FUNCTIONAL AND GENETIC CHARACTERIZATION OF SULFATASES IN *Salmonella enterica* serovar Typhimurium

by

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ABSTRACT

FUNCTIONAL AND GENETIC CHARACTERIZATION OF SULFATASES IN *Salmonella enterica* serovar Typhimurium

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Under the Supervision of Dr. Gyaneshwar Prasad

Salmonella enterica serovar Typhimurium is a gram-negative facultative intracellular food borne pathogen and accounts for $365 million in direct medical costs annually. The ability of *S.* Typhimurium to compete with the other microorganisms in the host gastrointestinal tract for nutrients and penetrate the epithelial mucosal layers is essential for its pathogenesis. The host mucosal glycans, the potential source of carbohydrates for gut microbiota, are heavily sulfated, making them resistant to digestion. The ability to produce sulfatases is important for utilizing the host-derived carbohydrates, intestinal adhesion, invasion of the host epithelium and systemic infection. The genomes of many enteric human pathogens contain annotated sulfatase genes, but very little is known about their regulation and physiological roles.

The genome of *S.* Typhimurium contains six annotated sulfatase genes. As a first step in elucidating the regulatory mechanisms of sulfatase expression, this study has

characterized two of them: an acid-inducible sulfatase, encoded by STM0084 (*aslA*), and a monoamine compound induced sulfatase encoded by STM3122.

The acid-inducible *aslA* is regulated by the EnvZ-OmpR and PhoPQ two-component regulatory system and is likely secreted by the TAT secretion system. The *aslA* mutant was also found to be defective in intracellular survival. This study represents the first report of acid stress regulated sulfatases.

The STM3122-encoded sulfatase was monoamine compound induced (MCI) and regulated by a transcriptional regulator, encoded by STM3124. The MCI sulfatase was found to be modified by the enzyme encoded by STM3123 and was localized in the periplasm. Interestingly the sulfatase was co-expressed with the monoamine regulon comprising a transporter and an oxidoreductase. It appears that the MCI sulfatase might be important for utilization of monoamine compounds as a carbon source. Infection experiments with zebrafish showed that the STM3122 mutant was defective in intracellular colonization. These results suggest that sulfatases are involved in pathogenesis, and sulfatase inhibitors might serve as potential therapeutic targets against *Salmonella* infections.

I dedicate my thesis to my loving husband, Shubhajit Mitra, for his immense support and constant encouragement. I could not have done it without him.

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ACKNOWLEDGEMENTS

I want to start by thanking my mentor Dr. Gyaneshwar Prasad for giving me a great platform to pursue research on *Salmonella.* When I joined the lab, I started with a project on *Escherichia coli* but it did not work as planned. So my husband told me that I should go and talk to him for a new project. I was little nervous because I did not know how he would react to it. So I finally went and spoke to him and was surprised to learn that he was also thinking on similar lines and had few things already in his mind that he wanted me to look at. So I was very excited and went ahead with preliminary experiments that we planned. The results for *Salmonella* were very exciting which led to my first publication. He always wanted me to present at different conferences, which I think gave me a great exposure to the big world out there. He was also always there when I was lost with some of my experiments. His continuous support and wonderful ideas allowed me to do what I always wanted to do and also helped me to realize what I want to do in the future. He was very supportive and instrumental in helping us take various big decisions about our future. We will be indebted to him as he gave us beautiful five years of togetherness.

I want to then thank my husband for allowing me to come to UWM. I was already married when I got accepted for my graduate studies. My parents and in-laws were all against me doing my Ph.D in a different country away from my husband. But he stood by me during the most difficult times and told me that I should go ahead for my graduate studies and promised me that he would follow me the next year. So my degree would not have been possible without him. It was always his dream. So I am happy for him. He was a great colleague to work and his suggestions helped me to troubleshoot easily. His

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I want to thank all my committee members Dr. Mark McBride, Dr. Daad Saffarini, Dr. Ching Hong Yang and Dr. Sergei Kuchin for their valuable feedback during the committee meetings.

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Lastly, I want to thank my parents and in-laws for their great support during the program. Their constant faith in me allowed me to complete it inspite of all the hurdles.

Chapter I – Introduction

Enteric pathogens are species of bacteria that invade the intestinal epithelium and initiate infections (1). Some of the common enteric pathogens include *Clostridium* species*, Salmonella enterica, Vibrio cholerae, Helicobacter pylori, Campylobacter jejuni, Yersinia* species*,* Enterotoxigenic *Escherichia coli* (ETEC) and enterohaemorrhagic *Escherichia coli* (EHEC) (2-4). These bacteria can cause a range of diseases from the less severe gastrointestinal infections to life threatening diseases like typhoid, gastric cancer, cholera, bacteremia and severe dehydrating diarrhea (4). CDC estimates that each year, approximately 128,000 people get hospitalized and 3000 deaths occur in the United States due to food borne illness caused by enteric pathogens.

Salmonella enterica serovar Typhimurium

Salmonella enterica serovar Typhimurium is a Gram-negative bacterium that belongs to the γ- group of Proteobacteria. It is the major cause of food borne illness worldwide each year, affecting around 94 million people (5). *Salmonella* outbreaks have often been linked to foods of animal origin; however, in the recent years produce-associated outbreaks have become common (6). *Salmonella* infection is contracted by consumption of contaminated food or water. The ingested bacteria get disseminated from the bloodstream to different host tissues and get excreted in the fecal matter (7). Outside the host, *Salmonella* can survive in the soil for prolonged periods and gain entry into the plants via lateral roots, wounded tissues, and stomata pores, and contaminate the fresh produce (Fig 1) (8-10).

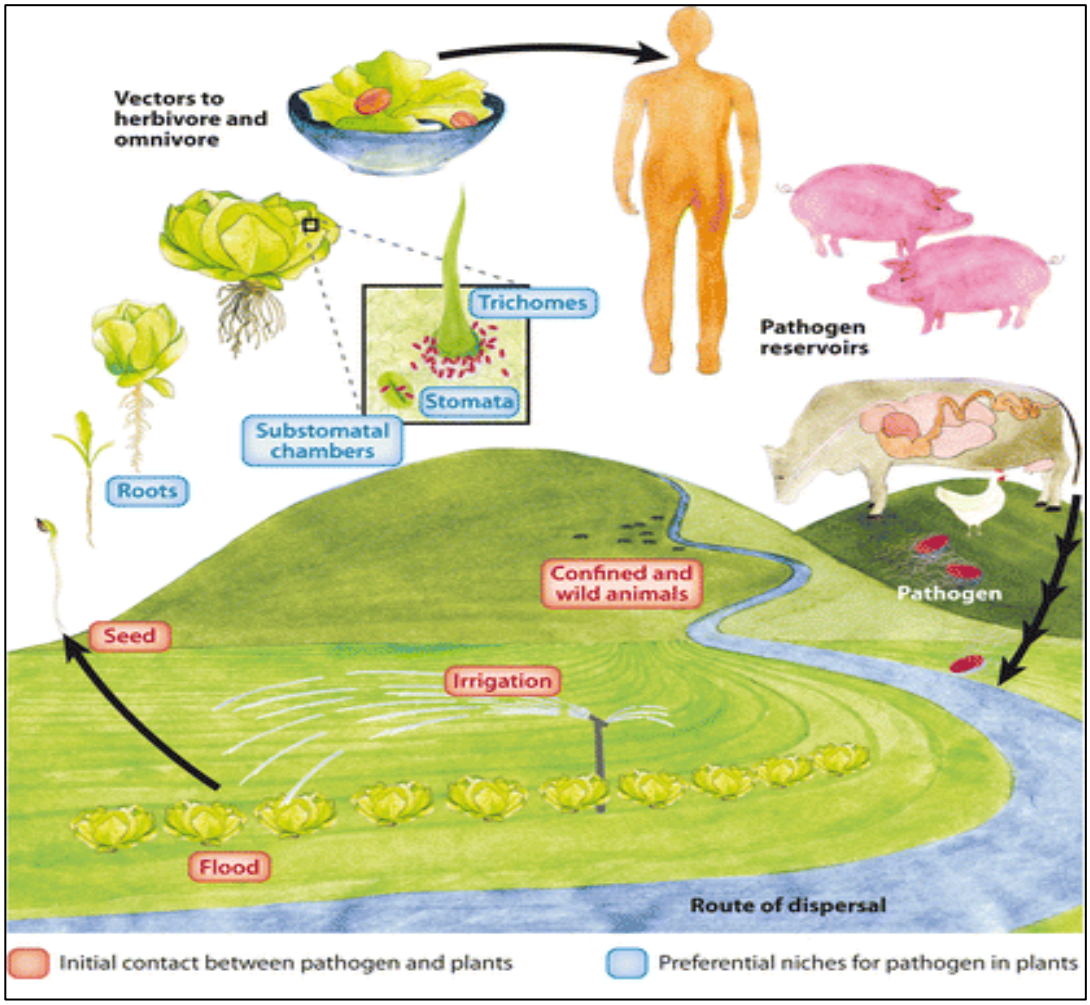

Figure 1. Life cycle of *Salmonella.* See text for details. [Adapted from (11)]

Role of nutrient assimilation in bacterial pathogenesis

Nutrient acquisition by the pathogen is a first step towards establishing successful infection. Unfortunately, not much has been documented about the nutritional preferences of *Salmonella* and the metabolic pathways that are critical for its persistence in the host. One of the major sources of nutrients in the mammalian gut is the mucus gel layer produced by the host goblet cells that cover the gastrointestinal epithelium (12). Mucin, the major constituent of mucus, is composed of oligosaccharide units. The four primary oligosaccharide units that are found in the mucin, N-acetylglucosamine, N-acetylgalactosamine, fucose and galactose and have terminal sulfate groups (12). In a recent study, it was shown that EHEC utilizes the mucus-derived carbohydrates in the bovine gut whereas the commensal *E.coli* does not (13). Thus, metabolic adaptations in the enteric pathogens confer a growth advantage and allow them to establish in the host environment.

Glycosaminoglycans (chondroitin sulfate, dermatan sulfate, and heparin) are linear polysaccharides comprising repeating disaccharide units of an amino sugar and uronic acid that are heavily sulfated to prevent their degradation by bacteria (14). However, by synthesizing sulfatases, they can hydrolyse sulfate from these sulfated carbohydrates. Bacterial strains like *Bacteriodetes thetaiotaomicron* and *Proteus vulgaris* have been reported to use glycosaminoglycans as nutrients (15, 16).

Sulfatases

Sulfatases represent a highly conserved gene family found in the three domains of life (18). These enzymes are characterized by a signature sequence "(C/S)XPXR" at the active site and undergo a unique post translational modification (19). Sulfatases require post-translational modification for the functionality of the enzyme that involves oxidation of the cysteine or serine at the active site to a Cα-formylglycine (FGly) residue. Based on the modified amino acid residue, these enzymes are broadly classified as Cys-type or Ser-type sulfatases (20). The formylglycine residue in its hydrate form attacks the sulfate ester group and forms an enzyme-sulfate intermediate. The enzyme-sulfate intermediate then dissociates to yield an alcohol and sulfate (Fig. 2) (21).

Sulfatases have been implicated in numerous biological processes such as assimilation of sulfur, hormone regulation, developmental cell signaling, cellular degradation and pathogenesis (22).

Genome wide analyses revealed the presence of putative sulfatase genes in bacterial species encompassing five phyla (Proteobacteria, Actinobacteria, Bacteroidetes, Planctomycetes and Cyanobacteria) (Fig 3). Surprisingly, most species have multiple genes annotated as sulfatases (23). Although genome analysis has revealed the presence of putative sulfatase genes in numerous bacteria they have only been studied and characterized in genera *Klebsiella, Salmonella, Pseudomonas, Sinorhizobium, Alteromonas* and *Mycobacterium* (24-31).

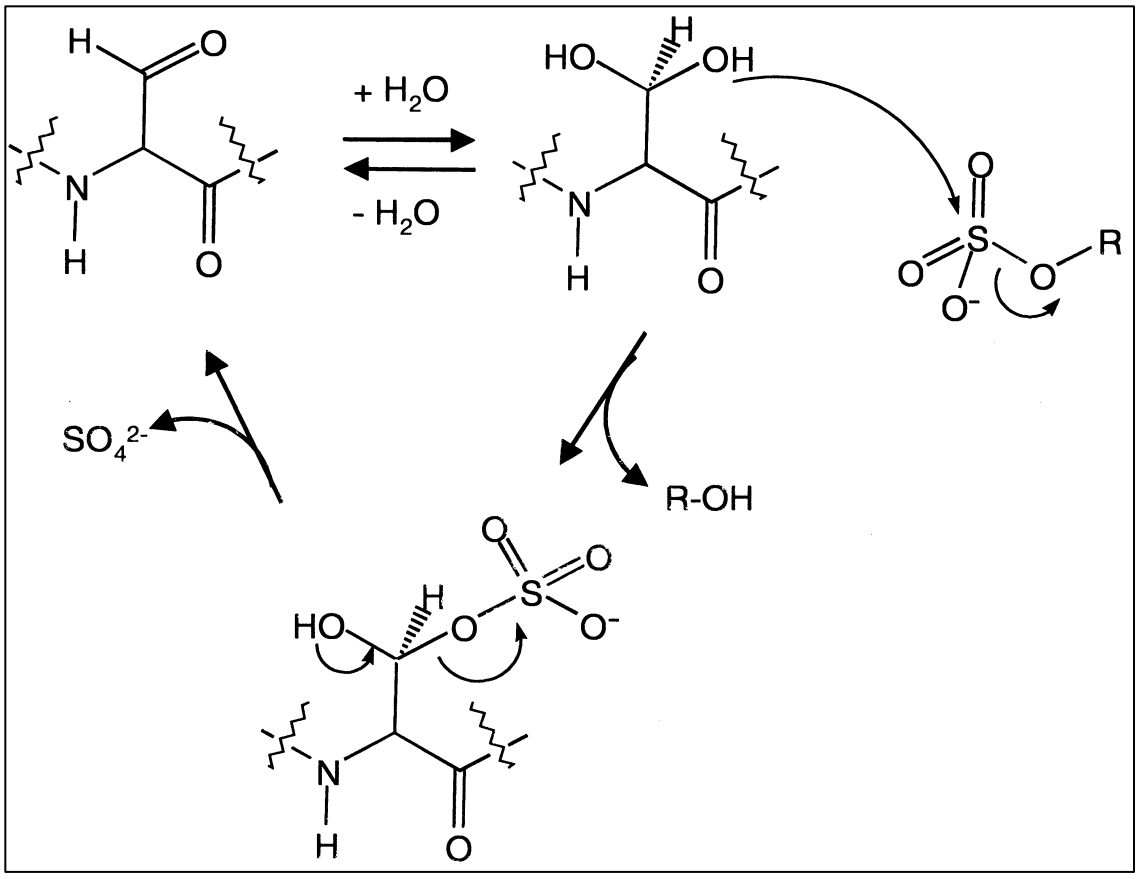

Figure 2. Mechanism of sulfate ester hydrolysis by sulfatases. [Adapted from (21)]

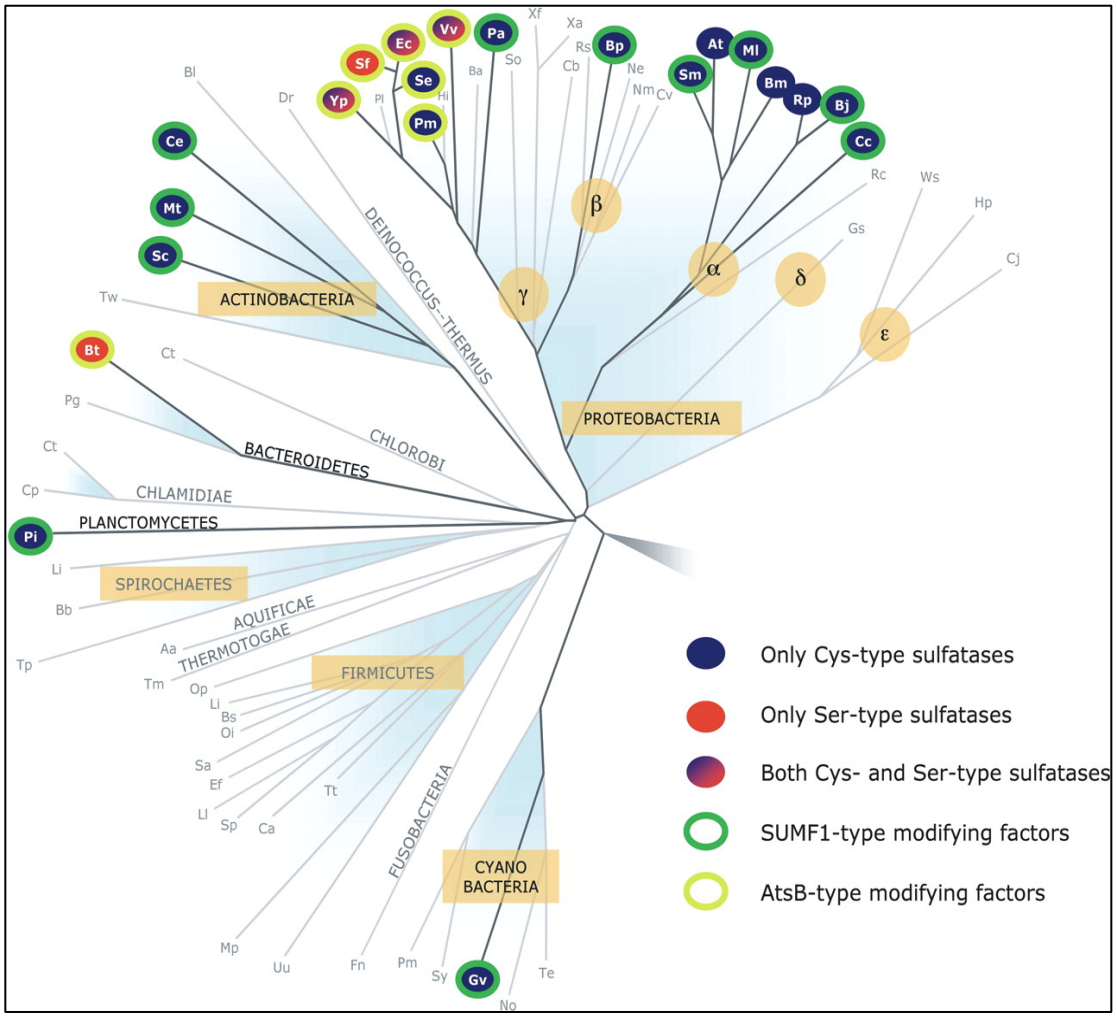

Figure 3. Phylogenetic tree showing the distribution of sulfatases in sequenced bacterial species. The phyla that lack sulfatase genes are shown in grey. [Adapted from (21, 23)]

Enzymatic desulfation by bacterial sulfatases facilitate processing of host mucosal glycans as nutrients (32). Desulfation by bacterial sulfatases not only allows access to the host nutrients, but also appears to be involved in host-microbe association (33). In addition, sulfatases have been shown to be involved in sulfate scavenging in soil bacteria, *Pseudomonas putida* and enteric bacteria, such as *Klebsiella aerogenes* (24, 34). In the marine bacterium, *Alteromonas carrageenovor*a, the sulfatase has been reported to hydrolyse the sulfated polysaccharides carrageenans from the red algae (31). These enzymes have also been used for taxonomic characterization of different members of Mycobacteria (35). Thus these enzymes have been implicated in diverse functions.

Sulfatases in *Salmonella*

The *Salmonella enterica* serovar Typhimurium genome has six genes annotated as sulfatases and four genes annotated as sulfatase regulators (Fig. 4); however, not much is known about the role of these genes. Initial analysis of the putative sulfatase genes was performed to predict the type of sulfatases in *S*. Typhimurium. Based on the predictions, there are four sulfatases that have the signature sequence (3 Cys- type, 1 Ser- type); however, two of the sulfatases in *S*. Typhimurium do not have a signature sequence (Table. 1). A Cys-type sulfatase without a signature sequence was reported in *Clostridium perfringens* (36).

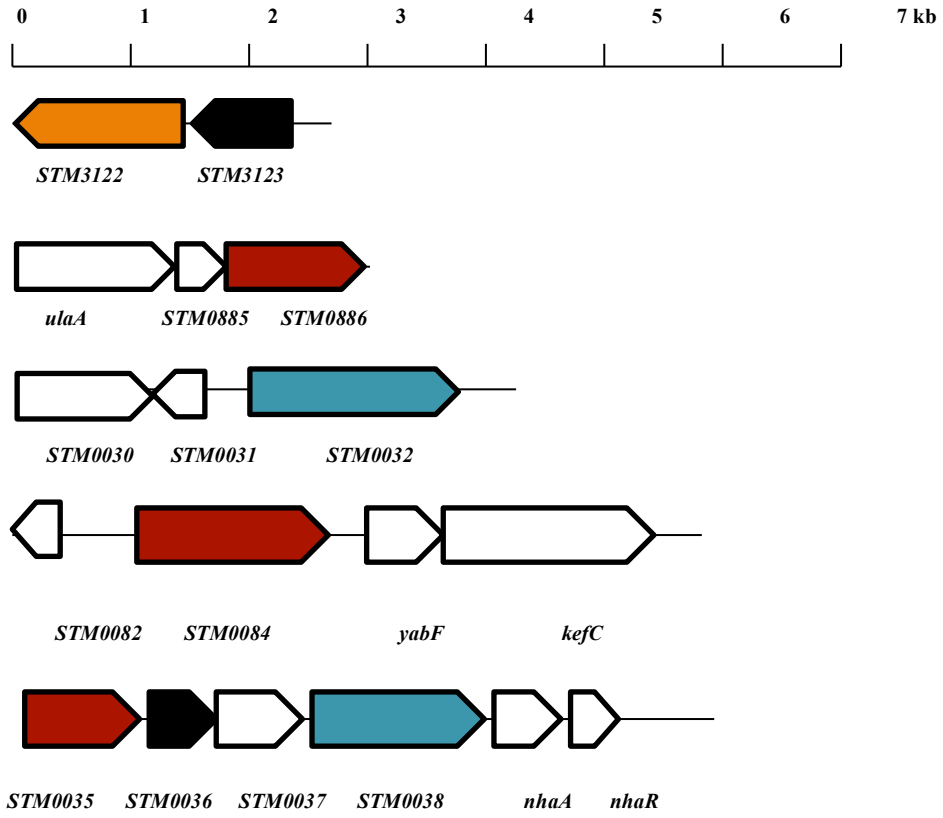

Figure 4. Genetic organization of annotated sulfatase gene clusters in *S*. Typhimurium. The Ser-type sulfatase is shown in orange, Cys-type sulfatases in red, and sulfatases without signature sequence in blue.

Table 1. Putative sulfatases in *S.* Typhimurium with the signature sequence

***Salmonella* locus**	**Signature Sequence (C/SXPXR)**
STM3122	**SAPAR**
STM0886	**C**MP**AR**
STM0032	-
STM0084	**C**TP**SR**
STM0035	**CSPSR**
STM0038	-

Note: (-) Signature sequence absent

Regulation of bacterial sulfatases

Studies in most bacteria have shown sulfatases to be regulated by sulfur availability (34, 37). These enzymes are derepressed when bacteria are grown in the presence of organosulfur sources such as methionine or taurine, and repressed when grown in presence of inorganic sulfate. Sulfur assimilation in enteric bacteria involves the *cys* genes under the control of the global regulatory protein CysB (38). The regulatory mechanism of sulfatase thus suggests that these enzymes might be involved in sulfur assimilation for the growth of bacteria and sulfatases might be an addition to the *cys* regulon. Interestingly, sulfatase from a marine bacterium *Alteromonas carrageenovora* is not regulated by sulfur supply (31).

Regulation of sulfatase by monoamine compounds like tyramine, dopamine and epinephrine has also been reported (39). The enzyme was also found to be co-regulated with the genes of the monoamine regulon via the regulatory protein MoaR (40). The expression of the monoamine regulon is subject to both catabolite and ammonium repression (41). However, the physiological link between the expression of the sulfatases in the presence of these compounds is not known. Recent studies have shown that bacterial pathogens perceive these compounds as host signals and respond by altering their gene expression (42, 43). Interestingly, on release from specific neurons these monoamine compounds are conjugated to sulfur to render them biologically inactive (44, 45).

Chapter II – AslA, an acid inducible sulfatase in *Salmonella enterica* serovar Typhimurium

Introduction

Salmonella enterica serovar Typhimurium is a facultative intracellular gut pathogen. It thrives at the low pH in the stomach and reaches the small intestine, where it invades the M cells and breaches the epithelial barrier (46). The type III secretion system 1 (T3SS1) encoded by *Salmonella* Pathogenicity Island 1 (SPI1) allows it to invade the M cells. Translocation of effector proteins by T3SS1 results in membrane ruffling and cytoskeletal rearrangement in the host cells, which facilitates endocytosis of the bacterium (47, 48). The bacterium reaches the basal surface of M cells where it is taken up by the macrophages. Within the macrophages, the bacterium establishes itself in replicative vacuole called *Salmonella* containing vacuole (SCV) (49). The translocation of effector proteins by T3SS2, encoded on the *Salmonella* Pathogenicity Island 2 (SPI-2) facilitates the intracellular survival of these bacteria (50). *S.* Typhimurium is then disseminated in the bloodstream and gets excreted in the fecal matter (Fig. 5).

The widespread occurrence of sulfatase and sulfatase modifying genes in *S.* Typhimurium genome suggests that they might be involved in survival and pathogenesis. The gastrointestinal tract harbors a dense population of microbes, which constantly compete for nutrients, and survival of the bacteria in this fierce environment is the first step towards successful host invasion. Of the various sources of nutrients available to the gut microbes, host mucosal glycans are important source of carbon. Some of the mucosal glycans are, however, heavily sulfated (e.g., colonic mucins and glycosaminoglycans)

(7), making them resistant to digestion and limiting the bacteria to not only for sulfur but also for carbon. To characterize the sulfatase genes, we hypothesized that these enzymes might be expressed at a pH they encounter within the host. In this chapter, we report an acid inducible sulfatase, discuss its regulation in *S.* Typhimurium and show that it might play a role in its survival within macrophages.

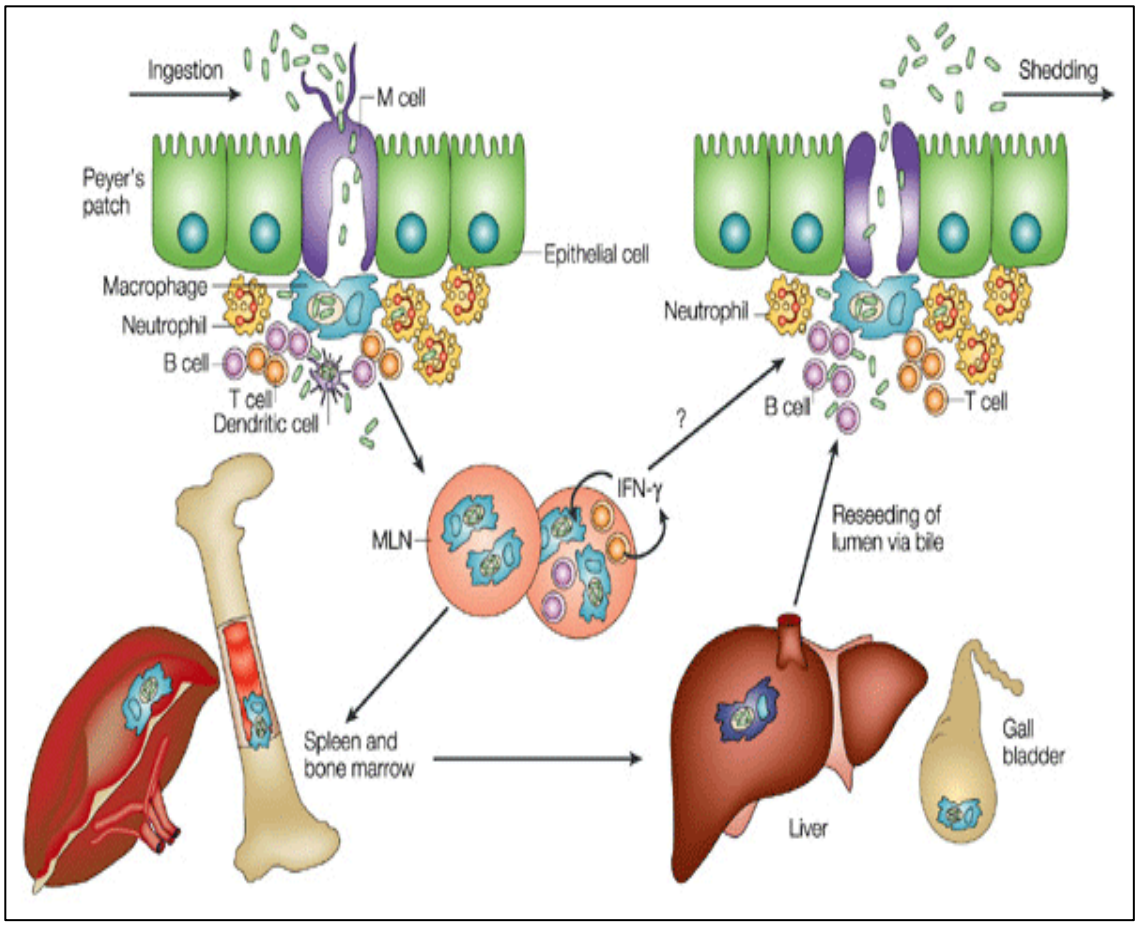

Figure 5. Complex pathogenesis exhibited by *Salmonella enterica.* See text for details. [Adapted from (7)]

Material and Methods

Bacterial strains, media and growth conditions

Bacterial strains and plasmids are summarized in Table 2. The *S.* Typhimurium and *Escherichia coli* strains were maintained on Luria Bertani (LB) agar at 37 °C. The following antibiotics were added as required: Kanamycin (75 μg ml^{-1}), Ampicillin (100 μg ml^{-1}), Spectinomycin (50 μg ml^{-1}), Tetracycline (10 μg ml^{-1}). MOPS Minimal medium MOPS Minimal medium was prepared as discussed by Neidhardt et al (51). The minimal media were filter-sterilized using a Millipore Express filter unit (0.22-μm pore diameter). The sulfated glycans heparin sulfate and chondroitin sulfate were used as the sole S source at 0.3% concentration.

Table 2: List of bacterial strains and plasmids used in this study

Strain/Plasmid	**Description**	**Source**
***Salmonella enterica* serovar Typhimurium strains**		
14028s	*Salmonella enterica* serovar Typhimurium ATCC1402S, Virulent isolate	(52)
LT2	Wild-type *Salmonella enterica* serovar Typhimurium, non-pathogenic isolate	(53)
SE1	LT2, ΔSTM3122	This work
SE2	LT2, ΔSTM0886	This work
SE3	14028s, ΔSTM0032	(52)
SE4	14028s, Δ*aslA*	(50)
SE5	14028s, ΔSTM0035	(50)
SE6	14028s, ΔSTM0038	(50)
SE7	14028s, Δ*cadC*	(50)
SE8	14028s, Δ*adiY*	(50)

SE9	14028s, Δ*rpoS*	(50)
SE10	14028s, Δ*phoP*	(50)
SE11	14028s, Δ*phoQ*	(50)
SE12	14028s, Δ*envZ*	(50)
SE13	14028s, Δ*ompR*	(50)
SE14	14028s, Δ*ompR* Δ*phoP*	This work, (54)
SE15	14028s, Δ*tatC*	(50)
SE16	14028s, ΔSTM0035	(50)
SE17	LT2, ΔSTM0035 ΔSTM3123	This work
SE18	LT2, ΔSTM0035 ΔSTM3123 ΔSTM1287	This work
SE19	LT2, ΔSTM0035 ΔSTM3123 ΔSTM1287 ΔSTM3966	This work
SE20	LT2, Δ*aslA* with pBAD18-*aslA*	This work, (54)
SE21	LT2, Δ*aslA* with pBAD18	This work, (54)
SE22	LT2, Δ*ompR* with pBAD18-*ompR*	This work, (54)
SE23	LT2, Δ*envZ* with pBAD18-*ompR*	This work, (54)
SE24	LT2, Δ*phoP* with pBAD18-*ompR*	This work, (54)
SE25	LT2, Δ*phoQ* with pBAD18-*ompR*	This work, (54)
SE26	LT2, Δ*ompR* with pBAD18	This work, (54)
SE27	LT2, Δ*envZ* with pBAD18-*aslA*	This work, (54)
SE28	LT2, with pBAD18-*aslA*	This work, (54)
SE29	LT2, Δ*phoP* with pBAD18-*aslA*	This work, (54)
***Escherichia coli* strain**		
DH5α	*supE44 recA1 lacZU169 (ϕ80 lacZ*ΔM15)	(55)
Plasmids		
pKD3	*Chl* cassette template, Amp^R	(56)
pKD4	*Kan* cassette template, Amp^R	(56)
pKD46	Red recombinase expression, Amp^R	(56)
pCP20	FLP recombinase expression, Amp^R	(57)
pBAD18	P_{BAD} expression vector, Amp^R	(58)
pHC60	Broad host range vector carrying *gfp,* Tet^R	(59)

Construction of mutant strains

S. Typhimurium deletion mutants were constructed using the method described by Datsenko and Wanner (56). Briefly, fragments consisting of an antibiotic cassette (Chloramphenicol or Kanamycin) with homology extensions of 50 nucleotides flanking the gene were amplified by PCR using template plasmids (pKD3 or pKD4 respectively). PCR products were purified and electroporated into *Salmonella* expressing Red recombinase from the helper plasmid pKD46. The mutants were selected on LB plates with the appropriate antibiotic. Mutations in the gene were confirmed using PCR and sequencing.

Flp excision of the antibiotic cassette

The antibiotic cassette was removed using Flp recombinase expressed on a temperature sensitive plasmid pCP20 (57). The strains were electroporated with pCP20 and selected on LB containing ampicillin at 30°C. The colonies were purified non-selectively at 42°C and finally checked for the loss of the plasmid and the antibiotic resistance in the strain.

P22 Phage transduction

The mutants were also constructed by using P22 phage transduction from the corresponding mutants of *S.* Typhimurium ATCC 14028. Bacteriophage lysates were prepared using methods described by Schmieger and Schicklmaier (60). Transductions were performed by incubating 100 µl of overnight grown recipient cells with 100µl of

phage lysates diluted to a titer of 10^8 PFU/mL at 37°C for 1 hour, and the mixture was plated on LB agar with appropriate antibiotics.

Rapid screening for sulfatase

The mutants were screened for sulfatase activity using the chromogenic substrate X-sulfate at 50 μg ml^{-1}. The substrate was added to the bacterial growth media. The activity was detected by the presence of blue coloration, indicating the hydrolysis of X-sulfate (61).

Quantitative sulfatase assay using p-nitrophenyl sulfate substrate

Sulfatase activity was determined by quantifying the release of p-nitrophenol from p-nitrophenyl sulfate using whole cells as described previously (24). Briefly, the overnight grown cells were harvested and resuspended in fresh growth medium, and the absorbance was read at 600 nm. For the assay, 1 ml of the cell suspension was diluted with 2 ml of the growth media, and permeabilized with 50 μl of chloroform by vortexing for 20 seconds. 1 ml of PNPS (6.4 mg/ 5 ml) was added and the cells were incubated at 37°C until a yellow colour developed. The reaction was terminated by adding 400 μl of 1N NaOH and the cells were spun down. The absorbance of the supernatant was measured at 550nm and 420nm. The enzyme activity was calculated in Miller Units (MU) using the formula:

Miller Units = $[1000\times \{OD_{420} - 1.75 \times OD_{550}\}] / T \times V \times OD_{600}$

T = Time in minutes

V = Amount of culture in ml

Complementation of mutants

The *ompR* and the *aslA* genes were amplified by PCR using the primers listed in Table 4 and high fidelity Phusion polymerase (Thermo Scientific). The amplified fragments were digested with SphI and HindIII, purified and ligated into pBAD18 (58). The ligations were transformed into DH5α, and the transformants were selected on LB plates containing ampicillin. The plasmids with inserts were confirmed using PCR and electroporated to *Salmonella* mutant strains. For the sulfatase assay, the complemented stains were grown with L-arabinose (0.2%) to induce the expression of the genes.

RNA isolation and RT-PCR

The strains were grown to mid-logarithmic phase in MOPS minimal medium at either pH 5.5 or pH 7.0. The cells were harvested and RNA was isolated using TRIzol reagent (Sigma). RNA concentrations were determined by UV/Vis spectrophotometry. RNA samples were further treated with RNase free DNase (Promega) to eliminate any DNA contamination.

cDNA synthesis and RT-PCR were performed using AccessQuick RT-PCR system (Promega) as follows. A DNase treated RNA sample (0.3 μg) was used as template for cDNA synthesis at 45°C for 45 min. PCR amplification was performed using primers listed in Table 3 as follows: 2 min of incubation at 94°C, followed by 25 cycles of amplification with denaturing at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min. Final extension was carried out at 72°C for 10 min. RNA samples that had not been subjected to reverse transcription were used as negative controls and *recA* was used as the internal control.

Table 3. List of primers used in this study

Primers	Sequence
Deletion of *S. enterica* STM3122	
STM3122FP	TTGCCCGCCGCTCTTGTACAACATCCGTAGTCTGAAATGGAGAATAAGC CGTGTAGGCTGGAGCTGCTTC
STM3122RP	TGAATGCGGTTATGTGAGCGTTGAATAATCATTTGAAACGTAACCAATAA CATATGAATATCCTCCTTA
Deletion of *S. enterica* STM0886	
STM0886FP	ATGTGGTAGAGAAAAATTTTCCGCACCTGCTCAACAAATGAGGGTGAGT CGTGTAGGCTGGAGCTGCTTC
STM0886RP	ACTTGCCGGGTGACGCTAACGCGCACCCGGCTTACAACATTGTTACGTT TCATATGAATATCCTCCTTA
Deletion of *S. enterica* STM3123	
STM3123FP	AAATGTGAGTCATGTCTCCGCATCAGAAATGATAACTACAGGACAGAAT AGTGTAGGCTGGAGCTGCTTC
STM3123RP	GACTACGGATGTTGTACAAGAGCGGCGGGCAAATGCGGCAAGAGGAAC CACATATGAATATCCTCCTTA
Complementation of *S. enterica* STM0084 (*aslA*)	
STM0084FP	ATTGACGCATGCACATATCCACGCTTCCTCGTACAGT
STM0084RP	ATTGACAAGCTTTCGCTGACCAATCGGTTTAAGGC
Complementation of *S. enterica ompR*	
*ompR*_FL_F	ATTGACGCATGCCACACGGGGTATAACGTGATCGTC
*ompR*_FL_R	ATTGACAAGCTTTGAACTTCGCGGTGAGAAGCG

Reverse Transcription

***recA* FP**	**GTCCAACACGCTGTTGATTTTC**
***recA* RP**	**GCAACGCCTTCGCTATCGT**
***aslA* FP**	**CCGGCAAACAGGCATCAATC**
***aslA* RP**	**TTTGTAGCGCAATAACGCGG**

Preparation of cell fractions

The bacterial cells were grown in MOPS minimal medium at pH 5.5 and cells were removed by centrifugation at 15,000g for 20 min to prepare the extracellular fraction. The harvested cells were used to prepare spheroplasmic and periplasmic fractions by PeriPreps-Periplasting Kit (Epicenter Biotechnology, Madison, WI).

***GFP labeling of S.* Typhimurium**

S. Typhimurium was GFP labeled using broad host range plasmid pHC60 (59). The plasmid was electroporated into *S.* Typhimurium and the transformants were selected on LB plates containing tetracycline

Infection of zebrafish embryos and microscopy

Bacterial strains were grown in MOPS minimal medium, pH 7.0. The cells were harvested, washed and resuspended in fish medium. Five zebrafish embryos (5 days post fertilization; gift of Dr. A. Udvadia, UW-Milwaukee) were placed in 20 ml of the fish medium with approximately 10^{11} CFU/ml bacteria in a 6 well- plate and incubated at 28°C for 3 hours. The embryos were removed from the wells and washed thoroughly and placed in fresh medium at 28°C on a 12 : 12 h light/dark cycle during the course of the experiment in the growth chamber (62). The zebrafish were removed and washed 3 times with sterile fish medium, anesthetized and mounted on low melting point agarose and visualized under laser confocal microscopy.

Isolation of bacteria from infected zebrafish embryos

All experiments involving zebrafish were done according to the protocols approved by the IACUC UW-Milwaukee. The bacteria were isolated from the infected zebrafish embryos by methods described previously (63). Briefly, the embryos were removed at different time points and washed thoroughly in embryo medium. The embryos (groups of five) were homogenized in 100 µl of PBS containing 1% Triton X-100 and incubated at 37°C for 20 min and serially diluted. The dilutions were plated on minimal media plates with appropriate antibiotics to get total cfu counts. For quantification of intracellular bacteria, the embryos (groups of five) were homogenized in 100 µl of PBS containing Trypsin-EDTA and incubated at 37°C for 20 min to get the intact host cells. The suspension was then split into two parts and one was treated with Gentamycin (100 µg ml^{-1}) at 37°C for 45 min and the other part was used as control. Both suspensions were then washed with PBS and resuspended in 100µl of PBS containing 1% Triton X-100 and incubated at 37°C for 20 min. The suspensions were serially diluted and plated on minimal media plates with appropriate antibiotics.

Results

Expression of sulfatase in *S.* Typhimurium requires a mild acidic pH

S. Typhimurium produced blue coloration on MOPS minimal media plates adjusted to pH 5.5 containing the chromogenic substrate X-gluc, indicating the presence of sulfatase activity. No sulfatase activity was observed on media plates adjusted to pH 7.0. *Escherichia coli* used as a negative control did not show any activity under tested conditions (Fig 6A). Further quantification of the enzyme activity by measuring the release of the p-nitrophenol detected the sulfatase in *S.* Typhimurium only in cells that were grown at pH 5.5 and assayed at pH 5.5. (Fig 6B)

STM0084 encodes the acid inducible sulfatase

Rapid screening of the mutants deleted for putative sulfatase genes (STM3122, STM0886, STM0032, STM0035, STM0038 and STM0084) identified that STM0084 mutant lacked sulfatase activity when grown in MOPS minimal medium adjusted at pH 5.5 with X-gluc (Fig 7A). STM0084 is annotated as *aslA* and encodes a putative arylsulfatase (64). The sulfatase activity in *aslA* mutant was restored to the wild type levels in the complemented strain (Fig 7B).

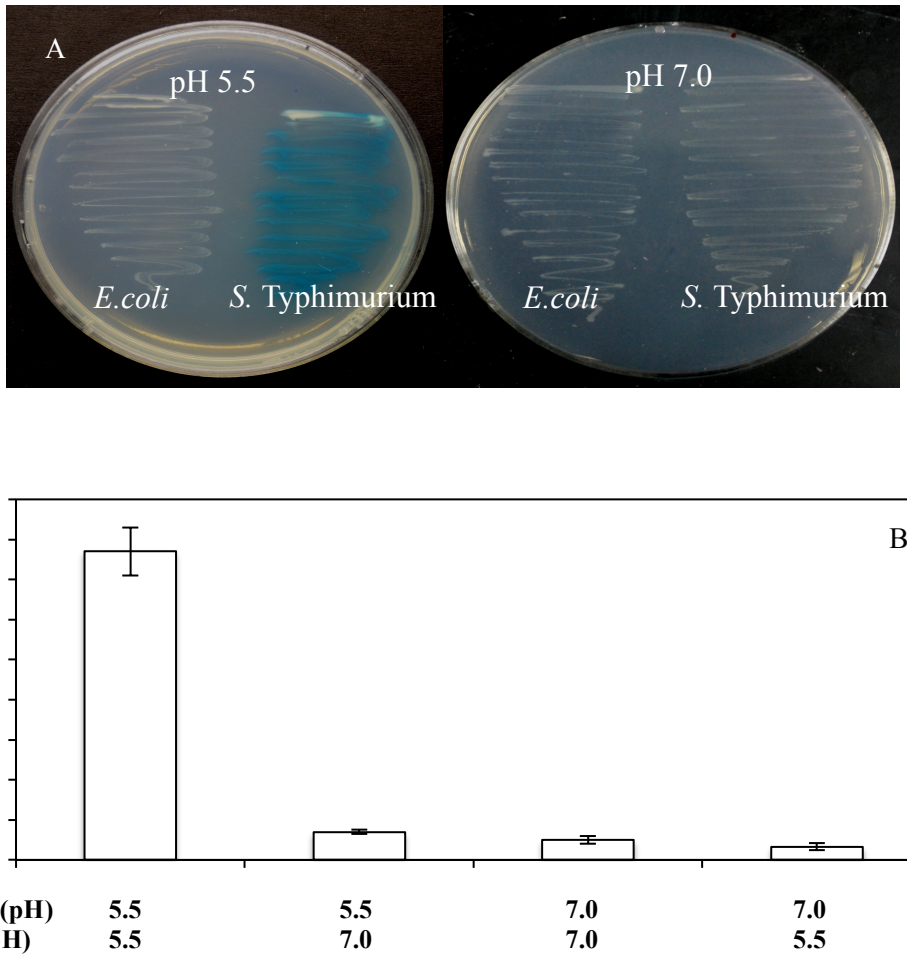

Figure 6. Expression of sulfatase in *S.* Typhimurium requires a mild acidic pH (A) Sulfatase activity was determined by hydrolysis of X-Sulfate on MOPS minimal media plates adjusted at different pH. *S.* Typhimurium showed sulfatase activity at pH 5.5 and no activity was detected at pH 7.0. *E. coli* used as a negative control did not show any sulfatase activity. **(B)** Sulfatase activity (in Miller Units) was determined by the hydrolysis of *p*-nitrophenyl sulfate. *S.* Typhimurium showed sulfatase activity only when it was grown and assayed at pH 5.5. The results are the means ± standard deviations of at least three independent experiments.

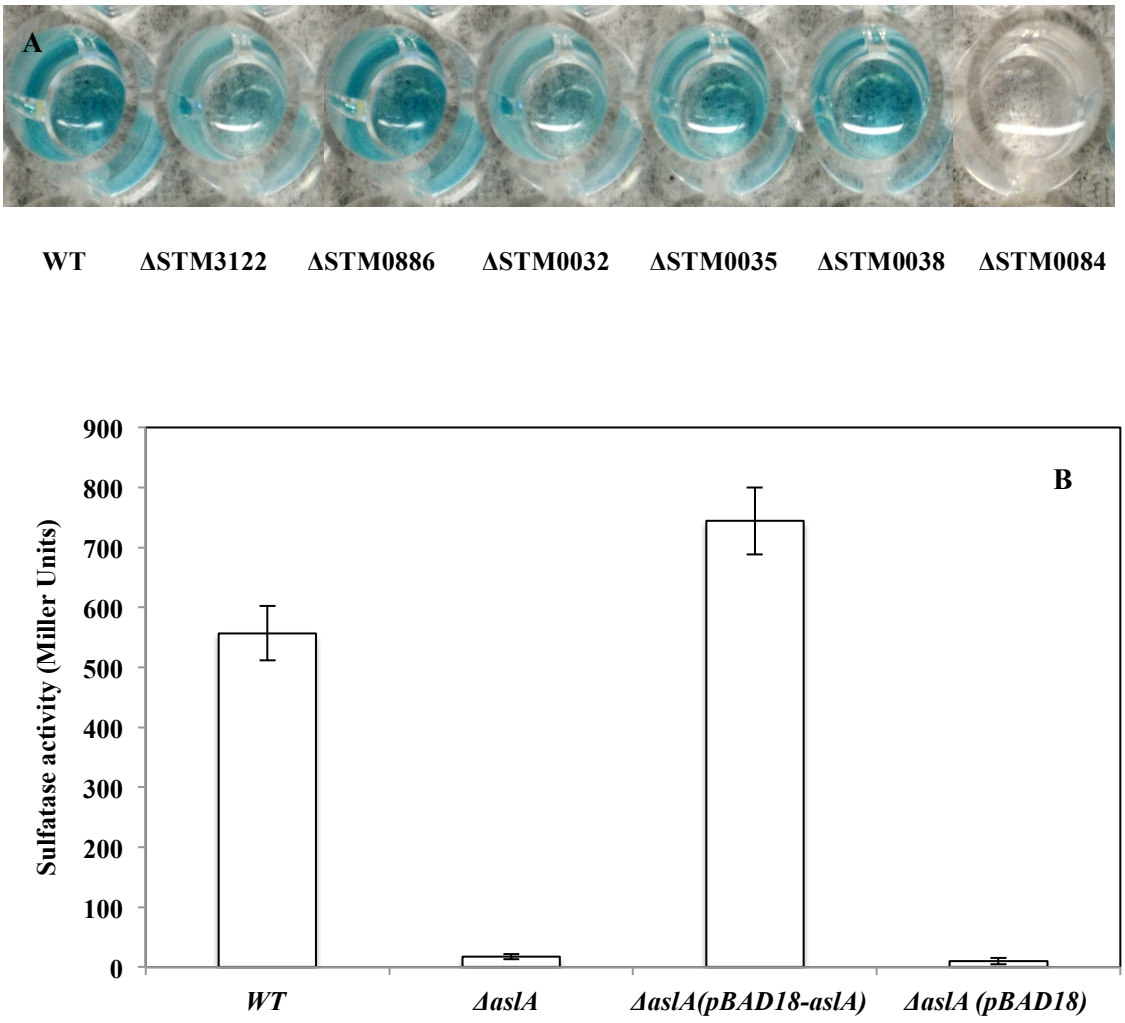

Figure 7. STM0084 encodes the acid inducible sulfatase. (A) Rapid screening of mutants identified that STM0084 encodes the acid inducible sulfatase **(B)** The *aslA* mutant lacked sulfatase activity and the mutant phenotype was restored by plasmid-based complementation. *aslA* mutant with the empty vector was used as a control. The results are the means ± standard deviations of three independent experiments.

Regulation of the acid inducible sulfatase in *S.* Typhimurium involves a novel mechanism

Previous studies had shown that expression of sulfatases in *S.* Typhimurium requires presence of monoamine compounds and is regulated by sulfate starvation and catabolite repression (39). To further determine if the acid inducible sulfatase was regulated by similar mechanisms, *S.* Typhimurium was grown with glycerol as carbon source and in minimal media supplemented with Tyramine as described earlier (39). For sulfur starvation experiments glutathione was used at 250 μM concentration. In contrast to the previous reports, the expression of the acid inducible sulfatase was not dependent on the presence of the tested monoamine compound Tyramine and was not affected by catabolite repression and sulfate starvation (Table 4).

Table 4. Acid inducible sulfatase in *S.* Typhimurium is not regulated by catabolite repression and sulfate starvation.

C source	S source	Sulfatase activity (Miller Units)
Glucose	Sulfate	557 ± 45
Glycerol	Sulfate	480 ± 50
Glucose	Glutathione	500 ± 38

aslA encoded sulfatase in _S._ Typhimurium requires EnvZ-OmpR and PhoPQ two component systems (TCS)

As *aslA* encoded sulfatase in *S.* Typhimurium required an acidic pH for its expression, we wanted to check the involvement of known acid response regulators in the expression of acid inducible sulfatase. The sulfatase activity in the mutants of known acid response regulators (*cadC, adiY, rpoS, phoP, phoQ, envZ, ompR)* (65-70) was assayed. The mutant lacking *phoP* or *phoQ* showed approximately 2-fold less activity, whereas mutants lacking *envZ* or *ompR* had 50-fold less activity than the wild type (Fig 8A). As the assay was performed with cells permeabilized with chloroform, the lack of sulfatase activity in the *envZ* or *ompR* mutants is not likely due to differential regulation of outer membrane porins in these mutants compared to the wild type. Sulfatase activity was restored to the wild-type level in the *ompR* mutant by complementation with *ompR* on a pBAD18 plasmid under the control of arabinose-induced promoter (Invitrogen Life Technologies) (Fig. 8B). However, there was significantly less sulfatase activity when OmpR was overexpressed in the *envZ*, *phoP*, or *phoQ* mutants (Fig. 8B). These results indicate that *aslA* expression is likely regulated by EnvZ- OmpR and require PhoPQ for maximal expression. No activity was observed in the *ompR* mutant containing only the vector *ompR* (VC) (Fig. 8B). It is also possible that these regulatory proteins might be controlling the expression of the sulfatase-modifying enzyme. To test this hypothesis the expression of *aslA* from arabinose inducible promoter was also checked in *ompR, envZ* and *phoP* mutants. The sulfatase activity was completely restored by the plasmid containing *aslA* in the *ompR*, *envZ* and *phoP* mutants. As a control, no sulfatase activity

was observed in cells containing only the vector (Table 5). These results suggest that these regulatory systems are required for expression of *aslA* and do not regulate a sulfatase modifying enzyme.

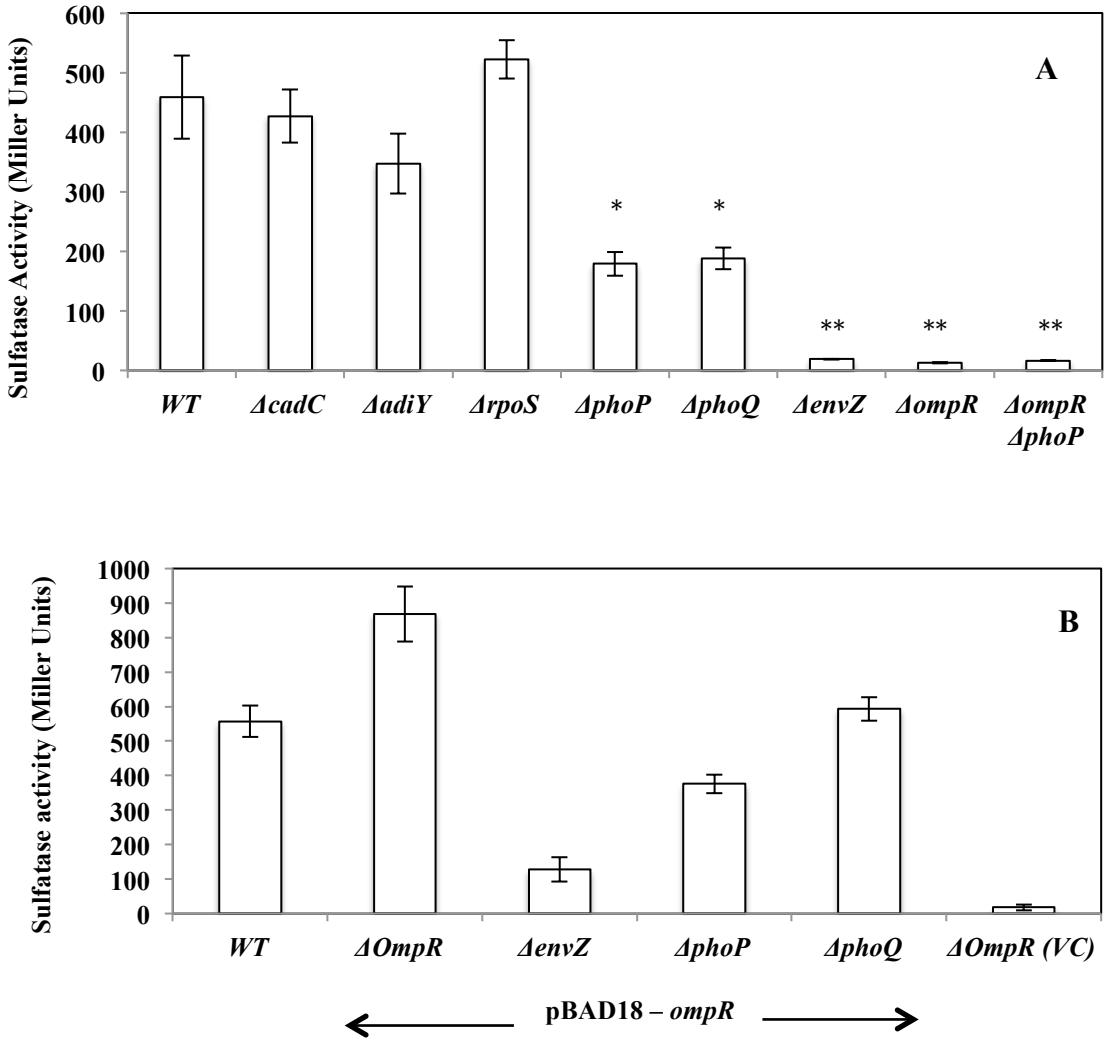

Figure 8. ***aslA*** **encoded sulfatase in** ***S.*** **Typhimurium requires EnvZ-OmpR and PhoPQ Two Component Systems (TCS) (A)** AslA is regulated by the PhoPQ and EnvZ-OmpR regulatory systems. The sulfatase activity was reduced 2-fold in mutants lacking *phoPQ* and was reduced 50-fold in mutants lacking *envZ-ompR* compared to the wild type. Mutants affected in *cadC*, *adiY*, and *rpoS* had activity similar to the wild type. **(B)** Complementation by plasmid based OmpR resulted in restoration of the sulfatase

activity in the *ompR* mutant, but was significantly less in the *envZ*, *phoP*, and *phoQ* mutants. *ompR* mutant with only vector was used as vector control (VC) did not show any activity. *, $P < 0.01$; **, $P < 0.001$. The results are the means ± standard deviations of at least 3 independent experiments.

Table 5. *aslA* encoded sulfatase in *S.* Typhimurium requires EnvZ-OmpR and PhoPQ two component systems (TCS). Sulfatase activity in *envZ*, *ompR* and *phoP* mutants containing the plasmid borne AslA suggests that the regulation of sulfatase modifying enzyme is independent of EnvZ-OmpR and PhoPQ TCS.

Strain	**Sulfatase Activity (Miller Units)**
WT	557 ± 45
Δ*aslA*	17 ± 4
Δ*aslA* (pBAD18-*aslA*)	744 ± 55
Δ*envZ* (pBAD18-*aslA*)	1046 ± 99
Δ*ompR* (pBAD18-*aslA*)	770 ± 23
Δ*phoP* (pBAD18-*aslA*)	975 ± 93
Δ*aslA* (pBAD18)	10 ± 5

OmpR regulates the expression of *aslA* via unidentified regulatory proteins

To further explore the role of OmpR in the expression of *aslA,* RT-PCR was performed. In accordance with the previous results, the *aslA* transcript was not detected in cells grown at pH 7.0 but was detected in cells grown at pH 5.5 (Fig 9). Expression of *recA* was used as a control. To evaluate if OmpR regulates *aslA* directly or indirectly, wild-type *S.* Typhimurium was grown at pH 7.0 and shifted to pH 5.5 with or without chloramphenicol (inhibitor of protein synthesis) and incubated for an additional 6 h. The cells were harvested and transcript levels of *aslA* and *recA* were determined. Although expression of *recA* was not affected, *aslA* transcript was not detected in chloramphenicol treated cells indicating that chloramphenicol did not affect the general transcriptional abilities of the cells in the assay (Fig 9).

AslA is not required for utilization of the heparin sulfate and chondroitin sulfate

As sulfatases in *Pedobacter heparinus* (previously *Flavobacterium heparinum*), *Bacteriodetes thetaiotamicron* and *Proteus vulgaris* have been reported to be involved in utilization of sulfated glycans (15, 16, 71, 72), the role of AslA in utilization of these glycans was examined. The wild type and the *aslA* mutant did not show any difference in growth with heparin sulfate or chondroitin sulfate as the sole sulfur sources in the MOPS minimal media at pH 5.5 (Fig 10). These results suggest that AslA is not required for the utilization of the tested sulfated glycans.

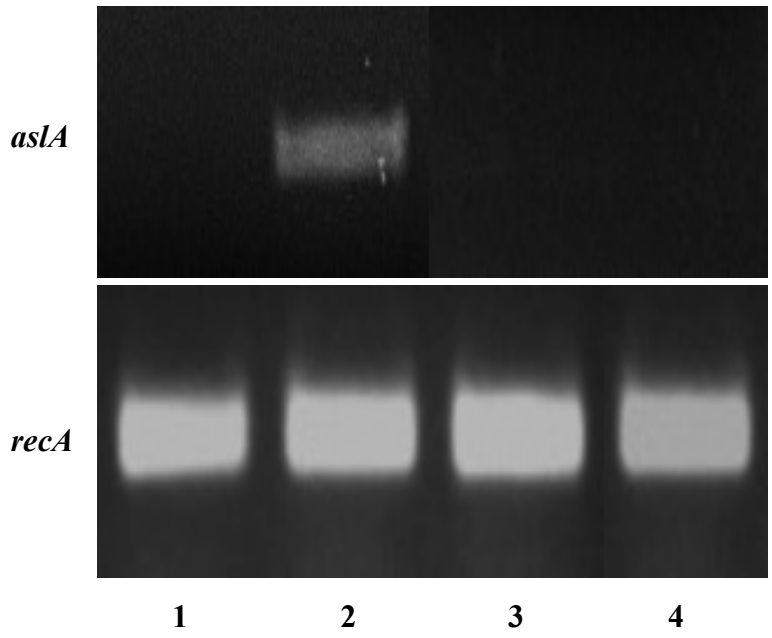

Figure 9. RT-PCR analysis of *aslA* expression. *aslA* was not expressed in cells grown at pH 7.0 (lane 1). The expression was induced when the cells were grown at pH 5.5 (lane 2). The *aslA* transcript was absent in *ompR* mutant (lane 3). Chloramphenicol treatment of wild type grown cells at pH 7.0 that were shifted to pH 5.5 resulted in repression of *aslA* (lane 4). *recA* was used as an internal control.

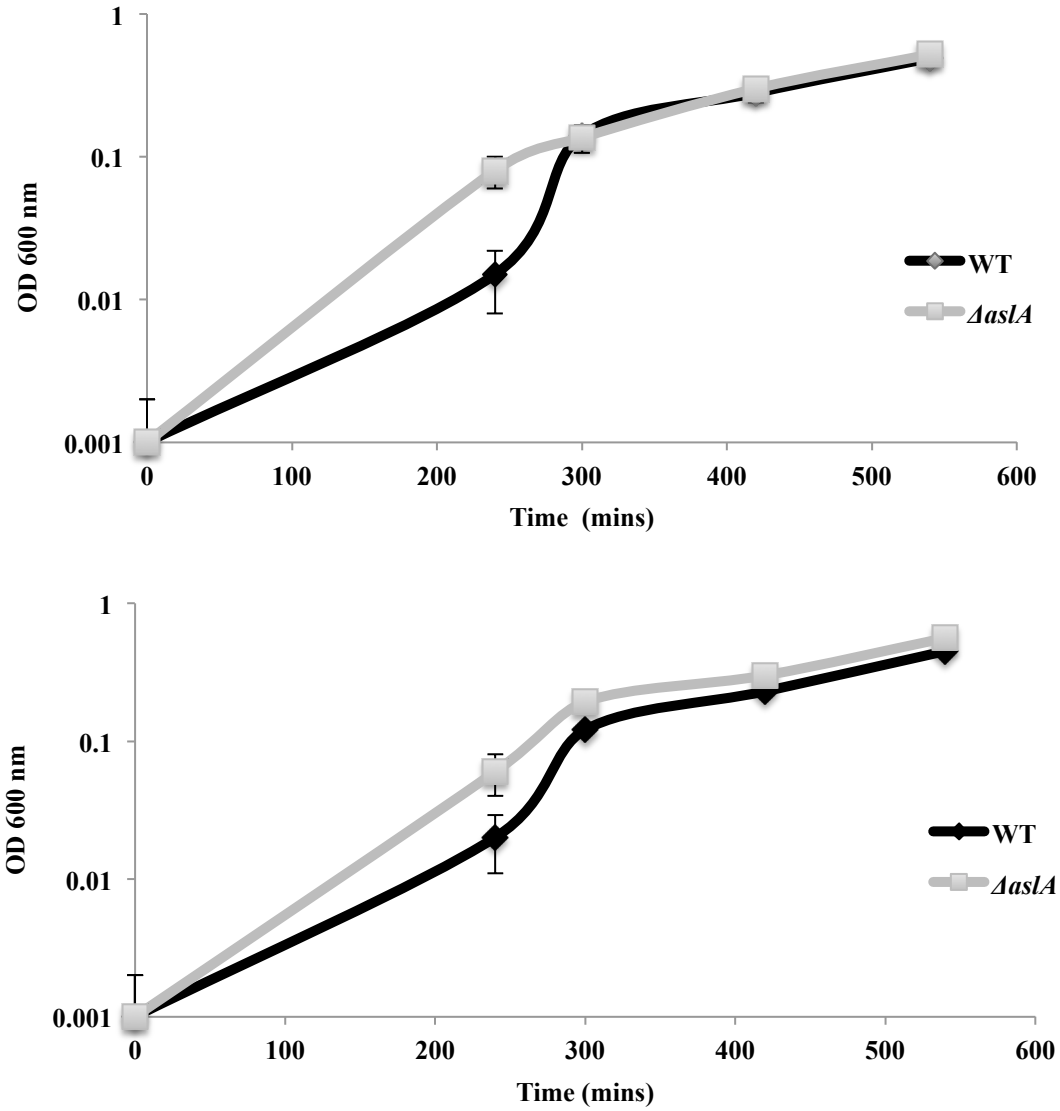

Figure 10. Growth of *aslA* on chondroitin sulfate (top) and heparin sulfate (bottom) as sole sulfur source. The *aslA* mutant did not show any growth defect on tested sulfur sources when compared to the wild type. The results are the means ± standard deviations of at least 3 independent experiments.

AslA might be secreted by the twin arginine translocation (TAT) system to the cytoplasmic membrane

To check the cellular localization of the acid inducible sulfatase in *Salmonella*, fractions were prepared and assay for sulfatase was performed. Sulfatase activity was not detected in the extracellular and the periplasmic fraction, but it was detected in the spheroplasmic fraction (data not shown). This finding suggests that it might be cytosolic or localized on the inner membrane.

AslA was predicted to be a twin arginine translocation (TAT) substrate (64). To confirm the secretion of the enzyme by the TAT system, the sulfatase activity was checked in strains with and without chloroform. Chloroform was used to permeabilize the inner membrane. The sulfatase activity was checked in the wild type strain with and without chloroform. No significant difference in the activity was observed. Interestingly, the sulfatase activity in the *tatC* mutant without chloroform was significantly reduced. However, the *tatC* mutant with chloroform showed similar activity as the wild type (Table 6).

AslA is likely modified by an unidentified sulfatase modifying enzyme

S. Typhimurium has four genes (STM0036, STM1287, STM3123 and STM3966) annotated as sulfatase modifying enzymes. To elucidate the role of these genes in the maturation of the acid inducible sulfatase, single mutants of these putative modifying enzymes were checked for their acid inducible sulfatase activity. No change in sulfatase activity was observed in the single mutants, suggesting the possibility functional redundancy. To test the functional redundancy of the genes, all genes were deleted one at

a time by removing the antibiotic cassette via Flp-recombinase mediated excision and the sulfatase activity was checked (Fig 11). Loss of all the putative modifying enzymes in the quadruple mutant did not affect the sulfatase activity suggesting that there is an unidentified modifying enzyme required for the maturation of the acid inducible sulfatase that remains to be elucidated.

aslA mutant is defective in macrophage survival

Zebrafish embryos exposed to *Salmonella* were removed at different time points, washed and euthanized. The ability of *Salmonella* to infect the embryos was initially checked under the Laser confocal microscope. We could detect the GFP labeled bacteria in the yolk sac at 3 days after infection (3DAI) and the gastrointestinal tract at 7 days of infection (7DAI) (Fig 12). Fishes without inoculation were used as control. The embryos were then disintegrated and processed by methods described in Methods section in Chapter II to determine both total and intracellular colony forming units. The wild type and the *aslA* mutant did not show any difference in the total colony forming units. Interestingly, no intracellular colony forming units could be detected from the embryos infected with the *aslA* mutant when compared to the wild type (Table 7), suggesting that AslA might be required for either infection or survival in the macrophages.

Table 6. Effect of the *tatC* mutation on the acid inducible sulfatase activity in *S.* Typhimurium

Strain	Chloroform	Sulfatase Activity (Miller Units)
WT	-	557 ± 45
WT	+	455 ± 90
Δ *tatC*	-	15 ± 5
Δ *tatC*	+	620 ± 55

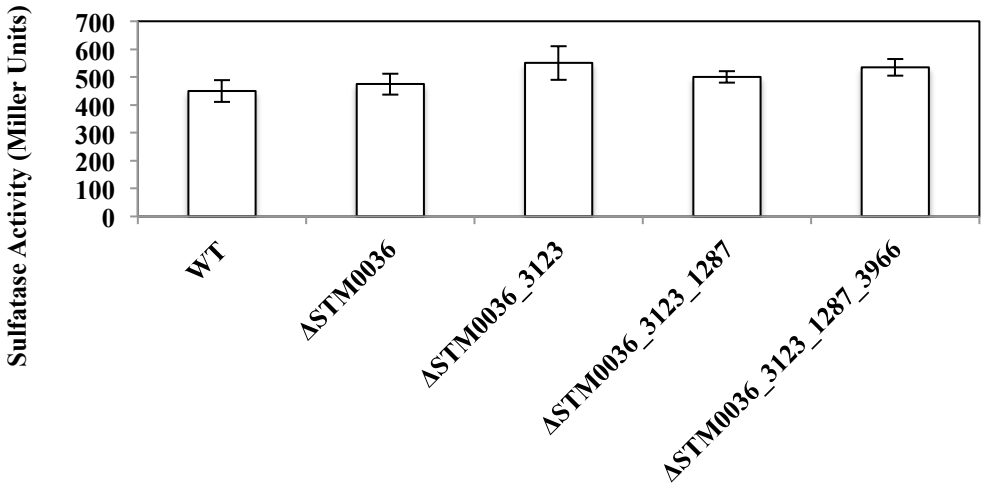

Figure 11. AslA is modified by an unidentified sulfatase modifying enzyme. Loss of the four putative sulfatase-modifying enzymes did not affect the acid inducible sulfatase activity in *S.* Typhimurium. The results are the means ± standard deviations of three

independent experiments.

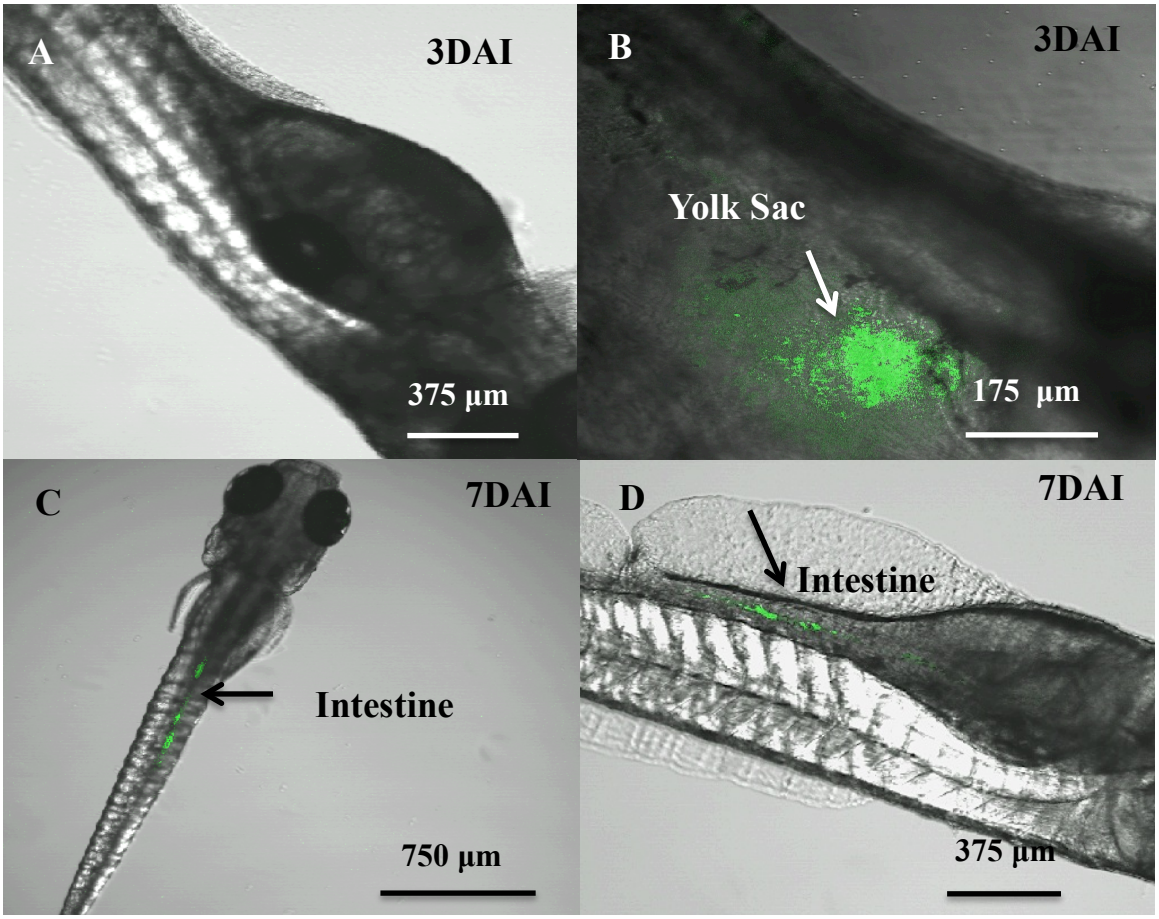

Figure 12. Detection of *S.* Typhimurium in zebrafish embryos. (A) Zebrafish embyros that were not exposed to *S.* Typhimurium were used as control. **(B-D)** GFP labeled *S.* Typhimurium detected in the yolk sac at 3DAI and the gastrointestinal tract at 7 DAI in the zebrafish embryos.

Table 7. Number of colony forming units of wild-type and *ΔaslA* mutant isolated from zebrafish embryos after 7 days of inoculation.

Strain	**Total** **Mean log_{10} cfu/embryo**		**Intracellular** **Mean log_{10} cfu/embryo**
	0 DAI	**7 DAI**	**7 DAI**
WT	6.7 ± 0.3	5.7 ± 0.1	4.0 ± 0.4
Δ*aslA*	7.1 ± 0.4	5.6 ± 0.3	None Detected

Discussion

We have established that the two-component system OmpR-EnvZ in *S.* Typhimurium regulates the acid inducible sulfatase AslA. In the gut lumen, *S.* Typhimurium is exposed to alkaline pH (8.0) and on entering the replicative niche, macrophages, it is exposed to mild acidic pH of 5.5 (73). This acidic pH is sensed by the sensor kinase EnvZ and the signal is transduced to the response regulator OmpR (74). Activated OmpR then regulates the expression of a two-component regulatory system, SsrA-SsrB, which in turn activates the type III secretion system encoded on the SPI-2 (75). The SPI-2 encoded T3SS is required for intracellular survival (76). The derepression of *aslA* at the pH that is encountered in the macrophages and involvement of OmpR-EnvZ two component system strongly suggests that AslA might be involved in survival in the hostile environment. It was recently reported that the *aslA* mutant was significantly attenuated and failed to colonise the chicken intestine (77). Our results with zebrafish embyros also show similar results as we were not able to retrieve any viable bacteria from the macrophages. These results suggest that AslA likely aids in survival within the macrophages, however further work needs to be done to understand the role of AslA in intracellular survival.

Another study had reported that a *tatC* mutant of *S.* Typhimurium showed impaired systemic colonization in mice (64). TAT secretion system transports folded proteins to the periplasm across the bacterial membrane and mainly consists of three components TatA, TatB and TatC (78). The TAT system in *S.* Typhimurium is predicted to transport at least 30 different proteins including AslA (79). We have shown that AslA is transported by the TAT system and is likely that it might have a physiological role in

S. Typhimurium survival and pathogenesis.

We have also shown that AslA is likely localized in the inner membrane and the subcellular localization of the protein allows us to predict its role in intracellular environment. It has been reported that the sulfated cell surface polysaccharides in *Mesorhizobium loti* are required to establish a symbiotic association with the host plant (80). It is possible that AslA might be involved in modifications of cell surface structures such as lipopolysaccharides or capsular polysaccharides. Such modifications in surface structures might help *Salmonella* to avoid the host defense and to replicate within the macrophages. Another possibility could be that AslA might be involved in utilization of sulfated glycans in the host. Further experiments need to be performed to confirm the role of AslA in intracellular survival.

As AslA is required for intracellular survival, it could be used as a potential therapeutic target against *S.* Typhimurium infections. The current findings will help to further understand *S.* Typhimurium pathogenesis and extend the knowledge to related human and animal pathogens.

Chapter III – Genetic evidence of monoamine compounds-induced sulfatase in *Salmonella enterica* serovar Typhimurium

Introduction

Derepression of sulfatase in *S.* Typhimurium in the presence of tyramine, a monoamine compound, has been reported (25). Nearly two decades after the report of monoamine compound-regulated sulfatase, an interdisciplinary branch of science was introduced which is now referred as "Microbial Endocrinology" (81). This branch of science entails the inter-kingdom signaling between host and bacteria via the host hormones. Recently, there have been many reports of host-bacterial communication that involves catecholamine stress hormones (42, 43). The catecholamines are monoamine compounds that include the neurotransmitters dopamine, epinephrine and norepinephrine (82). The gastrointestinal tract is innervated by the enteric nervous system (ENS) and is abundant in dopamine and norepinephrine (83). Epinephrine is primarily synthesized in the adrenal medulla and transported by the bloodstream (84). Interestingly, the catecholamines and their derivatives are also widespread in the plant kingdom. Some produce that are rich in catecholamines include beans, banana, citrus and avocado (85, 86). As bacteria come in contact with these hormones, they have evolved mechanisms to respond to them by expressing the genes that are indispensable for pathogenesis and survival within the host. An adrenergic receptor, QseBC was reported in EHEC that resembled a two component system. QseC is a sensor kinase which senses the

catecholamines (epinephrine and norepinephrine) and autophosphorylates which transfers a phosphate group to the cognate reponse regulator, QseB. The phosphorylated QseB is activated which then regulates the virulence genes under its control (87, 88). It was recently reported that EHEC possesses a secondary adrenergic receptor, QseEF that also resembles a two component system where QseE is the sensor kinase and QseF is the cognate response regulator (89). In recent years, there have been numerous reports on catecholamines promoting the growth and virulence of bacterial pathogens including *E. coli*, *Salmonella*, *Helicobacter*, *Listeria, Campylobacter*, and *Yersinia* (90-95).

In this chapter we report that in *S.* Typhimurium, STM3122 encodes the catecholamine-induced sulfatase. *In silico* analysis of the STM3122 neighbouring genes showed the presence of a gene encoding a sulfatase-modifying enzyme, STM3123, upstream of STM3122. Further upstream of STM3123, a regulatory gene STM3124, a gene for amino acid transporter (STM3126) and a gene for oxidoreductase (STM3128) were found (Fig 13). We also report the role of STM3123 and STM3124 in the expression of the catecholamine-induced sulfatase.

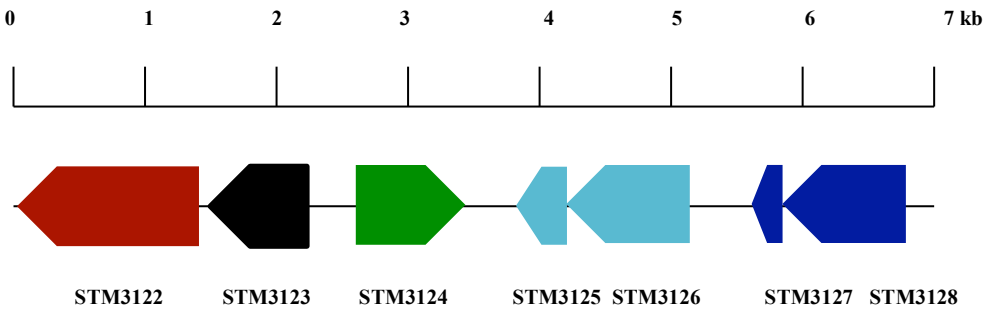

Figure 13. Organization of STM3122 and the neighbouring genes in *S.* Typhimurium. The gene number is based on the *S.* Typhimurium strain LT2 genome annotation. STM3122 - sulfatase; STM3123 - sulfatase modifying enzyme; STM3124 – regulator; STM3125 – orf; STM3126 – amino acid transporter; STM3127- orf and STM3128 – oxidoreductase. The genes shown here will be referred to as the putative monoamine regulon in the text.

Material and Methods

Bacterial strains, media and growth conditions

Bacterial strains and plasmids used in the study are summarized in Table 8. The media and growth conditions used for the maintenance of the strains are same as described in Materials and Methods section in Chapter II. A basal minimal media (sulfatase inducing medium) described previously was used for the induction of the sulfatase (25). Briefly, the media contained the following per liter of distilled water: K_2HPO_4, 7 g; KH_2PO_4, 3 g; $MgC1_2$, 0.1g; K_2SO_4,0.2g; $Na_3C_6H_50_7$-$2H_20$,0.5g; NH4C1,1 g; and glucose,2g. Monoamine compounds were freshly prepared by filter sterilizing through a 0.2-μm pore size syringe unit and added at 10mM concentration (tyramine-hydrochloride) and 200 μM concentration (dopamine and epinephrine) to the minimal media adjusted to pH 6.7.

Table 8: List of bacterial strains and plasmids used in this study

Strain	**Description**	**Source**
***Salmonella enterica* serovar Typhimurium**		
LT2	Wild type	**(52)**
SE30	LT2, ΔSTM3124	This work
SE31	LT2, ΔSTM3123	This work
SE32	LT2, Δ*qseB*	This work
SE33	LT2, Δ*qseC*	This work
SE34	LT2, Δ*qseE*	This work
SE35	LT2, Δ*qseF*	This work
SE36	LT2, Δ*qseBF*	This work
SE37	LT2, ΔSTM3126	This work
SE38	LT2, ΔSTM3128	This work
SE40	LT2, ΔSTM3122 with pBAD18-STM3122_3123	This work

SE41	LT2, ΔSTM3122 with pBAD18	This work
SE42	LT2, ΔSTM3124 with pBAD18-STM3124	This work
SE42	LT2, ΔSTM3124 with pBAD18	This work

Construction of mutant strains

The *Salmonella* deletion mutants were constructed using the same method described as in the Materials and Methods section in Chapter II. Mutations in the gene were confirmed using PCR and sequencing.

Flp excision of the antibiotic cassette

The antibiotic cassette was removed using Flp recombinase expressed from a temperature sensitive plasmid pCP20 as described before in the Methods section in Chapter II.

P22 Phage transduction

The mutants were also constructed by using P22 phage transduction as described before in the Methods section in Chapter II.

Rapid screening for sulfatase

As monoamine compound-induced sulfatase could not be detected using chromogenic substrate X-sulfate, we grew the cells overnight in the inducing medium with p-nitrophenyl sulfate. The sulfatase was detected by yellow coloration of the medium on addition of 1M NaOH, indicating the release of p-nitrophenol.

Quantitative sulfatase assay using p-nitrophenyl sulfate substrate

Sulfatase activity was determined by quantifying the release of p-nitrophenol from p-nitrophenyl sulfate using whole cells described before in Methods section in Chapter II with slight modifications. The cells used for the assay were grown overnight in basal minimal media with monoamine compounds (tyramine-hydrochloride, dopamine or epinephrine) and the enzyme activity was calculated in Miller Units (MU).

Complementation of mutants

The STM3122_3123 fragment and STM3124 gene were amplified by PCR using the primers listed in Table 9 with high fidelity Phusion polymerase (Thermo Scientific). The STM3122_3123 and STM3124 amplified fragments were digested with EcoRI and XbaI, and XbaI and HindIII respectively, purified and ligated into pBAD18 (58). The ligations were transformed into DH5α and the transformants were selected on LB containing ampicillin. The plasmids with inserts were confirmed using PCR and electroporated to SE30. For the sulfatase assay, the complemented strain was grown with L-arabinose (0.2%) to induce the expression of the gene.

Table 9: List of primers used in this study

Primers	**Sequence**
Deletion of *S. enterica qseB*	
***qseB*FP**	**CGGCGACAAGGTTAACTGACGGCAACGCGAGTTACCGCAAGGAAGAACAGGTGTAGGCTGGAGCTGCTTC**
***qseB*RP**	**CAGGAAAATAAGCGTCAGCCTGACGCGCAGGCTGAGACGTTGCGTCAATTCATATGAATATCCTCCTTA**

Deletion of *S. enterica qseC*

***qseC*FP**	**AGCGAATTTATTCGCACCGTGCACGGCATCGGCTACACCCTGGGTGACG CGTGTAGGCTGGAGCTGCTTC**
***qseC*RP**	**GAAATTAGCAAAATGTGCAAAGTCTTTTGCGTTTTTGGCAAAAGTCTCT GCATATGAATATCCTCCTTA**

Deletion of *S. enterica qseE*

***qseE*FP**	**GTTGTATCTGGCGCTTCCCTCGGTTAGCATCTTTTTTATTCTTCTTTTAT GTGTAGGCTGGAGCTGCTTC**
***qseE*RP**	**TAATAGTTTGGACGAAAACGTGTGACATACGCACCAGGCTTAAATTCAT ACATATGAATATCCTCCTTA**

Deletion of *S. enterica qseF*

***qseF*FP**	**ACGTCTGAAGATGGCGCGGCGCCGTCGCCGTCACAAGATGAGGTAACG CCGTGTAGGCTGGAGCTGCTTC**
***qseF*RP**	**TTTTGATCGGTTAAACGTAACATATTTCGCGCTACTTTACGGCATGAAAA CATATGAATATCCTCCTTA**

Complementation of *S. enterica* STM3124

STM3122_3123 FL_F	**ATTGACGAATTCTGGCCAGTAGTTGATAAGGCTG**
STM3122_3123 FL_R	**ATTGACTCTAGACATCGCCAACGCTGAGAAAC**

Complementation of *S. enterica* STM3124

STM3124_FL_F	**ATTGACTCTAGAACCGCAACATACGTCACATT**
STM3124_FL_R	**ATTGACAAGCTTACCAGCGTGTTCTTCTGCTT**

Reverse Transcription

STM3122 FP	**GTCCAACACGCTGTTGATTTTC**
STM3122 RP	**GCAACGCCTTCGCTATCGT**
STM3123 FP	**CCGGCAAACAGGCATCAATC**

STM3123 RP	**TTTGTAGCGCAATAACGCGG**
STM3126FP	**GCGCAGATTACCACCTTTGC**
STM3126RP	**CATCAAGAGGTGCGCCAAAC**
STM3128FP	**CCTACAACCAGCGGCAGTTA**
STM3128RP	**GGGACTGATCCGCTCAACAA**

RNA isolation and RT-PCR

The strains were grown to mid-logarithmic phase in basal minimal media with and without tyramine. The cells were harvested and RNA was isolated using TRIzol reagent (Sigma). RNA concentrations were determined by UV/Vis Spectrophotometry. RNA samples were further treated with RNase free DNase (Promega) to eliminate any DNA contamination. cDNA synthesis and RT-PCR were performed using AccessQuick RT-PCR system (Promega) as described in the Methods section in Chapter II.

Infection of zebrafish embryos

Zebrafish embryos were exposed to the bacterial strains using methods described previously in the Methods section in Chapter II.

Isolation of bacteria from infected zebrafish embryos

The bacteria were isolated from the infected zebrafish embryos by methods described earlier in the Methods section in Chapter II.

Results

Monoamine compound-induced sulfatase in *S.* Typhimurium is encoded by STM3122

The sulfatase activity was checked in the presence of different concentrations of monoamine compounds like tyramine, dopamine and epinephrine. Similar levels of sulfatase activity in the wild type could be detected in the presence of 10 mM tyramine or 200 μM dopamine but not in the presence of epinephrine. No sulfatase activity was detected in the wild type grown without tyramine. (Fig 14A).

For further characterization of the gene encoding the sulfatase all the putative sulfatase mutants (ΔSTM3122, ΔSTM0886, ΔSTM0032, ΔSTM0035 and ΔSTM0038) in *Salmonella* were screened for the sulfatase activity in presence of Tyramine. A mutation in STM3122 resulted in the absence of the monoamine compound-induced sulfatase in *Salmonella* (Fig 14B). Protein sequence analysis of STM3122 predicted it as a Ser-type sulfatase (Table 1). The sulfatase activity in the STM3122 mutant was restored back to the wild type levels by plasmid based complementation.

STM3124 positively regulates the putative monoamine regulon

To test if STM3124 acts as the positive regulator of the putative monoamine regulon, RT-PCR was performed on RNA from tyramine grown STM3124 mutant. The RT-PCR analysis revealed that the level of mRNA for STM3122, STM3126 and STM3128 was significantly higher in the tyramine grown wild type strain when compared to the tyramine grown STM3124 mutant (Fig 15A). These results confirm the role of STM3124 as a positive regulator of the monoamine regulon. The sulfatase activity in the STM3124 mutant was further checked to confirm its regulatory role. The

STM3124 mutant lacked the monoamine compound-induced sulfatase activity. The sulfatase activity in STM3124 mutant was restored to the wild type level in the complemented strain (Fig 15B).

STM3122 is co-transcribed with STM3123 a sulfatase modifying enzyme

To check if STM3122-encoded sulfatase is modified by STM3123, the sulfatase activity was checked in the ΔSTM3123 mutant. The strains were grown in the basal minimal media containing tyramine. The ΔSTM3123 mutant lacked the sulfatase activity when compared to the wild type. To further determine if STM3122 and STM3123 were in an operon, RT-PCR was performed using primers to amplify the intergenic region. Amplification of the intergenic region confirms that STM3122 is co-transcribed with STM3123 and the genes are organized in a single transcriptional unit (Fig 16).

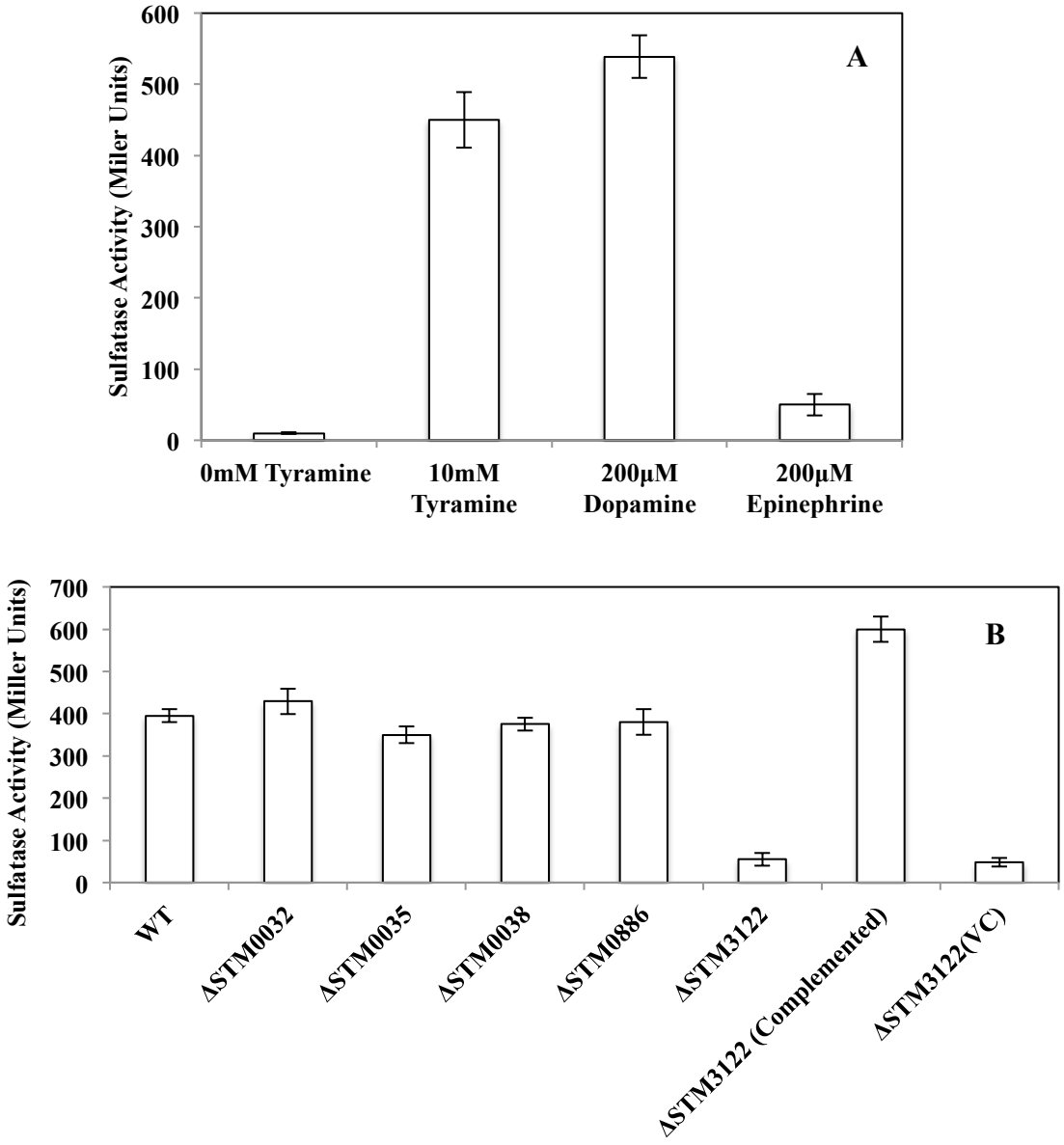

Figure 14. Monoamine compounds-induced sulfatase in *S.* Typhimurium is encoded by STM3122 (A) Similar levels of sulfatase activity in the wild type detected in presence of tyramine and dopamine. The sulfatase activity was significantly low in epinephrine grown cells. Cells grown without tyramine was used as negative control. (**B**) Screening of the sulfatase activity in the mutants identified STM3122 as the gene encoding the

monoamine compound-induced sulfatase. The activity in the STM3122 mutant was The results are the means ± standard deviations of at least three independent experiments.

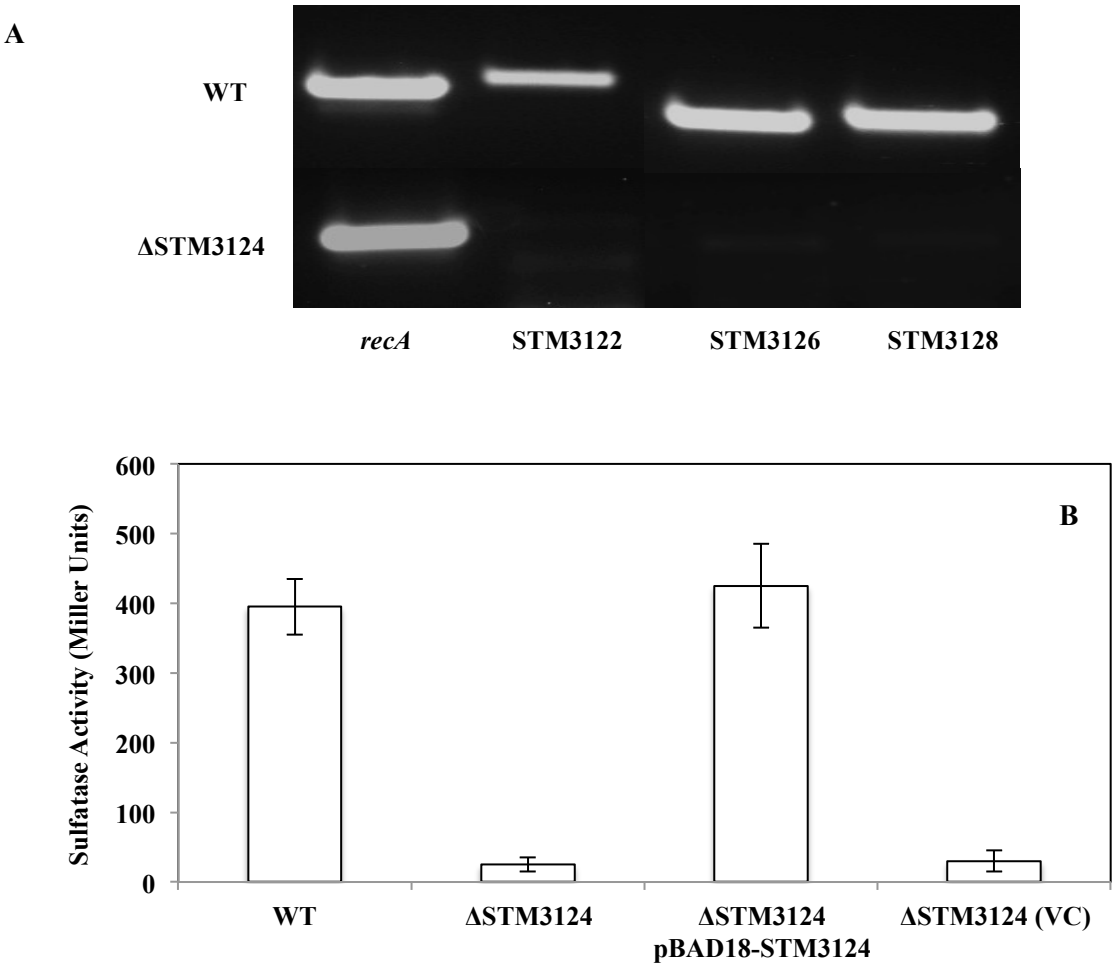

Figure 15. STM3124 positively regulates the putative monoamine regulon (A) RT-PCR analysis of gene expression (putative monoamine regulon) in wild- type and STM3124 mutant cells. The transcripts for the STM3122, STM3126 and STM3128 were induced in the wild type cells grown with tyramine (top panel) when compared to the STM3124 mutant. *recA* was used as an internal control. **(B)** The STM3124 mutant lacked

sulfatase activity and the mutant phenotype was rescued by plasmid-based complementation. STM3122 mutant with the empty vector was used as a control. The results are the means ± standard deviations of three independent experiments.

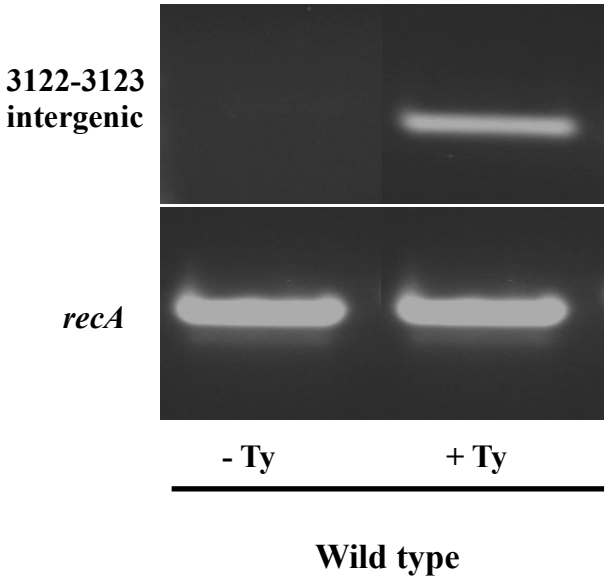

Figure 16. RT-PCR analysis of the STM3122-3123 intergenic region. The intergenic region was amplified in tyramine grown cells (top right). The transcript for the intergenic region was absent in cells grown without tyramine (top left). *recA* was used as an internal control.

Monoamine compound-induced sulfatase is not controlled by the adrenergic receptors

The adrenergic receptors (QseBC and QseBF) in bacteria have been shown to respond to catecholamines, so we wanted to test the role of these receptors on the STM3122 encoded sulfatase. The sulfatase activity in *qseB, qseC, qseE* and *qseF* mutants was determined by using p-nitrophenyl sulfate (PNPS). We found that the sulfatase activity in these mutants was not affected. To further exclude the role of the adrenergic receptors in the monoamine compound-induced sulfatase expression, a double mutant *qseBF* was constructed and the sulfatase activity was assayed. The sulfatase activity in the *qseBF* mutant was the same as observed in the wild type (Fig 17).

Localization of the monoamine compound-induced sulfatase in the periplasm

Initially SecretomeP was used to predict the localization of the catecholamine-induced sulfatase in *Salmonella*. The prediction program gave a high secP score of 0.77 (threshold score is 0.5) suggesting it to be a secreted protein. In order to confirm the subcellular localization of the protein, cell fractions were prepared from the tyramine grown wild type cells and assayed for sulfatase activity. High sulfatase activity was detected in the periplasmic fraction, suggesting the localization of the sulfatase in the periplasm. Low level of activity was also detected in the spheroplastic fraction suggesting that it might be modified in the cytoplasm. No sulfatase activity was detected in the extracellular fraction (Table 10).

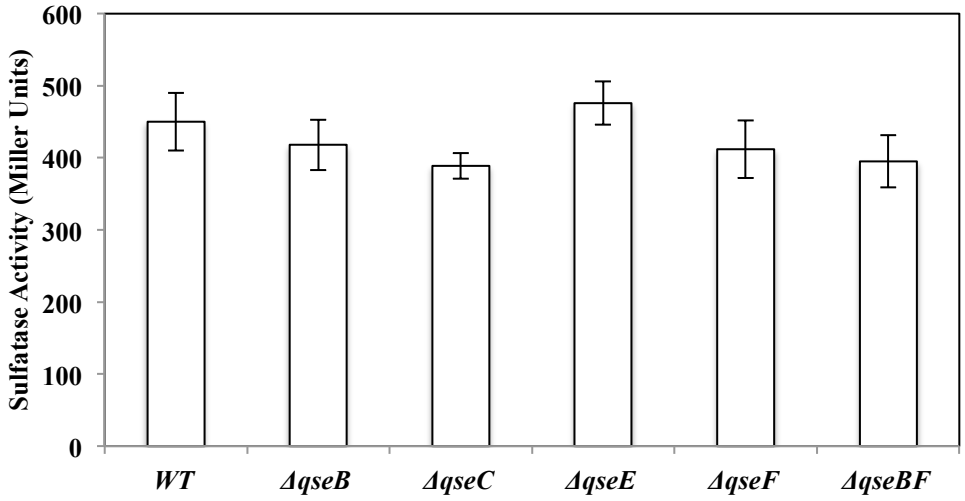

Figure 17. Mutation of adrenergic receptor genes does not affect the monoamine compound-induced sulfatase in *S.* Typhimurium. The sulfatase activity in the mutants for the adrenergic receptors was same as the wild type. The results are the means ± standard deviations of three independent experiments.

Table 10. Monoamine compound-induced sulfatase activity in cell fractions

Cellular Fraction (Wild type)	**Sulfatase activity (nMoles/mg protein)**
Extracellular	Not Detected
Periplasmic	503 ± 75
Spheroplastic	62.5 ± 15

S. Typhimurium can use tyramine as a sole carbon source

Since the genes STM3126 and STM3128 were induced when cells were grown with tyramine we wanted to test the possibility of tyramine being used as a nutrient source by *Salmonella.* We found that *Salmonella* could use tyramine as a sole carbon source. The role of the genes of the monoamine regulon in the utilization of tyramine as carbon source was also determined. The STM3128 (oxidoreductase) mutant was unable to grow on tyramine as a sole carbon source (Fig 18). However, mutation of STM3126 (amino acid transporter) had no effect on the utilization of tyramine as sole carbon source in *Salmonella*. Since the STM3126 mutation had no effect on the metabolism of tyramine, it is possible that STM3126 likely transports a different metabolite with similar structure, and probably tyramine could enter by other nonspecific transporters.

STM3122 mutant is defective in macrophage survival

To determine both total and intracellular colony forming units, orally infected zebrafish embryos were processed as described in the Methods section in Chapter II. No intracellular colony forming units could be detected from the embryos infected with the STM3122 mutant when compared to the wild type (Table 9), suggesting that the STM3122-encoded sulfatase might be required for intracellular survival.

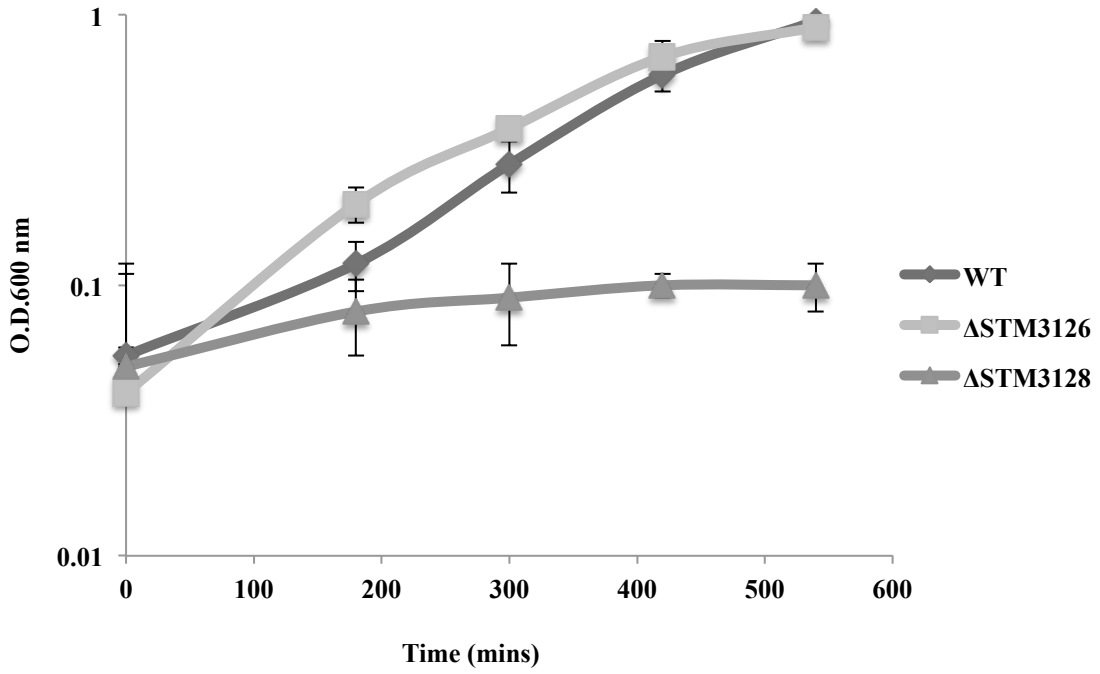

Figure 18. Growth of *S.* Typhimurium on tyramine as a sole carbon source. The STM3128 mutant is unable to grow on tyramine as a carbon source. The results are the means ± standard deviations of at least three independent experiments.

Table 11. Number of colony forming units of the wild type and ΔSTM3122 mutant isolated from zebrafish embryos after 7 days of inoculation.

Strain	Total Mean log_{10} cfu/embryo		Intracellular Mean log_{10} cfu/embryo
	0 DAI	7 DAI	7 DAI
WT	6.7 ± 0.3	5.7 ± 0.1	4.0 ± 0.4
ΔSTM3122	6.1 ± 0.2	4.8 ± 0.4	None Detected

Discussion

We show that *S.* Typhimurium produces sulfatase in presence of monoamine compounds. These compounds are abundant in the animal and plant kingdoms. Derepression of sulfatase in the presence of monoamine compounds suggests that they might be important in the lifestyle of *S.* Typhimurium.

Bacterial pathogens are limited for iron in the host cells as it is tightly bound to the host proteins such as transferrin, lactoferrin, etc (96). Iron is essential for the growth of bacteria, thus iron sequestration from the bacteria is a key host immune response against the pathogen (97). It has been proposed that the monoamine compounds interact with the host protein-bound ferric iron and reduce it to ferrous iron (97). The ferrous iron has lower affinity towards the host protein and thus is released from the complex and made available to the bacteria (98-100). Interestingly, the catecholamines are conjugated to sulfur and are inactive and do not show iron loss from the mammalian iron binding proteins (98). Thus desulfation of the catecholamines might be essential for their role in supply of iron to the bacteria. However, the subcellular localization of the STM3122-encoded sulfatase in the periplasm suggests that it may not be involved in the supply of iron to the pathogens.

We have shown that the monoamine compound tyramine induces the expression of the STM3122-encoded sulfatase and can also be used as a carbon source by *S.* Typhimurium. So it is likely that these catecholamines might be signaling the bacteria of the host environment and also serve as a nutrient source. These physiologically important monoamine compounds exist as sulfoconjugates, and it is possible that

desulfation might be an important step for these compounds to be processed as a carbon source. But the question, how *S.* Typhimurium senses the presence of monoamine compounds and activates the expression of the monoamine regulon still remains unanswered.

We also show that the STM3122 mutant is defective in macrophage survival. It was recently reported by Haneda et al (101) that the *pheV*-tRNA-located genomic island comprising STM3117-STM3138 is a pathogenicity island that is required for virulence in the mouse infection model. It is also interesting to note that the monoamine regulon is a part of the *pheV*-tRNA-located genomic island. Another study by Chaudhari et al (77) showed that STM3122, STM3123 and STM3124 mutant strains are significantly attenuated in intestinal colonization. These observations suggest that the STM3122-encoded sulfatase aids in *S.* Typhimurium pathogenesis.

Concluding Remarks

Several enteric pathogens have genes in their genomes annotated as sulfatases, but not much is known about their physiological importance and their function. In our study we have characterized a Cys and a Ser type sulfatase in *S.* Typhimurium and have shown their role in pathogenesis. However, there are four additional putative sulfatase genes in *S.* Typhimurium, which can be explored in the future. The presence of more than one sulfatase gene in the genome suggests that these enzymes play a major role in the lifecycle of *S.* Typhimurium and they might be necessary at different stages of infection.

Inhibitors of sulfatases can be promising therapeutic targets to treat bacterial infections. Drugs like Nitecapone and Sucralfate have been shown to counteract the sulfatases in *Helicobacter pylori* (102, 103). The current findings in *S.* Typhimurium can be used to characterize unknown sulfatases in other pathogens. Many studies on bacterial pathogens have focused on secretion systems and the effector molecules that are involved in pathogenesis, but a good understanding of the nutrient requirements and metabolic adaptations of the pathogen is also necessary to fight it.

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Curriculum Vitae

Seema Das

EDUCATION

University of Wisconsin, Milwaukee, Wisconsin, 2008- Present
Ph.D. Biological Sciences, Area of Specialization: Molecular Microbiology

Bangalore University, Bangalore, India, 2002-2007

M. Sc. in Biotechnology, 2005-2007
B. Sc. in Biotechnology, 2002-2005

Jain Institute of Vocational and Advanced Studies, Bangalore, India, 2005-2006
Advance Diploma in Genetic Engineering

AWARDS AND HONORS

Best Graduate Student Poster award, First Place, 72nd Annual Meeting NCB-ASM, 2012

UWM Graduate School Travel Award, 2013

UWM Department of Biological Sciences Travel Award, 2012 and 2013

Chancellors Graduate Student Award, UWM, 2008-2012

Ruth Walker Graduate Student Grant- In Aid Award 2012 and 2013

Ruth Walker Award, 2011

Topper of the Post graduating class for Master of Science, 2007

RESEARCH EXPERIENCE

Doctoral Research: Department of Biological Sciences, University of Wisconsin-Milwaukee, 2009-Present (Advisor: Dr. Gyaneshwar Prasad)

- Characterization of sulfatases in *Salmonella enterica* serovar Typhimurium LT2.
- Regulatory networks involved in the expression of sulfatases.
- Investigation of the role of the sulfatases in the *Salmonella* pathogenesis.

TECHNICAL SKILLS

Isolation of DNA and RNA, Primer Design, PCR, RT-PCR, Semi-quantitative RT-PCR, Southern Blotting, Protein expression and Purification, Protein Detection, DNA cloning, Random and Gene Specific Mutagenesis, Promoter Expression Assay, Virulence Assays, LPS Profiling, Laser Confocal Scanning Microscopy.

INVENTION DISCLOSURE

Invention Disclosure (Approved): Gyaneshwar Prasad and Seema Das

Characterizing Sulfatase in Salmonella typhimurium for development as drug targets and markers for food contamination. UW-Milwaukee Office of Technology Transfer. 2012.

PUBLICATIONS

Das, S., S. Singh, M. McClelland, S. Forst and G. Prasad. 2013. Characterization of an acid-inducible sulfatase in *Salmonella enterica* serovar Typhimurium. Applied and Environmental Microbiology. 79(6):2092-2095.

Das, S. and G. Prasad. Genetic evidence of monoamine compounds regulated sulfatase in *Salmonella enterica* serovar Typhimurium. (Manuscript in Preparation)

Mitra, S., A. Mukherjee, E.K. James, **S. Das**, H. Owen, P.M. Reddy, J.M. Ane and G. Prasad. *Rhizobium* (Agrobacterium) sp. IRBG74 requires rhamnose rich LPS for endophytic colonization of rice as well as nodulation of *Sesbania cannabina*. (Manuscript in Preparation)

ABSTRACTS

Characterization of an Acid-Inducible Sulfatase in *Salmonella enterica* serovar Typhimurium LT2.113th ASM General Meeting, Denver, Colorado 2013.

OmpR Regulates the Acid Inducible Arylsulfatase AslA in *Salmonella enterica* serovar Typhimurium LT2, 72nd Annual Meeting of the North Central Branch American Society for Microbiology, Fargo, North Dakota, 2012**.**

Genetic Regulation of Arylsulfatase in *Salmonella enterica* serovar Typhimurium LT2, 19th Annual Midwest Microbial Pathogenesis Conference, Milwaukee, 2012.

PRESENTATIONS

OmpR Regulates the Acid Inducible Arylsulfatase AslA in *Salmonella enterica* serovar Typhimurium LT2, 72nd Annual Meeting of the North Central Branch American Society for Microbiology, Fargo, North Dakota, 2012. (Poster Presentation)

Genetic Regulation of Arylsulfatase in *Salmonella enterica* serovar Typhimurium LT2, 19th Annual Midwest Microbial Pathogenesis Conference, Milwaukee, 2012. (Poster Presentation)

Dissecting the Regulation of Sulfatases in *Salmonella enterica* serovar Typhimurium LT2. Biological Sciences Research Symposium,UWM, Spring 2012. (Oral Presentation)

Regulation of Sulfate and Sulfonate Utilization Genes in *Escherichia coli* by S- Adenosyl Methionine. Biological Sciences Research Symposium,UWM, Spring 2011. (Poster Presentation)

Physiological and Regulatory Mechanisms of Extended Homeostatic Response of *Escherichia coli* to Nitrogen Limitation. Biological Sciences Research Symposium, UWM, Spring 2010. (Poster Presentation)

Mechanism of Growth Co-ordination during Nitrogen Limitation in *Escherichia coli* K12. Biological Sciences Research Symposium, UWM, Spring 2009. (Poster Presentation)

TEACHING EXPERIENCE

BioSci 150. Foundations of Biological Sciences I Lab section. (2008-2011)
BioSci 152. Foundations of Biological Sciences II Lab section. (2012-Present)