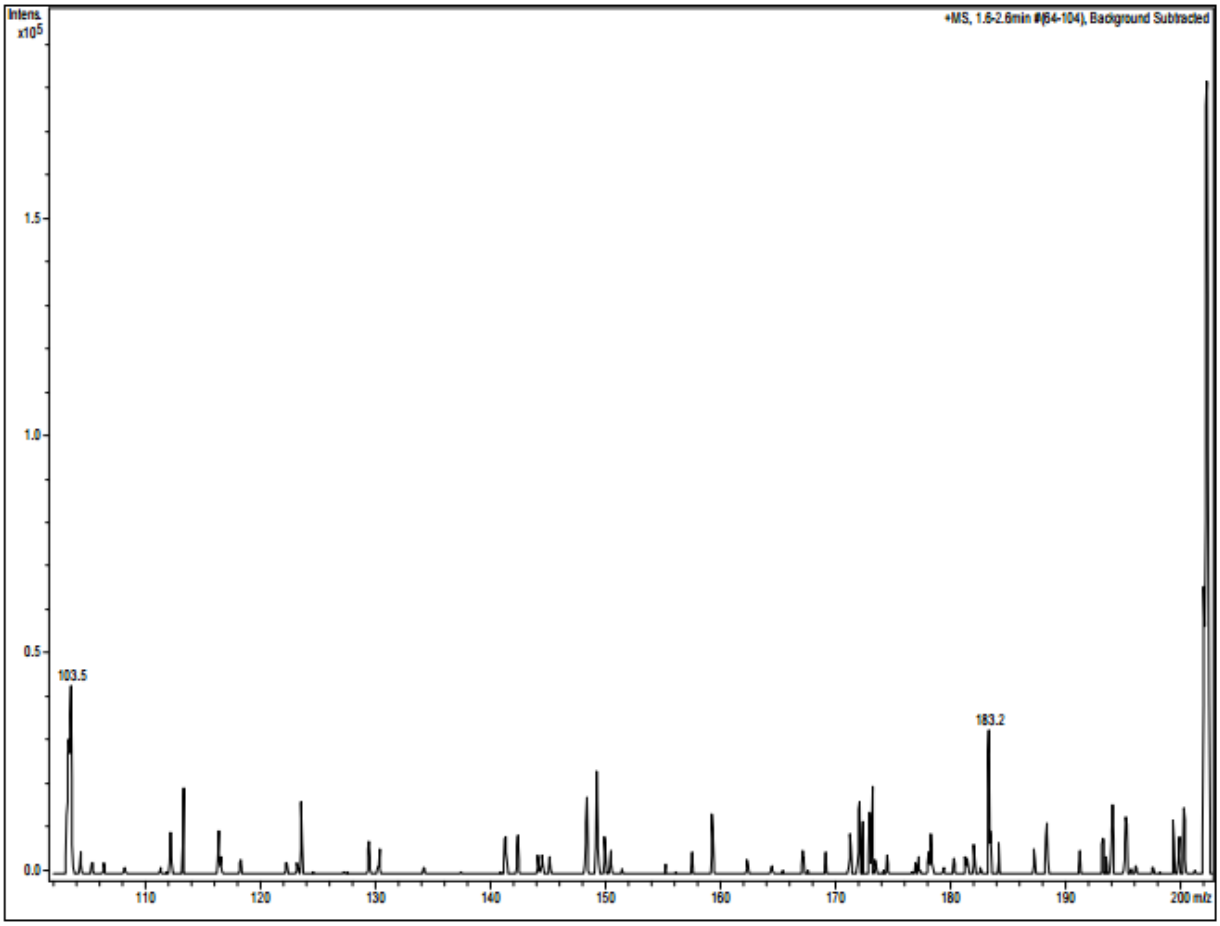

Figure 6.2. Mass spectrum of extracts from cells grown on fructosemethionine. *S.* Typhimurium 14028s was grown in MOPS minimal medium containing 20 mM fructosemethionine as the sole carbon and nitrogen source. One ml of culture was subjected to 10 rounds of freeze-thaw in an ethanol-dry ice bath. Cell debris was removed by centrifugation for 1 min at 6,000 x g, and the resulting supernatant liquid was analyzed by mass spectrometry using electrospray mass spec on a Burker Esquire 3000 Plus machine. A prominent peak in the mass spectrum corresponding to the expected mass for methionine (m/z 150.2, $(M+1)^+$) was not evident in the sample suggesting that *S.* Typhimurium intracellular levels of methionine are not elevated during growth on fructosemethionine.

Characterization of the STM2357-STM2361 operon

Gene in the STM2357-STM2360 locus constitute a putative RpoN-dependent operon that is likely under control of the uncharacterized bacterial enhancer-binding protein (bEBP) encoded by STM2361 (Fig. 6.3). Two genes in this operon, STM2357 and STM2359, are predicted to encode amino acid transporters. The product of STM2358 belongs to the alanine racemase, N-terminal domain protein family (PF01169). Further analysis of the STM2358 protein sequence using the SEED annotation/analysis tool revealed that the protein groups closely with ornithine racemases (EC 5.1.1.12) from bacteria belonging to the phyla Firmicutes and Thermotogae (Fig. 6.4). Since most of these bacteria are thermophiles it is possible that the *Salmonella* protein has a role in either heat shock or heat resistance. STM2360 is annotated as a putative diaminopimelate decarboxylase and belongs to the Group IV pyridoxal (PLP)-dependent decarboxylase, C-terminal sheet domain (PF00278) and Group IV PLP-dependent decarboxylase, pyridoxal binding domain (PR02784) protein families. Further analysis of the STM2360 protein sequence using the SEED annotation/analysis tool indicated that the protein is not closely related to other non-*Salmonella* proteins, other than an ornithine/diaminopimelate/arginine decarboxylase family protein from *Clostridium novyi* NT. We purified his-tagged versions of the STM2358 and STM2360 proteins and showed that the proteins have arginine/ornithine racemase and potential D-arginine transaminase activities, respectively. We were unable, however, to ascertain physiological roles for these enzymes in *S*. Typhimurium.

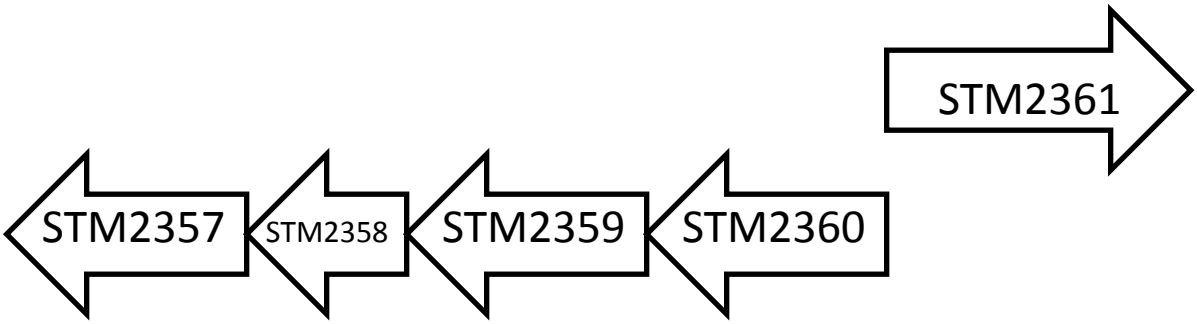

Figure 6.3. Operon organization of STM2357-60. STM2361 encodes an RpoN-dependent activator. My preliminary data suggest that STM2360 encodes a PLP-dependent D-arginine transaminase and STM2358 encodes a PLP-dependent arginine/ornithine racemase. STM2359 and STM2357 encode putative amino acid transporters.

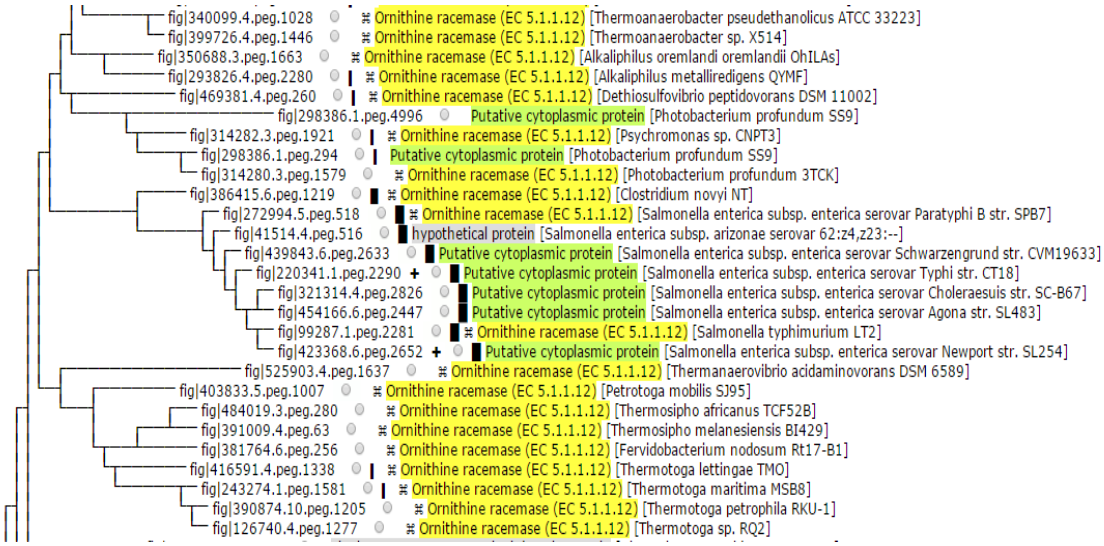

Figure 6.4. Portion of the SEED tree for STM2358. The tree suggests that STM2358 most closely pairs with ornithine racemases, many of which are found in thermophiles.

As indicated above, the proteins encoded by STM2358 and STM2360 are PLP-dependent enzymes. PLP-dependent enzymes are capable of catalyzing a couple of reactions involving the α-carbon of amino acids including transamination, racemization and decarboxylation (13). In

these reactions, when the alpha amino group is transferred to PLP it forms pyridoxamine phosphate (PMP). PLP and PMP have different light absorption spectra which can be used to measure reactions of PLP-dependent enzymes in vitro. Transamination reactions involve the exchange of an amino group between an amino acid and a keto acid (13). In this reversible reaction, the amino acid in is converted to the corresponding keto acid, and the keto acid is converted to its corresponding amino acid. Racemizations occur by the abstraction and subsequent return of the hydrogen to the α-carbon resulting in a change in the chirality (13). Amino acid racemizations are reversible and the equilibrium constant of the reaction is 1. Decarboxylation reactions involve the removal of the α-carboxyl group as CO_2 followed by a rearrangement of electrons (13).

Nothing is known about the physiological role of the STM2357-2360 operon in *S.* Typhimurium. Kröger and co-workers used RNA-seq analysis to examine global gene expression in *S.* Typhimurium under 20 different conditions, but none of these conditions resulted in expression of measurable transcription levels of any of the genes within the STM2357-2360 operon (14). These researchers did notice, however, that expression of the gene encoding the putative activator of the STM2357-2360 operon (STM2361) was up-regulated ~130-fold following pH 3 shock (14), indicating that the STM2357-2360 operon may have a role in acid stress. A possible explanation for why the STM2357-2360 operon was not similarly up-regulated upon pH 3 shock is that the inducer that is sensed by the activator was not present in the growth medium.

STM2358 encodes an arginine/ornithine racemase

To identify the substrates for the STM2358 protein, the purified enzyme was incubated with various amino acids and the absorbance spectra of the reactions were monitored with time. A side reaction of racemization is a transamination that results in the depletion of PLP (390 nm, but the enzyme is red shifted making the PLP peak appear around 420) and the formation of PMP (325 nm). The only amino acids which resulted in an apparently stoichiometric conversion of PLP to PMP were the D- and L-forms of arginine, ornithine and cysteine (Fig. 6.5; data only shown for L-lysine). Cysteine reacts non-enzymatically with PLP (15), and we postulate that this was the case for the STM2358 protein as the absorbance spectrum looked somewhat different than that of D- or L- arginine or ornithine (Fig. 6.6; data only shown for D-cysteine). Specifically, the addition of cysteine appeared to result in a much more rapid conversion of PLP to PMP than the addition of arginine or ornithine.

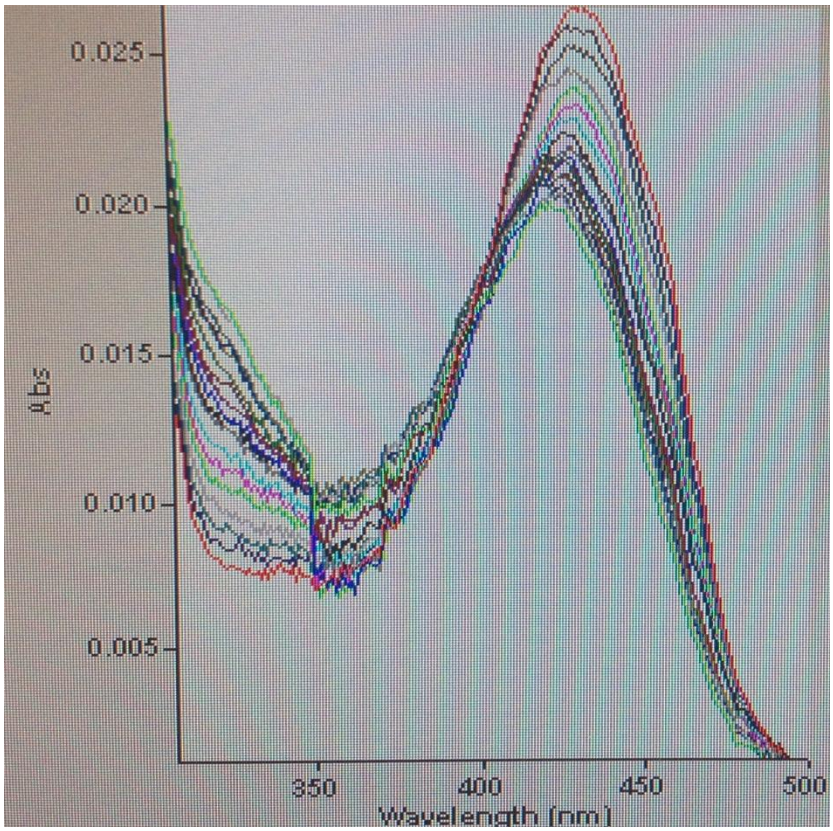

Figure 6.5. Changes in the absorbance spectrum of the STM2358 enzyme upon incubation with L-arginine. Changes in the absorbance spectrum of the STM2358 protein were very similar upon incubation with D-arginine, D-ornithine or L-orninthine. The time first scan which is from time 0 to 5 minutes is indicated by the red line, which starts with PLP at about 420 nm. The last time point of 8 hours is indicated in green with the top peak of PMP at 325 nm.

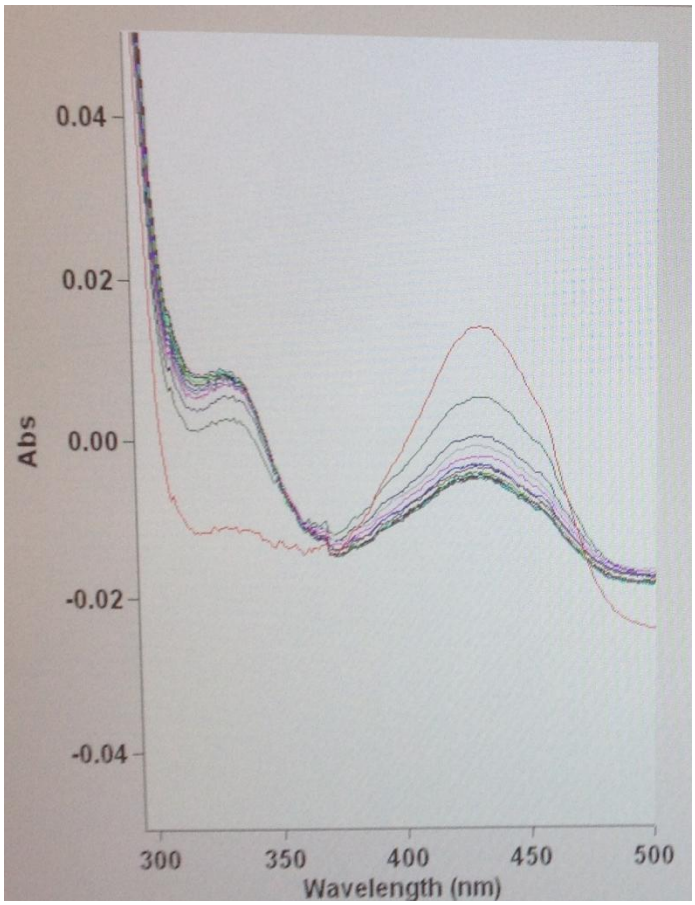

Figure 6.6. Changes in the absorbance spectrum of the STM2358 enzyme upon incubation with L-cysteine. Changes in the absorbance spectrum of the STM2358 protein were very similar upon incubation with D-cysteine. The time first scan which is from time 0 to 5 minutes is indicated by the red line, which starts with PLP at about 420 nm. The last time point of 8 hours is indicated in green with the top peak of PMP at 325 nm.

To confirm that arginine is a substrate for the STM2358 enzyme, we used a polarimeter to monitor the change in the optical rotation of L-arginine as it was converted to D-arginine. For these reactions, purified enzyme was incubated with L-arginine and aliquots were removed at various times and then spun down before the supernatant was measured. To determine the optimal pH of the reaction, assays were done at various pH values (pH 4 to 8). Since the

activator was expressed under acid shock, we wish wished to test lower pH values. For some racemases, the optimal pH is more basic, so we wished to test them as well. For the assay, the optical rotation should decrease over time to zero, at which point the reaction has reached equilibrium (i.e, a 1:1 ratio of D- to L- arginine). Racemization of L-arginine occurred under all pH conditions tested in the assay and appeared to occur fastest at pH 4, but ceased after 1.5 h of incubation at this pH (Table 6.3). We postulate that the enzyme was unstable at this pH which accounted for the reaction not proceeding to equilibrium. The temperatures differ in Table 5.1 depending on room temperature since the polarimeter was not temperature controlled.

Table 6.3. Results from the polarimeter assay with L-arginine and purified STM2358.

Time	pH 4	pH 5	pH 6	pH 7	pH 8	temp
0 hr	0.65	0.65	0.7	0.7	0.75	22.3
1.5 hr	0.2	0.65	0.55	0.45	0.5	26.3
3 hr	0.2	0.6	0.6	0.4	0.15	26.1
4.5 hr	0.15	0.2	0.2	0.35	0.15	26.3

STM2360 encodes an enzyme that appears to have D-arginine transaminase activity

STM2360 is annotated as encoding a putative diaminopimalate decarboxylase. We were unable to detect, however, decarboxylase activity for purified STM2360 protein with diaminopimalate or any other amino acid. When D-arginine was incubated with the enzyme though, PLP was converted to PMP very rapidly with nearly complete conversion occurring with 5 min at 37^{o}C. The speed of the reaction suggested that the enzyme functioned as a D-arginine

transaminase and that transamination was not a side reaction as that observed for the arginine/ornithine racemase. Both L-forms and D-forms of all of the common amino acids (with the exception of proline), plus some uncommon amino acids (D- and L- norleucine and *meso*-diaminiopimelate) were tested as potential substrates for the STM2360 enzyme. Of these other amino acids, only D-cysteine caused a spectral shift when incubated with the enzyme, but this shift may have resulted from a non-enzymatic reaction of D-cysteine with PLP.

To identify potential keto acid substrates for the D-arginine transaminase, the purified enzyme was incubated with D-arginine to form PMP, after which various ketoacids were added to the assay mixture to see if PLP was reformed. Addition of 3-mercaptopyruvate, which is the corresponding ketoacid for cysteine, did not result in the conversion of PMP associated with the enzyme to PLP. This finding suggested that the apparent D-cysteine transaminase activity we had observed was an artifact. It is possible that 3-mercaptopyruvate is labile, which might have accounted for its failure to serve as an enzyme substrate in the assay. Other ketoacids which we tested included α-ketoglutatate, oxaloacetate and pyruvate, all of which also failed to convert PMP to PLP.

Characterization of the STM0649-STM0651 operon

The products of STM0649-50 are annotated as a putative altronate dehydratase, while the product of STM0651 is annotated as a putative 2-keto-3-deoxy-gluconate permease (Fig. 6.7). Altronate is an intermediate in the pathway for catabolism of galacturonate in *E. coli* and is converted to 2-keto-3-deoxygluconate by altronate dehydratase (http://www.uniprot.org/uniprot/P42604). The resulting 2-keto-3-deoxygluconate is subsequently phosphorylated by KdgK to form 2-keto-3-deoxy-6-phosphogluconate (Fig. 6.8), an intermediate

in the Entner-Doudoroff pathway. *S.* Typhimurium is unable to utilize galacturonate as it is missing at least one of the enzymes in the pathway for galacturonic acid catabolism. STM0652 encodes a predicted an RpoN-dependent activator which presumably activates transcription of the STM0649-0651 operon in response to an unknown signal. Consistent with this hypothesis, there is a consensus RpoN-dependent promoter sequence (5'-TGGCACGCCTTTTGAT-3') located 35 bp upstream of the predicted translational start site of STM0649. Moreover, Samuels *et al*. observed that the STM0649-0651 genes were up-regulated in a *S.* Typhimurium strain that expressed a truncated form of the RpoN-dependent activator DctD which activated transcription constitutively and promiscuously (16). In addition to the RpoN-dependent promoter upstream of STM0649, Kröger *et al*. used differential RNA-seq (dRNA-seq) to identify a transcriptional start site upstream of STM0651 and internal to STM0650 which corresponded to a potential sigma70-dependent promoter (17). For dRNA-seq analysis, the 5′-monophosphate–dependent terminator exonuclease (TEX) is used to specifically degrade 5′-monophosphorylated RNA species, while 5′-triphosphorylated RNA species (primary transcripts) remain intact and are subsequently sequenced. For the dRNA-seq analysis done by Kröger *et al*., RNA was prepared from *S.* Typhimurium SL1344 cultures grown aerobically in a rich medium (Lennox broth) to early stationary phase (17).

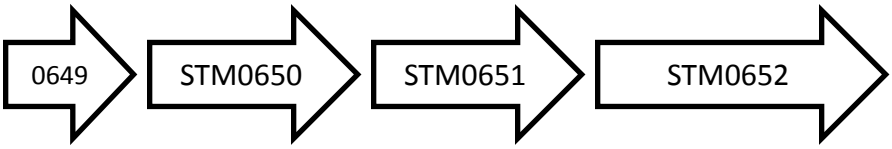

Figure 6.7. STM0649-0651 operon organization. STM0649-50 encodes a putative altronate dehydratase. STM0651 encodes a putative 2-keto-3-deoxy-gluconate (KDG) permease. STM0652 encodes an RpoN-dependent activator that presumably activates transcription of the STM0649-0651 operon.

To determine if STM0649/0650 encodes altronate dehydratase, we introduced the genes into an *E. coli uxaA* (encodes altronate dehydratase) mutant to determine if they would complement the *uxaA* mutation and restore the ability of the strain to grow on galacturonate. STM0649/0650 were amplified from S. Typhimurium 14028s genomic DNA and cloned into the expression vector pLAC22 (7) to place them under control of the *lac* promoter/operator. The resulting plasmid (pLAC22+STM0649-50), was introduced into an *E. coli* strainJW3062-1 (Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*uxaA781::kan*, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*) obtained from the Coli Genetic Stock Center. The pathways for utilization of galacturonate and glucuronate in *E. coli* are very similar and both are converted into 2-keto-3-deoxygluconate before entering the Entner-Doudoroff pathway. *E. coli* JW3062-1 grew well with glucuronate as a carbon source, but not galacturonate (data not shown). Introduction of pLAC22+STM0649-50 into *E. coli* JW3062-1 failed to restore growth of the strain on galacturonate under aerobic or anaerobic growth conditions. For these complementation experiments various concentrations of IPTG ranging from zero to 1 mM were included in the medium to induce expression of STM0649/0650. These results suggest that STM0649/0650 does

not encode altronate dehydratase. A caveat of these results, however, is that we did not visualize protein bands corresponding to the predicted products of STM0649/0650 following SDS-polyacrylamide gel electrophoresis of extracts of cells in which we induced expression of STM0649/0650 (data not shown). Thus, it is possible the STM0659/0650 protein was not expressed in the complementation assays.

Kröger *et al*. used RNA-seq to examine global gene expression in *S*. Typhimurium SL1344 under a variety of conditions. Expression of STM0649 and STM0650 was only observed under anaerobic growth or anaerobic shock (for anaerobic shock cultures were shaken vigorously and then shaking was ceased and mineral oil was added to the top of the medium to impede diffusion of oxygen into the medium) (Fig. 6.3) (14). From these observations we postulated that expression of the STM0649-0651 operon requires anaerobic conditions and that the substrate for the STM0649/0650 enzyme induces expression of the STM0649-0651 operon and is present in the medium (Lennox broth) used by Kröger *et al*. in their study. We further hypothesized that the STM0649/0650 enzyme is a dehydratase that converts its substrate into a compound that can feed directly into a well characterized pathway. Based on these assumptions, we believe gluconate, mannonate and altronate to be likely substrates for the permease encoded by STM0651 and the enzyme encoded by STM0649/0650 since a dehydratase could convert any of these compounds to 2-keto-3-deoxygluconate (Fig. 6.9).

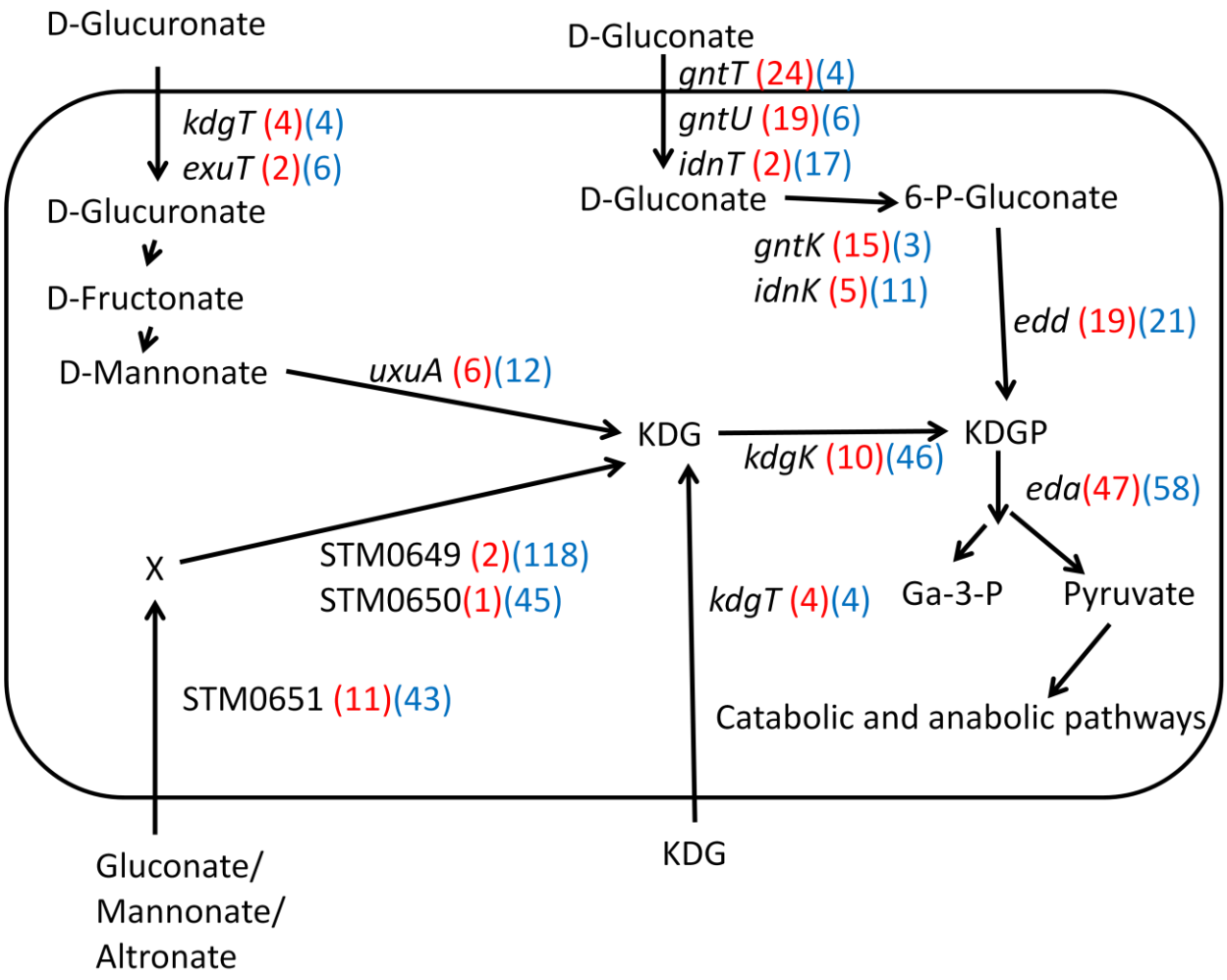

Figure 6.8. Sugar acid metabolism in *Salmonella*. STM0649-51 operon is turned on during anerobic growth. We hypothesize that this operon is being used to metabolize gluconate, mannonate or altronate. Transcript levels for the indicated genes determined from RNA-seq analysis are from samples of *S.* Typhimurium SL1344 cultures grown to early exponential phase under aerobic conditions (in red) or grown under anaerobic conditions (in blue) and are from Kröger *et al*. (14). The numbers refer to number of specific transcripts per million reads. KDG is 2-keto-3-deoxygluconate, KDGP is 2-keto-3-deoxy-6-P-gluconate, and Ga-3-P is glyceraldehyde-3-P.

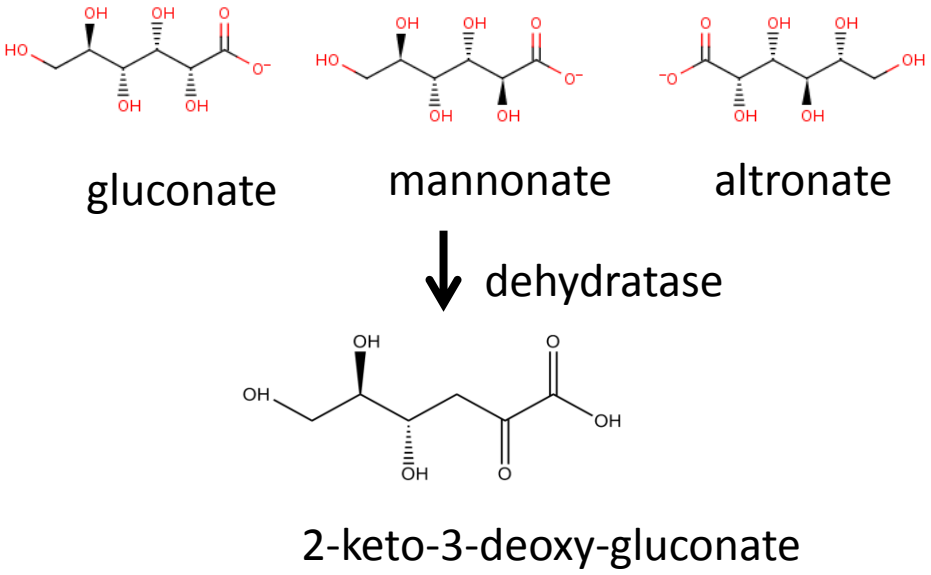

Figure 6.9. Potential substrates for STM0649-50 dehydratase that would result in 2-keto-3-deoxy-gluconate. Removal of a water molecule from the C-2 and C-3 positions of D-gluconate, D-mannonate or D-altronate would produce 2-keto-3-deoxy-gluconate which could feed into the Entner-Doudoroff pathway.

As seen in Figure 6.7, genes known to be involved in gluconate utilization were generally down-regulated under anaerobic conditions, while expression of the STM0649-0651 operon was up-regulated under anaerobic conditions (14). Since gluconate is more common than mannonate or altronate, we postulated that the STM0649-0651 operon played a role in utilization of gluconate under anaerobic conditions. To test this hypothesis, we knocked out the genes encoding the two known gluconate kinases in *S.* Typhimurium SL1344, *gntK* and *idnK* (Fig. 6.7) as well as STM0649/0650 and examined the abilities of the resulting mutants to grow with gluconate as the sole carbon source under aerobic or anaerobic conditions. We created a double mutant of *gntK* and *idnK*, as well as a triple mutant that lacked STM0649/0650, *gntK*, and *idnK*,

and accessed the phenotypes of these mutants. Preliminary analysis of the mutants revealed that the triple mutant was unable to use gluconate as a sole carbon source when cultured under fermentative growth conditions, whereas the *gntK*/*idnK* double mutant and wild-type strains grew equally well under these conditions (Table 6.4). The triple mutant was able to grow with gluconate under anaerobic conditions when nitrate was included in the medium as a terminal electron acceptor for anaerobic respiration. The triple mutant also grew with gluconate under aerobic conditions, although the doubling times for the triple mutant and the *gnt*/*idnK* double mutant with gluconate as the sole carbon source under aerobic conditions were somewhat longer than the doubling time for wild type. We infer from these data that STM0649/0650 has a role in gluconate utilization under anaerobic conditions, but another undefined route for gluconate utilization that is not reliant on the gluconate kinases GntK or IdnK is able to operate in *S.* Typhimurium under aerobic or anaerobic respiration growth conditions.

Table 6.4. Growth rate on MOPS with gluconate and ammonium. Cultures were grown either aerobically or anaerobically with gluconate as the sole carbon source and ammonium as the sole nitrogen source. There was no measureable growth for the triple mutant under anaerobic conditions.

	Doubling time under aerobic growth conditions (min)	Doubling time under anaerobic growth conditions (min)	Doubling time under anaerobic growth conditions with 50 mM sodium nitrate
Wild-type	72 ± 5.5	71 ± 6.4	140 ± 18
Δ*gntK* Δ*idnK*	98 ± 7.3	76 ± 8.3	210 ± 24
ΔSTM0649-50 Δ*gntK* Δ*idnK*	87 ± 6	No measurable growth	180 ± 24

Time permitting and depending on the results of the planned experiments, we wish to further characterize the STM0649-0651 operon through the following experimental approaches. First, we plan to examine transcript levels of STM0649 qRT-PCR to test the hypothesis that expression of the operon is up-regulated by gluconate under anaerobic conditions. We also plan to see if the STM0649/0650 enzyme is involved in mannoate utilization and have recently obtained mannonate for these experiments. It may be neccessary to delete *uxaA* to demonstrate that STM0649/0650 is involved in mannonate utilization since this gene is involved in turning over mannonate generated during glucoronate catabolism. In addition, we plan to purify the STM0649/0650 products and examine their enzymatic activities in vitro. Additional studies may

include: i) repeating the complementation assay with the *E. coli uxuA* mutant; and ii) determining if STM0651 transports gluconate by knocking out the genes encoding known gluconate transporters (*gntT*, *gntU*, and *idnT*) and then determining if STM0651 allows *S.* Typhimurium to utilize gluconate in this mutant background.

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CHAPTER 7: CONCLUSIONS

The goal of my dissertation research has been to identify activities for RpoN-dependent genes in *S.* Typhimurium of unknown function. Toward this end, I identified substrates for two mannose family PTS permeases and demonstrated roles for catabolic enzymes associated with these PTS permeases. Specifically, I discovered that one of the PTS permeases transports D-glucosaminate and that enzymes associated with the permease (i.e., genes encoding these enzymes are in an operon with genes encoding the PTS permease) convert D-glucosaminate-6-phosphate to 2-keto-3-deoxy-D-gluconate-6-phosphate, which is subsequently converted to pyruvate plus glyceraldehyde-3-phosphate. It is not evident where *Salmonella* might encounter D-glucosaminate in nature. It seems likely that D-glucosaminate is produced from the oxidation of D-glucosamine, a relatively abundant amino sugar. D-glucosamine could be oxidized non-enzymatically, or could be oxidized by the action of glucose oxidase or other hexose oxidases. Interestingly, glucose oxidase is a PQQ-dependent enzyme that is localized in the periplasmic space of *E. coli* and possibly other gram-negative bacteria. Thus, any D-glucosaminate produced from the oxidation of D-glucosamine by glucose oxidase would be free to diffuse across the outer membrane and would be available for transport by *Salmonella*.

I discovered that a second mannose family PTS permease in *S.* Typhimurium was responsible for transporting fructoselysine and glucoselysine. Deglycase enzymes associated with fructoselysine/glucoselysine PTS permease cleave the phosphorylated substrates of the PTS

permease to release lysine and glucose-6-phosphate or fructose-6-phosphate, which are further catabolized. Fructoselysine is an example of Amadori product, and since it involves the reaction of glucose with a primary amine it is known as a glycation product or fructosamine. Glucoselysine is an example of a Heyns products, and since it involves the reaction of fructose with a primary amine it is referred to as a fructation product. Amadori and Heyns products are widespread in nature and it is likely that *Salmonella* encounters these compounds in the gastrointestinal tracts of its hosts.

My preliminary data suggest the third mannose family PTS permease, which is encoded within the STM0572-0577 operon, transports fructosemethionine and possibly other α-glycated amino acids. We are only beginning to appreciate the significance of fructosamines as a nutrient for Salmonella. Ali *et al*. recently reported that the ability to utilize fructose-asparagine was important for successful colonization of *S.* Typhimurium in a mouse inflamed intestinal model. Thus, it seems quite probable that the ability of *Salmonella* to utilize other glycation products is important for efficient host colonization. Consistent with this hypothesis, results from my chicken colonization study revealed that at least one of the three mannose-family PTS permeases operons is important in the colonization of the chicken jejunum, ileum, and ceca. Moreover, some of the genes encoding these mannose family PTS permeases and associated enzymes have been implicated as being important for colonization of the chicken, pig, and calf (1). Further studies are needed in order to determine which of the mannose family PTS permeases are important for colonization of the chick intestinal tract.

An unexpected result of my research on fructosamine utilization in *Salmonella* was that fructoselysine and fructosemethionine serve as good nitrogen sources for the bacterium whereas lysine and methionine do not. The physiological basis for this paradox is not known. It may be

simply that the transport systems for lysine and methionine are not sufficient for supporting growth of *Salmonella* on these amino acids as sole nitrogen sources. Further investigation into the pathways responsible for the degradation of lysine and methionine derived from fructoselysine and fructosemethionine, respectively, may help to resolve the paradox surrounding the ability of *Salmonella* to use these fructosamines as nitrogen sources.

While the physiological role of the STM2357-2360 operon remains to be elucidated, I found that at least two of the gene products of the operon utilize arginine as STM2358 is an arginine/ornithine racemase and STM2360 has D-arginine transaminase activity. It is possible that the STM2357-2360 operon is involved in acid stress since expression of the activator (STM2356) is induced by pH shock (2). My attempts to show that the STM2357-2360 operon has a role in acid tolerance were unsuccessful, although it was not clear if the operon was expressed under the conditions of my assay. Future studies for this operon which might lead to further insight into its physiological role include determining the corresponding keto acid for the arginine transaminase, identifying conditions that result in induction of the operon, and discovering substrates for the transporters encoded bySTM2357 and STM2359.

The products of the STM0649-51 operon appear to be involved in utilization of gluconate under anaerobic conditions which may make it important for colonization since the gastrointestinal tract is anaerobic. Future directions for the study of this operon include determining if compounds that are structurally similar to gluconate, such as altronate or mannonate, are substrates for the products of the STM0649-0651 operon. In addition, purification of the putative dehydratase encoded by STM0649/0650 and characterization of its enzymatic activity in vitro will provide valuable information on the role of the STM0649-0651 operon in *S.* Typhimurium.

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