# The influence of *Salmonella* genomic island 1 on global gene expression of multi-drug resistant *Salmonella* Typhimurium DT104 in mid-log and early stationary growth phases

By

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A Thesis submitted to the Faculty of Graduate Studies of the

University of Manitoba in partial fulfillment of the requirements for the degree of

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# The Influence of *Salmonella* Genomic Island 1 on Global Gene Expression of Multi-Drug Resistant *Salmonella* Typhimurium DT104 in Mid-Log and Early stationary Growth Phases

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**Abstract**

A once rare strain, *Salmonella* Typhimurium DT104 (DT104) acquired multiple drug resistance and has emerged globally and has been associated with increased morbidity and mortality. Increased death rates associated with MDR DT104 may be due to ineffective antibiotic treatments; however,they may also result from a hypervirulent phenotype exhibited by this strain that often harbours *Salmonella* Genomic Island 1 (SGI1). SGI1 is a 43 kb chromosomal element containing 44 ORFs including genes that confer multi-drug resistance in addition to those with unknown and putative regulatory functions. SGI1-influenced gene expression was assessed in mid-log and early stationary growth phases using microarray analysis with an SGI1 isogenic strain pair of DT104 to determine if it influences genes attributed to virulence and/or increased fitness. In mid-log phase, SGI1 influenced genes involved in O and H antigen variation. A larger portion of the DT104 transcriptome was influenced by SGI1 in early stationary phase including invasion genes activated by and including *hilA* (SPI1 and SPI4). Up-regulation of invasion genes is supportive evidence that SGI1 is involved in MDR DT104 associated hypervirulence. Future studies should confirm if the invasiveness of MDR DT104 harbouring SGI1 to mucosal cells reflects the invasive gene expression profile when grown to early stationary phase, and the involvement of specific SGI1 ORFs, particularly those with putative regulatory functions, in the up-regulation of *hilA*.

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## List of Abbreviations

| | |
|---|---|
| **A** | ampicillin |
| **BLAST** | Basic Local Alignment Search Tool |
| **bp** | base pair |
| **C** | chloramphenicol |
| **cDNA** | complimentary DNA |
| **CFU** | colony forming unit |
| **CGH** | comparative genomic hybridization |
| **CS** | conserved sequence |
| **Ct** | critical threshold |
| **Cy3** | cyanine 3 |
| **Cy5** | cyanine 5 |
| **ddH2O** | double distilled water |
| **DMD** | digital micromirror device |
| **DNA** | deoxyribonucleic acid |
| **ds** | double stranded |
| **EDTA** | ethylene diamine tatra-acetic acid |
| **kb** | kilobases |
| **KEGG** | Kyoto Encyclopedia of Genes and Genomes |
| **Km** | kanamycin |
| **FDR** | false discovery rate |
| **gDNA** | genomic DNA |
| **GEI** | genomic island |
| **IVOM** | Interpolated Variable Order Motifs |
| **MAS** | maskless array synthesizer |
| **MDR** | multi drug resistant |
| **MM** | mismatch |
| **µg** | micrograms |
| **µl** | microlitres |
| **µm** | micrometres |
| **ml** | mililitres |
| **nm** | nanometres |
| **$OD_{600}$** | optical density at a wavelength of 600 nm |
| **ORF** | open reading frame |
| **PAI** | pathogenicity island |
| **PAMP** | pathogen associated molecular pattern |
| **PCR** | polymerase chain reaction |
| **PHB** | polyhedral body |

| | |
|---|---|
| **PM** | perfect match |
| **RPz** | rumen protozoa |
| **RNA** | ribonucleic acid |
| **RT-qPCR** | reverse transcriptase quantitative PCR |
| **S** | streptomycin |
| **SAM** | Significance Analysis Microarray |
| **SNP** | single nucleotide polymorphism |
| **SGI1** | *Salmonella* Genomic Island 1 |
| **SPI1** | *Salmonella* Pathogenicity Island 1 |
| **SPI2** | *Salmonella* Pathogenicity Island 2 |
| **SPI3** | *Salmonella* Pathogenicity Island 3 |
| **SPI4** | *Salmonella* Pathogenicity Island 4 |
| **ss** | single stranded |
| **Su** | sulfonamide |
| **T** | tetracycline |
| **Tm** | trimethoprim |
| **TBE** | tris-borate-EDTA |
| **TE** | tris EDTA |
| **TLR** | toll like receptor |
| **TOSS** | type one secretion system |
| **TTSS** | type three secretion system |
| **V/cm** | volts per centimeter |
| **x g** | relative centrifugal force |

# 1 Introduction

## 1.1 *Salmonella* Classification

*Salmonella* are Gram-negative rods that are facultative anaerobes. The complex genus of *Salmonella* is a subsidiary of the family *Enterobacteriaceae* and is comprised of more than 2500 members based on species, serotypes, phagetype, and biotype (122). The annually updated Kauffmann-White scheme is the main system used for *Salmonella* nomenclature and classification of all members of the genus (96). According to this system, the genus of *Salmonella* is comprised of only 2 species; *Salmonella bongeri* and *Salmonella enterica*. The *S. enterica* species is further subdivided into 6 subspecies; *entericae, salamae, arizonae, diarizonae, houtenae*, and *indicae*. These subtypes can be further divided into serovars based on antigenic properties (O and H antigen). Based on antigenic properties such as the O (somatic) and H (flagellar) antigens, subspecies are subdivided into several serovars (122). *Salmonella enterica* subspecies *entericae* are further subdivided into serovars such as Typhimurium, Agona, Typhi and Enteritidis, which can be further differentiated into subgroups based on phagetype, biotype and other methods of differentiation.

### 1.1.1 Serotypes and Host.

In humans *Salmonella* are common foodborne pathogens that cause salmonellosis, typhoid fever, and to a lesser extent, bacteraemia (133). *S. enterica* subspecies *entericae* are responsible for approximately 99% of infections in humans and warm blooded animals (118, 122). Other *Salmonella* spp. and subspecies are associated with cold blooded animals and the environment (122). Several serovars such as Enteritidis and

Typhimurium have broad host ranges where others are restricted to a narrow host range such as Typhi and Paratyphi, which are exclusively human pathogens (100, 133).

Serovars with a broad host range may be host-adapted where they cause systemic infections such as enteric fever or bacteraemia in one specific host and localized gastrointestinal infections in others (100). Some host-adapted serovars are Typhimurium (murine-adapted), Choleraesius (swine-adapted), Dublin (Bovine-adapted), Gallinarium (Chicken), and Typhi (human adapted).

*Salmonella enterica* subsp. *entericae* serotype Typhimurium (herein referred to as *S.* Typhimurium) is comprised of multiple strains based on phagetype (definitive types) such as DT120, DT193, DT104, and U302 (72). *S.* Typhimurium has a broad host range with the ability to infect humans, birds, pigs, sheep, cattle, and rodents (100). *S.* Typhimurium is murine-adapted in which the disease manifestation in mice is typhoid fever (systemic disease) whereas in other hosts such as humans and cattle it causes enterocolitis. This thesis will focus on *S.* Typhimurium with a special emphasis on phagetype DT104.

## 1.2 Pathogenesis of *Salmonella*

### 1.2.1 Clinical Presentation

*Salmonella* serovars are associated with 2 clinically different diseases known as enterocolitis (salmonellosis) and typhoid fever (enteric fever) (133). Typhoid fever is a more severe disease than enterocolitis however, enterocolitis infections from *Salmonella* are amongst the most frequent causes of food bourne illness with approximately 1.4 million cases and 550 deaths annually in the US (133). *S.* Typhi has been eradicated

from North America aside from sporadic cases in travelers (133). However; outbreaks of multidrug resistant *S.* Paratyphi also capable of typhoid fever were reported in Canada and France (85, 124).

*S.* Typhimurium and *S.* Enteritidis are common pathogens implicated in enterocolitis. In humans, enterocolitis caused by *S.* Typhimurium is characterized by a rapid onset (12 – 72 hours) of diarrhea and other symptoms include vomiting, dehydration, abdominal pain, and fever (133). The pathology of this disease is normally localized to the mesenteric lymph nodes and intestinal mucosa where there is a large influx of neutrophils and epithelial cell necrosis. Salmonellosis is commonly self limiting and resolves within 8 days (90). Typhoid fever is a systemic infection that is caused by *S.* Typhi and *S.* Paratyphi in humans. This disease has a longer onset of 'milder' symptoms (5 to 9 days) and diarrhea is not typical of the disease (90, 133). Lesions and high bacterial loads are detected in Peyers patches, mesenteric lymph nodes, liver and spleen. The hallmark of typhoid fever is enlargement of parts of the endoreticular system and the influx of mononuclear cells in the intestinal lumen (90). Low level bacteraemia is common in typhoid fever whereas clinical isolates of enterocolitis causing *S.* Typhimurium from humans are rarely found in blood. The mortality rate of typhoid fever is 10 – 15 % when infections are left untreated (90). Long term chronic fecal shedding of *S.* Typhi is seen in 5 % of patients that have had typhoid fever.

### 1.2.2 Progression of Salmonellosis

Salmonellosis is initiated by the ingestion of contaminated food or water. Oral inoculation of $10^4$ to $10^7$ CFU of *S.* Typhimurium in cattle results in diarrheal disease that

resolves in 8 days, where doses of $10^8$ to $10^{11}$ are lethal (111, 133). The bacteria must be able to survive the low acidity of the stomach. After entering the small intestine, *Salmonella* must transverse the mucous layer of the lumen and adhere to the apical side of epithelial cells of the intestinal mucosa. *Salmonella* then secretes several effector proteins via a Type Three Secretion System (TTSS) into the attached cell that triggers cytoskeleton rearrangement. The rearrangements disrupt the epithelial brush border and generate large membrane ruffles that internalize the bacterium into a vacuole (90, 114). The brush border reconstitutes after invasion. The infected epithelial cells then secrete interleukin-8 which in turn recruits neutrophils that translocate across the mucosal barrier into the lumen (90). The neutrophils secrete chemokines that increase vascular permeability of the mucosa allowing fluid to diffuse from blood plasma to the intestinal lumen. The accumulation of fluid in the intestinal lumen results in diarrhea, the characteristic symptom of salmonellosis. Neutrophils also secrete chemokines that attract more neutrophils and an inflammatory response is triggered resulting in the necrosis of cells of the epithelial lining. Salmonellosis infections typically do not progress to a systemic infection.

*S.* Typhi, the causative agent of typhoid fever in humans, has a preference for M cells of Peyer's patches over epithelial mucosa (90). In typhoid fever, mononuclear cells are predominantly recruited over neutrophils, likely in response to necrosis of Peyer's patches from the selective invasion of M cells. Systemic invasion occurs when *S.* Typhi translocates to the basolateral side of M cells, is taken up by macrophages, and transported to reticuloendothelial organs (133).

### 1.2.3 Virulence Factors

For *Salmonella* spp. to successfully infect a host, they must be able to survive low pH, have motility, adhere to host cells, invade, persist, and evade host defences (90, 133). *Salmonella* have an arsenal of virulence factors including adhesins, TTSS, toxins, and flagellae that are dynamically regulated where their expression is coordinated in response to several host environment conditions encountered during pathogenesis (90). Virulence genes in *Salmonella* spp. are typically clustered together on pathogenicity islands (PAI), plasmids, and operons.

#### 1.2.3.1 Classical Virulence Determinants

Fimbriae (pili) are filamentous surface structures that are involved in adhesion and colonization of host cells (119). *Salmonella* may harbour several types of fimbriae that range in size and can be up to 8 nm wide and 10 μm long. Fimbriae are typically encoded by operons and are assembled via the chaperone usher pathway. Over 10 fimbriae operons including *fim*, *lpf*, and *pef* have been detected in *S.* Typhimurium based on homology to fimbriae of other bacteria.

Motility is important in *Salmonella* pathogenesis since translocation of the intestinal mucous is essential for a successful infection. Flagella are surface structures that confer motility, are larger than fimbriae, and are peritrichously arranged in *Salmonella* (119). *S.* Typhimurium encodes genes involved in flagellar biosynthesis on 4 regions of the chromosome termed *fli, flh, flg* and *flj* (55). The flagellum shaft of *S.* Typhimurium is composed of either FliC (phase 1) or FljB (phase 2) flagellar antigens that are alternately expressed (134). Flagella are a type of pathogen associated molecular

pattern (PAMP) recognized by host immune cells and are potent stimulators of the immune response (65). Modification of flagellar antigen expression pattern might be beneficial for immune evasion (46).

Expression of either FliC or FljB on flagella is governed by a phase switching mechanism mediated by an invertible chromosomal DNA segment harbouring the *fljBA* transcriptional promoter (55). Hin recombinase mediates the inversion of this segment through DNA recombination. The phase 1 flagellar antigen (FliC) is expressed when the orientation of the *fljAB* transcriptional promoter is in the opposite direction of transcription of the *fljAB* region. In this orientation, the repressor of the phase 1 flagellin gene, *fljA* and the phase 2 flagellin gene *fljB* are not expressed, whereas *fliC* is constitutively expressed. Phase 2 flagella are expressed where inversion of the *fljAB* promoter allows for induction of *fljAB* expression. FliA inhibits FliC resulting in the expression of phase 2 flagella and repression of *fliC* by FliA.

*Salmonella* may encode several toxins that cause cytopathic effects in hosts. This organism harbours the endotoxin lipid A, a component of lipopolysaccharide found in Gram-negative cell membranes, along with several exotoxins (119). Exotoxin Clg, which is a collagenase, was implicated in a rare cytopathic phenotype of a MDR *S.* Typhimurium DT104 isolate from a bovine source, that is capable of inflicting cytotoxic damage on cultured HEp-2 and murine mucosal cells (129). Several *Salmonella* encoded enterotoxins are involved in target cell invasion (eg. SopB and SopE2), inflammation, and fluid accumulation in the intestinal mucosa (eg. SopA, SopB, SopE2, and SipA) (133). These enterotoxins are encoded in different chromosomal locations and in concert they induce cytopathic effects of the host cells.

In Gram-negative bacteria, membrane bound secretion systems enable the transport of proteins into the extracellular environment. The Type One Secretion System (TOSS) and the TTSS play a role in secretion of virulence factors by *Salmonella* (52). The TOSS enables proteins to be secreted in the extracellular environment and is composed of heterotrimeric complexes, consisting of an ATP-binding cassette (ABC) exporter, membrane fusion protein and a pore forming outer membrane protein (OMP) (52). The TTSS is architecturally more complex than the TOSS, where 20 different proteins comprise a membrane bound needle structure. TTSSs penetrate eukaryotic cell membranes in close proximity allowing translocation of secreted virulence factors from bacterial to host cytoplasm (52). Translocation of secreted *Salmonella* effectors involved in cytoskeletal rearrangement (described above) into the cytoplasm of host cells is mediated by a TTSS during invasion.

#### 1.2.3.2 Genomic Islands

Genomic islands (GEIs) are defined as large (10 – 200kb) segments of DNA that differ in G + C content, codon usage, or have other genomic properties that differ from the host chromosomal DNA (70). In addition, GEIs are often inserted into tRNA genes and flanked by 16-20 bp direct repeats, which arise from site specific integration. GEI elements are believed to be transmitted horizontally amongst bacteria since they often carry genetic mobility factors such as phage genes, integrases, and those involved in conjugation. Insertion elements such as transposons can be found on GEIs which may be involved in the incorporation or deletion of genetic material from the element. GEIs

usually harbour genes that are beneficial and provide a selective advantage for the host, such as antibiotic resistance, symbiosis, metabolic and pathogenicity islands.

#### 1.2.3.2.1 Pathogenicity Islands

Pathogenicity islands (PAI) are GEIs that carry one or more virulence genes (50). Currently 10 PAIs have been identified in *Salmonella* spp, and are named *Salmonella* Pathogenicity Island (SPI) 1 through 10 (119). SPI1 through 5 are found in *Salmonella* Typhimurium and other serovars of *Salmonella* subspecies *enterica*. SPI6 through 10 were detected in serovar Typhi CT18 in 2001 (93).

*Salmonella* pathogenicity island 1 (SPI1) is an important virulence determinant factor for *S.* Typhimurium induced enterocolitis that facilitates mucosal invasion (133). SPI1 encodes a TTSS that secretes effector proteins involved in epithelial cell invasion and fluid accumulation. SPI1 encoded effector proteins SipB, SipC, and SipD form a translocation complex on mucosal cell membranes that facilitate delivery of AvrA and SptP as well as non SPI1 encoded effector proteins such as SopA, SopB, SopD, SopE1, SopE2, SspHI, and SlrP. In concert, the effector proteins disrupt normal cellular processes and induce membrane ruffling and cytoskeleton rearrangements resulting in the internalization of *S.* Typhimurium (90). SPI1 also encodes virulence regulator proteins HilA and InvF that are important in the facilitation of invasion (6, 30).

*Salmonella* pathogenicity island 4 (SPI4) is implicated in adhesion to epithelial cells and was recently found to be coregulated with SPI1 (51). SPI4 encodes a TOSS that secretes a large redundant protein SiiE which functions as a non-fimbral adhesin found to

adhere specifically to polarized epithelial cells (54). Functions of the SPI1 virulence regulator HilA are implicated in the secretion of SiiE (53).

*Salmonella* pathogenicity island 2 (SPI2) is a virulence determinant factor in systemic infections and is essential for replication in epithelial cells and intracellular survival in macrophages (46). This PAI is activated inside phagosomes of macrophages and encodes a TTSS which translocates effector proteins into the cytosol that prevents killing due to phagolysosome maturation (90). Like SPI2, *Salmonella* pathogenicity island 3 (SPI3) is also involved in intracellular survival in macrophages (46). SPI3 harbours 10 ORFs including the *mgtCB* operon that is involved in survival in low $Mg^{2+}$ environments such as macrophages.

### 1.3 Multi-Drug Resistant *S.* Typhimurium DT104 and Global Dissemination

*S.* Typhimurium DT104 was first identified in the 1960's and was rarely encountered until the 1980's, when multiple drug resistant strains emerged (86). Multi-drug resistant (MDR) *S.* Typhimurium DT104 rapidly became the most frequently isolated phagetype (definitive type) of *S.* Typhimurium worldwide. A MDR *S.* Typhimurium DT104 (MDR DT104) epidemic is of concern since invasive infections can be difficult to treat, especially in children, due to the multidrug resistance phenotype of this organism. The emerging MDR DT104 encountered are resistant to a minimum of 5 antibiotics including ampicillin (A), Chloramphenicol (C), Florfenicol (F) Streptomycin (S), Sulfonamides (Su), and Tetracycline (T). This penta-resistance profile is known as the ACSSuT phenotype (R type) and the first recorded incident of penta-resistant *S.* Typhimurium DT104 occurred in the United Kingdom in 1984 and was associated with

imported exotic birds (59, 64). The first human case of ACSSuT DT104 was in the UK in 1989 (86, 116). By 1992 this organism was encountered from human or animal sources in the United States, France, Germany, and Italy, and by 1996, ACSSuT *S.* Typhimurium DT104 was found in Canada, Belgium, Japan, Israel, Czech Republic, and Denmark (56, 59, 64, 99). Throughout the 1990's, ACSSuT *S.* Typhimurium DT104 was increasingly isolated from humans and many animal species, especially those of agricultural importance. By 1996, DT104 became the dominant *S.* Typhimurium phagetype encountered and the second most isolated *Salmonella* strain in the UK (97).

Since emerging in the 1980s, the number of MDR *S.* Typhimurium DT104 has increased world wide while the total number of *S.* Typhimurium were unaffected, excluding Italy and Israel where the incidents of serovar Typhimurium increased with the emergence of ACSSuT DT104 (44, 83). As seen previously with other *Salmonella* serovars including Typhimurium, phagetype DT104 has emerged and displaced other phagetypes as the most common isolate (59). This trend was noted worldwide, and alarming since the majority of DT104 isolates were multidrug resistant where the previous strains of *S.* Typhimurium DT104 were generally antibiotic susceptible.

Furthermore, MDR DT104 has been associated with increased morbidity and mortality relative to antibiotic susceptible *Salmonellae*. In Denmark a higher probability of death from gastroenteritis was observed in infections caused by the pentaresistant DT104 as compared to susceptible isolates (61). An enhanced severity of gastroenteritis was also noted in the aforementioned study. Additional studies have reported increased hospitalizations and death rates resulting from MDR DT104 infections (56, 61, 81, 121). In addition to human infections, this strain was found to be more virulent in cattle, where

Evans *et al.* (1996) reported that calves infected with this strain were 13 times more likely to die (43). The increased morbidity of this strain may be due to ineffective antibiotic treatments, however, may result from an enhanced virulence phenotype. The resistance genes of the majority of isolates of the emerging DT104 strain were later determined to be clustered together on a chromosomal element known as the *Salmonella* Genomic Island 1 (SGI1) (21). This genomic island has been hypothesized to play a role in MDR DT104 associated hypervirulence (20, 85, 86). SGI1 and variants were also found to be distributed in *S.* Typhimurium and other *Salmonella* serovars (20, 78).

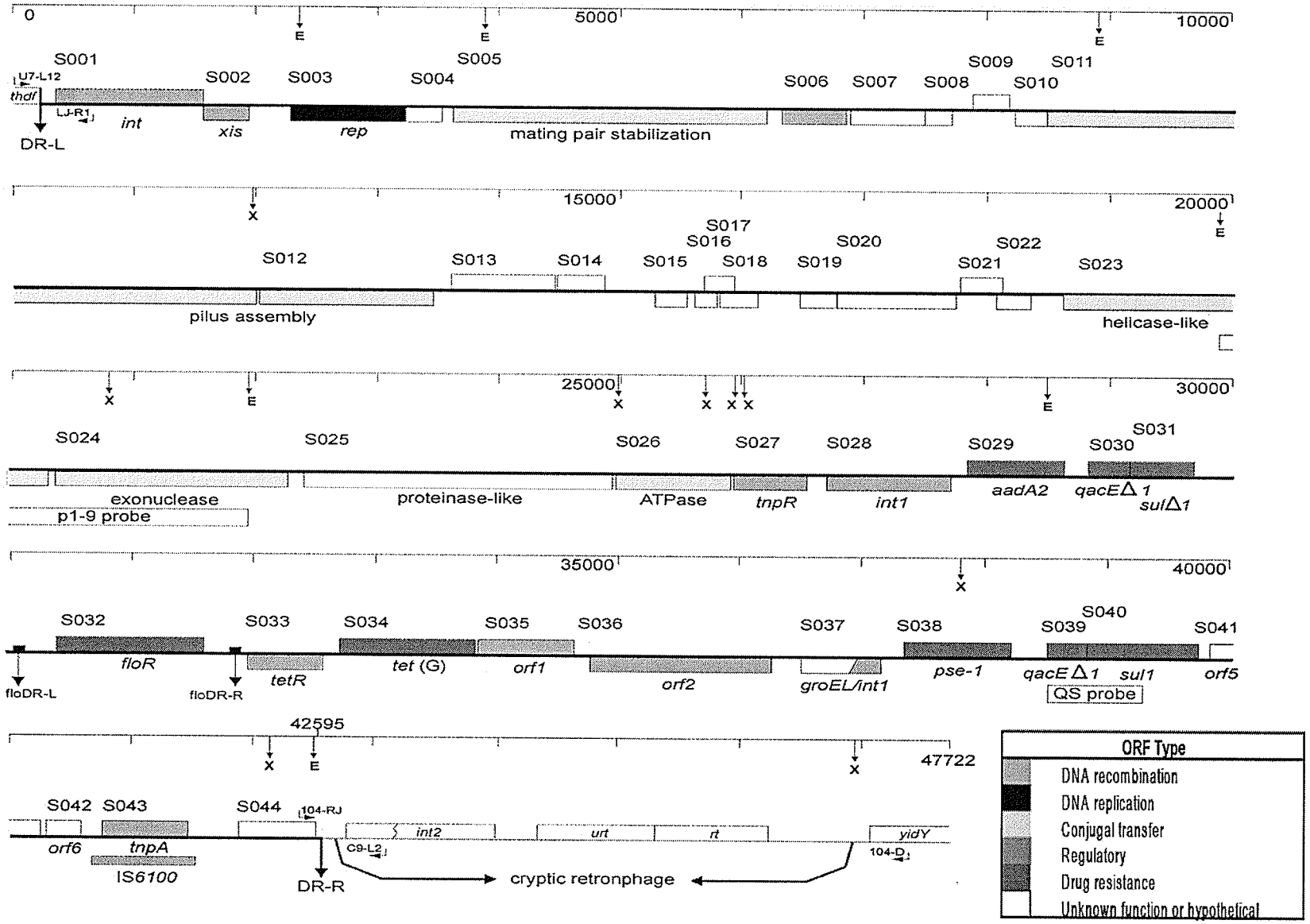
## 1.4 Salmonella Genomic Island 1

### 1.4.1 Genetics

The genetic elements that confer the ACSSuT MDR phenotype were found to be colocalized to the same region on the *S.* Typhimurium DT104 chromosome (23, 106). The MDR genes were associated with a 43 kb genomic element termed SGI1 illustrated in Figure 1 (21). The complete nucleotide sequence revealed that SGI1 had 44 ORFs named S001-S044 (20). The 44 ORFs were catalogued into 7 major gene classes based on homology to other genes, which are as follows: DNA recombination (8 ORFs: S001-2, S020, S027-28, S036-37, and S043); DNA replication (1 ORF: S003); conjugal transfer (6 ORFs: S005, S011-12, S023-24, and S026); regulation (5 ORFs: S004, S006-7, S033, and S035); drug resistance (8 ORFs: S029-32, S034, S038-40); other functions (2 ORFs: S025 and S026); and ORFs that are hypothetical or have an unknown function (15 ORFs: S008-10, S013-19, S021-22, S041-42, and S044) (20, 86).

**Figure 1: Genetic map of *Salmonella* Genomic Island 1 (SGI1) and flanking regions in *S.* Typhimurium DT104**

The location of ORFs and restriction enzyme cleavage sites on SGI1 are indicated by the bp ruler above the SGI1. Functions of ORFs are differentiated by colour and are listed in the legend at the bottom left. ORF boxes above the line indicate transcription from left to right and boxes below the line indicate transcription in the opposite direction. SGI1 flanking regions DR-L and DR-R are indicated as well as the cryptic retron sequence located at the 3′ end that is found in *S.* Typhimurium strains only. This figure was used with permission from (20)


0
5000
10000
E
S001
S002
S003
S004
S005
S006
S007
S008
S009
S010
S011
U7-L12
thdf
LJ-R1
int
xis
rep
mating pair stabilization
DR-L
15000
20000
X
S012
S013
S014
S015
S016
S017
S018
S019
S020
S021
S022
S023
pilus assembly
helicase-like
25000
30000
S024
S025
S026
S027
S028
S029
S030
S031
exonuclease
proteinase-like
ATPase
tnpR
int1
aadA2
qacEΔ1
sulΔ1
p1-9 probe
35000
40000
S032
S033
S034
S035
S036
S037
S038
S039
S040
S041
floR
tetR
tet (G)
orf1
orf2
groEL/int1
pse-1
qacEΔ1
sul1
orf5
floDR-L
floDR-R
QS probe
42595
47722
S042
S043
S044
104-RJ
orf6
tnpA
IS6100
int2
urt
rt
yidY
C9-L2
104-D
DR-R
cryptic retronphage
ORF Type
DNA recombination
DNA replication
Conjugal transfer
Regulatory
Drug resistance
Unknown function or hypothetical

SGI1 was the first identified genomic island in *S.* enterica that contains a cluster of antibiotic resistance genes (20, 21). All of the resistance genes are localized within SGI1 in a 13 kb region at the 3′ end associated with a complex class 1 integron called In104, which is bordered by inverted repeat segments called IRi and IRt (20, 34, 86). In104 consists of a central region that is an R plasmid like element flanked by two class 1 integron cassettes previously known as InC and InD on the 5′ and 3′ end of the of the 13 kb MDR region, respectively (20, 34, 86).

Three of the 8 antibiotic resistance genes *qacEΔ1* (2 copies) and *sulΔ1* are non functional, and the remainder constitute the penta-resistant ACSSuT phenotype of DT104 harbouring SGI1. The R plasmid-like region contains *floR* encoding resistance to chloramphenicol (C) and florfenicol (F), *tet(G)* encoding resistance to tetracycline (T), and *tetR* which is a regulator of *tet(G)*. As class 1 integrons, both InC and InD contains a resistance gene for sulphonamides (*sul1*), however, the *sul1* gene in InD is truncated and non functional (*sulΔ1*) (20). They also contain the *qacEΔ1* gene encoding a non functional (truncated) resistance gene to quaternary ammonium compounds commonly used as antiseptics or disinfectants (20, 47). Each integron region in In104 has an *attI1* (*attB*) site that is capable of incorporating gene cassettes that have an *attC* sequence. InC and InD have an integrated resistance gene cassette where the former contains *aadA2* encoding resistance to both streptomycin (S) and spectinomycin (Sp), and the latter contains a $bla_{PSE\text{-}1}$ encoding a beta-lactamase conferring resistance to ampicillin (A). As seen in variants of SGI1, resistance gene cassettes can be incorporated, deleted or switched at either *attI1* site (34, 86).

#### 1.4.1.1 Class 1 Integrons and Gene Cassettes

Integrons are non-mobile genetic elements similar to transposons that can capture genes in the form of cassettes (47). Class 1 integrons are one of four integron classes and are differentiated based on the type of integrase contained within it. The structure of class 1 integrons consists of 2 regions, the 5′ conserved sequence (5′-CS) and a 3′ conserved sequence (3′-CS). The 5′-CS contains a site-specific integrase gene (*intI1*) and a promoter region ($P_{ANT}$) that initiates expression of elements found upstream of an adjacent *attI1* site, which is a recognition site for integration of gene cassettes. The 3′-CS region is variable in length and typically contains a copy of *sul1*, encoding resistance to sulphonamides and a non functional copy of the *qacE1* denoted *qacEΔ1* encoding quaternary ammonium compound (e.g. antiseptics) resistance.

Gene cassettes are mobile promoterless elements that consist of a single ORF and an *attC* site for recognition of the integron attachment site *att1* (47). Most gene cassettes carry ORFs for antibiotic resistance. Integration or deletion of gene cassettes on the integron occurs by homologous recombination facilitated by the 5′-CS *int* gene. Preservation of the *att1* site of integration enables the integron to acquire several gene cassettes. Class 1 integrons may be void of cassettes, or contain one or multiple cassettes integrated in tandem.

### 1.4.2 SGI1 Location

The last 18 bp of *thdF,* encoding the thiophene and furan oxidation protein was determined to be the integration site for SGI1 likely by homologous recombination (21). To date, all natural *Salmonella* isolates harbouring SGI1 (or a variant) have contained the

genomic island in the 3′ end of the *thdF* gene (20, 34). In all *S.* Typhimurium serovars SGI1 is described to be inserted into the *thdF* gene and adjacent to the 5′ end of *int2*, a gene associated with a retron sequence upstream of *yidY* (20). All other characterized *Salmonella* spp. lack this retron sequence and therefore SGI1 is then inserted in *thdF* and adjacent to *yidY*. The 18 bp chromosomal SGI1 attachment site is known as *attB* whereas the corresponding attachment sequence on SGI1 is termed *attP*. Chromosomal SGI1 is flanked by 18 bp imperfect direct repeats with 1 mismatched nucleotide. Doublet *et al.* (2007) has identified homologous *attB* regions in *Shigella* spp. and *Pseudomonas* spp., which suggests the potential for SGI1 to be horizontally transferred to these organisms (which are known to be pathogenic) (37, 38). Recently a secondary *attB* site between *sodB* and *purR* was identified in *S.* Typhimurium and SGI1 was shown to insert in this region by conjugation with the aid of a helper plasmid R55 *in vitro* (36).

### 1.4.3 Variants of SGI1

ACSSuT SGI1 was first documented in DT104 and now has been reported world wide in other Typhimurium phagetypes, and other *S. enterica* serovars including Agona, Albany, Paratyphi B, Cerro, Derby, Dusseldorf, Emek, Haifia, Infantis, Kentucky, Kiambu, Meleagridis, Newport, and Tallahassee (3, 19, 20, 34, 39, 40, 77, 78, 84).

Excluding the original, there are currently 15 variants of SGI1 named SGI1-A through SGI1-O. Each variant is genetically different in the MDR region resulting in an altered resistance phenotype. For example, SGI1-A first isolated from *S.* Agona has the typical ACSSuT phenotype with additional resistance to trimethoprim (Tm) (19), and SGI1-K, first isolated from *S.* Kentucky, imparts mercury resistance encoded by a *mer*

module located at the 3′ end of SGI1 upstream of ORFSO44 (79). The variants of SGI1 apparently resulted from insertions, deletions, or switching of gene cassettes at either, or both *attI1* sites (eg. SGI1-A and F), whereas others may have resulted from homologous recombination resulting in deletions (SGI1-B) or inversion of some genes (SGI1-E) (19).

Between 1998 and 2002 in Canada there was a rapid emergence of *S.* Paratyphi B dT+ with the MDR phenotype (85). Several isolates were found to contain SGI1, and clustered into a triad of closely related groups indicating 3 independent events of the serovar acquiring this genomic element. Another observation was that the incidence of *S.* Paratyphi dT+ strains harbouring SGI1 was rapidly increasing whereas those that displayed the ACSSuT phenotype with no SGI1 were not.

A variant of SGI1 was reported in *Proteus mirabilis* in 2007 in Palestine, and was the first report of SGI1 in a bacterium other than *Salmonella* (1). This incident resulted in the discovery of SGI1-L. More recently in China there were several incidents of *P. mirabilis* harbouring yet another variant termed SGI1-O (22). To date *P. mirabilis* is the only bacterium outside of the *Salmonella* genus with occurrences of SGI1 variants.

### 1.4.4 Mobility of SGI1

SGI1 shares common features of GEIs and therefore harbours genes that indicate this genomic element can be horizontally transferred (20, 70). SGI1 and variants have been detected in several *Salmonella* serovars as noted above as well as in *P. mirabilis*, which indicates it is transmissible. Furthermore, three epidemic strains of *S.* Paratyphi B dT+ harbouring SGI1 were detected in Canada between 2000 and 2002 (84) indicating 3 separate events of SGI1 acquisition (85). Boyd *et al.* (2001) has detected several ORFs

with homology to genes involved in DNA recombination and conjugation genes, including integrase, excisionase, and a cryptic retrophage (in Typhimurium only), however SGI1 appears to lack a complete compliment of mobility genes (20). Transduction may be implicated in SGI1 mobility since Schmieger *et al.* (1999) was able to transmit the MDR region (later to be described in SGI1) by a P22-like phage (106). Doublet *et al.* (2005) demonstrated that SGI1 can be transmitted by conjugation into *E. coli* K12 in the presence of the *Klebsiella* plasmid R55 (33). They have also detected SGI1 a circular form. SGI1 has been classified as an integrative mobilizable element (IME) since it is transmissible but not self mobilizable (33).

Horizontal transfer of SGI1 is of concern since relatively rare serovars have rapidly emerged after acquiring it, and aside from *P. mirabilis*, other bacterial species bear potential SGI1 recognition sites such as *Shigella* spp., *Vibrio* spp., and *Pseudomonas* spp. (34, 37, 59, 85). Also the stability of the chromosomal location may allow SGI1 to persist even if selective pressure (eg. antibiotic use) is removed (86).

### 1.4.5 SGI1 and Virulence

DT104 was a once rare strain of *S.* Typhimurium, that emerged in the 1980's with an MDR (ACSSuT R-type) phenotype and has since disseminated worldwide. MDR DT104 has been associated with increased morbidity and mortality relative to antibiotic susceptible strains. It is currently not known if the morbidity of this organism is due to ineffective antibiotic treatments, however, MDR DT104 has been hypothesized to be more virulent (20, 85, 86).

Studies have been conducted to assess if MDR DT104 strains are hypervirulent relative to susceptible strains with inconclusive results. Carlson *et al.* (2000) found that MDR DT104 were not more invasive or adherent to HEp-2 cells than susceptible strains and they were not more infectious based on murine oral $LD_{50}$ values (25). Also MDR DT104 were not more invasive to HEp-2 cells than susceptible strains in the presence of antibiotics (28). Allen *et al.* (2001) did not find that MDR DT104 were more virulent than susceptible strains based on survival in peritoneal macrophages, epithelial cell invasion, resistance to nitrogen compounds and reactive oxygen, and infectious dose (murine oral $LD_{50}$) (4).

It was unknown whether the MDR DT104 strains used in the above studies contained SGI1. Aside from genes that confer the ACSSuT R-type, SGI1 encodes several ORFs with putative functions that may play a role in MDR DT104 associated hypervirulence (20). SGI1 is most likely transmitted by horizontal means and there is concern that if other pathogens acquire it, they will exhibit the hypervirulent phenotype associated with MDR DT104 (33, 34). More recent studies have been conducted to assess the issue of hypervirulence in MDR DT104 harbouring SGI1. Wu *et al.* (2002) demonstrated that SGI1 has been implicated in a cytopathic phenotype atypical of salmonellosis observed in an MDR DT104 strain isolated from cattle (129). The collagenase gene *clg* responsible for this cytopathic phenotype is repressed by SlyA, a transcriptional regulator. SGI1 was implicated in the repression of *slyA* resulting in the induction of *clg* (26). Rasmussen *et al.* (2005) found MDR DT104 were transiently hyperinvasive *in vitro* (HEp-2 cells) and *in vivo* (bovine infection model) after exposure to rumen protozoa (RPz) commonly found in cattle, which was linked to the presence of

SGI1 (101). Carlson *et al.* (2007) reported that SGI1 ORF S013 was involved in this RPz enhanced virulence *in vitro* and *in vivo* (27).

SGI1 encodes 16 ORFs that have unknown functions and 4 ORFs with potential regulatory functions. These ORFs may influence expression of virulence or fitness genes outside of SGI1, that may contribute to the hypervirulent phenotype associated with MDR DT104 (20, 57). To determine if SGI1 effects global gene expression, an isogenic strain pair of *S.* Typhimurium LT2 was constructed. Microarray analysis using the isogenic strain pair of *S.* Typhimurium LT2 revealed that SGI1 did indeed influence the expression of chromosomal genes based on analysis with microarrays. Thirty-six genes were differentially expressed where up-regulated genes included those for iron and sialic acid utilization and those down-regulated included genes involved in chemotaxis and motility in mid-log growth phase (57). No classical virulence genes were observed to be influenced by SGI1 in *S.* Typhimurium LT2 which may have resulted form the growth phase used, or the fact that this strain is avirulent due to a non-functional *rpoS* gene (57, 113). In this project the influence of SGI1 on DT104 gene expression was assessed using a fully virulent MDR DT104 strain with a functional *rpoS* gene.

## 1.5 Microarrays

### 1.5.1 Overview

DNA microarrays enable researchers of many disciplines to perform high throughput analyses involving genomics. The concept of microarrays was conceived in the early 1980's and advancements in information technology, robotics, and genomics enabled the technology to emerge (69). Microarrays are based on the same principle as

Southern blots. The concept is that single stranded DNA in solution will specifically hybridize to complimentary DNA fixed to a solid substrate. Microarrays are a tool that can quantify expression of up to several 100 000 ORFs or assess genomic content.

A microarray consists of an ordered arrangement of oligonucleotide spots on a solid 2-dimensional substrate (10). High density microarrays can consist of over 100 000 spots per $cm^2$ (10). Each spot consists of a large number of copies of a particular nucleic acid representing a probe for a complete, or portion of a specific transcript. Microarrays may contain a few hundred spots to several hundred thousands of spots representing all known ORFs from an organism's genome. The length of the nucleic acid probes may range from 18 bp to 1000 bp and are fixed directly to the solid substrate such as a glass microscope slide or a silicon chip (69, 110). Entire microarrays range in size from less than 20 $mm^2$ to the area of a microscope slide. Two common types of microarrays are spotted arrays or oligonucleotide arrays which are discussed in section 1.5.4 of this document.

Comparative genomic hybridizations (CGH), single nucleotide polymorphism (SNP) detection, species identification, assessing phylogenetic relationships, and gene expression are some analyses to which microarrays have been applied. In the former analyses mentioned, microarrays are used in DNA-DNA hybridizations and the targets are prepared from genomic DNA. In expression microarrays, cDNA targets are derived from RNA transcripts of a sample. The following discussion will focus on expression microarrays since they were the type used in this project.

### 1.5.2 The Hybridization Process in Brief

Outlined herein is the stepwise flow of a typical microarray expression experiment from sample preparation to hybridization and analysis. A simple experimental design for a whole genome microarray analysis is a 2 condition experiment, where differential expression of the transcriptome can be observed between reference samples and test samples. Target samples are derived from RNA, which is extracted from each sample after experimental treatment. RNA is unstable and susceptible to degradation so extracts are commonly reverse transcribed into complimentary DNA (cDNA) which is less likely to degrade in subsequent labeling and hybridization manipulations (69). Sample labeling involves the enzymatic incorporation of fluorescently labeled nucleotides. Cyanine 3 (Cy3) and Cyanine 5 (Cy5) are commonly used fluorescent dyes in microarray experiments that are detected at different wavelengths. The hybridization step involves adding the target sample in aqueous solution to the microarray where the labeled transcripts bind specifically to one of the many complimentary probe sequences fixed on the solid substrate. The hybridized arrays are digitized by scanning with a laser at a particular wavelength enabling the dye to fluoresce. The degree of fluorescence for a probe spot is recorded as intensity value, which is directly proportional to the amount of labeled target oligonucleotides bound. The intensity values measured are representative of the degree of expression of the gene or ORF represented by the probe spot.

Relative expression data are generated with spotted DNA microarrays, where the reference sample and test sample are labeled with different fluorophores, mixed, and hybridized to the same array (2 colour array) (69). The use of different fluorescent dyes

allows intensity values to be generated for each sample by scanning with two different wavelengths to eliminate cross-interference of the fluorophores. A ratio is calculated for each probe spot from the intensity values for the reference and test sample, enabling differential expression to be detected. Competitive binding occurs when two samples are hybridized to one microarray, therefore absolute expression can not be measured (109). Absolute expression can be quantified for oligonucleotide arrays where each sample is hybridized onto an individual microarray (1 colour array), allowing the comparability of expression data between arrays (69).

Visual inspection of the digital image and normalization of the raw data are essential for extrapolation of meaningful expression data. The former ensures that hybridization and extrapolation of intensity data from the digital array image is satisfactory, and the latter ensures that the expression data between samples is comparable. Normalization is critical since variation between microarrays can be introduced from array fabrication or sample handling, which introduces technical error in data analysis resulting in false biological conclusions (71). Several methods of normalization have been proposed, however, many are based on assumptions and may also introduce bias. Currently no "gold standard" of normalization exists.

Detection of differentially expressed genes between reference and test samples involves mining large lists of expression data for several thousand genes. Several software packages exist to mine these large data sets using statistical analyses (69). Multiple testing for differentially expressed genes may yield false positive results that can be minimized by increasing the stringency of statistical parameters, however true biological differences may be overlooked. Confirmation of microarray data is typically

done using an independent gene expression assay such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) (80).

### 1.5.3 Common Types of Microarrays

#### 1.5.3.1 Spotted Microarays

Spotted microarrays are easily customized since they can be developed "in house" and are relatively inexpensive to produce. The probe spots are generated from a large collection of cDNA or PCR products which are spotted and covalently fixed onto the array substrate. The exact sequence of the probes is not a requirement for array manufacture since transcripts of unknown ORFs may be included in the probe set which may elucidate a possible function or role. The spots are typically arranged in several grids and up to 80 000 probes may be "printed" onto the microarray slide (69). The probe sets on spotted arrays are determined by the researcher who may focus on a specific set of ORFs or the whole genome (109). Nucleic acid probes range in length from 25 to 1000 bp depending on the size of transcript or PCR product that the probes are derived from. The potential for cross-hybridization increases with the length of the probe resulting in decreased specificity. Typically one probe represents one ORF. Experiments using spotted microarrays quantify relative expression since test and reference samples are co-hybridized on the same slide. This technique is termed a two colour array, and generates a ratio for each probe. Gene expression is not directly comparable between arrays due to low accuracy of digital grid alignment on spots of the hybridized microarray image, and also slide to slide variation during array printing (109). Array to array

comparisons of relative expression may be done only if the same reference sample is used (109).

### 1.5.3.2 Oligonucleotide Microarrays

Oligonucleotide microarrays are comprised of synthetic nucleic acids and may have a higher probe density than spotted microarrays where one array may contain over 500 000 spots in an area less than $20mm^2$ (60, 69). Since a high number of spots are possible, one ORF may be represented by multiple short probes. Probe sequences are typically designed *in silico*. Probe sequences are shorter than those of spotted arrays, which range from 20 to 75 nucleotides long where cross hybridization is less likely. Knowledge of nucleic acid sequences is necessary for oligonucleotide microarrays since probes are not derived from expressed transcripts. The number of ORFs of a particular organism represented on the microarray is also limited by genome sequence data (69). All nucleic acid probes on a microarray are synthesized simultaneously *in situ* on a solid substrate through a process involving photolithography and solid state DNA synthesis (48). Producers of commercially available microarrays commonly use this process for large scale production of robust microarrays. Highly reproducible microarrays enable single sample hybridizations or "one colour" arrays which allow direct comparison between arrays, eliminating the need for co-hybridization of a reference sample as in two colour arrays described above. Since competitive binding between target samples is absent in one colour arrays absolute expression can be quantified.

### 1.5.3.3 Commercially Available Microarrays

Miniature biological assays or "chip" technology emerged in the mid 1990's where original high throughput assays were limited to a 96 well plate (48). Increased density and miniaturization are goals sought by researchers for highly repetitive multiple assays to minimize both buffer quantity and processing time (48). The manufacture of oligonucleotide microarray chips requires specialized equipment and there is less flexibility in customization of microarrays (69). Commercially available microarray chip technology has become more prevalent in laboratory use where robust high density microarrays are designed, mass produced, and available through a catalog (60). Several companies have developed platforms for microarray analysis and supply equipment and buffers or provide a service by performing hybridizations on submitted samples. A number of platforms exist including those from Affymetrix and Roche-Nimblegen, which were used in this project. Each platform utilizes different probe set designs, probe sizes, internal controls, and methods of hybridization that provide reproducible data individually (60). Currently no inter-platform standardized methods of array design exist and data generated from different platforms may not be directly comparable. Validation of results acquired from any high throughput experiment with an independent method of expression analysis is good practice. This is especially important when comparing data from multiple data sets conducted over time using rapidly evolving and updated high throughput technology.

High density microarrays developed by Affymetrix are known as GeneChips® that are comprised of greater than 100 000 probes 25 bp long fixed on a silicon substrate.

Each ORF is represented by 11 to 20 short probes to improve signal to noise ratios and minimize cross hybridization (60, 69). Perfect match (PM) oligonucleotides completely complimentary to target sequences are partnered with mismatch (MM) oligonucleotides. MM oligonecleotides differ from the former by a single nucleotide in the centre of the probe sequences (60). MM probes detect non specific hybridization that is subtracted from the PM probe signal to minimize experimental error.

Researchers from Affymetrix pioneered biological chip development where several oligonucleotides of different sequence are simultaneously synthesized *in situ* using a process that involves photolithography and solid state DNA synthesis in the production of miniature high density arrays (48, 60). Light directed synthesis involves repetitive cycles of illumination and nucleotide chemical coupling (49, 94). Light is used to remove a photolabile protection group on the reactive site allowing chemical coupling of the next nucleotide in the subsequent chemical coupling step. Masks are used in the illumination step to block photodeprotection of specific oligonucleotides so that probes requiring the incoming nucleotide at that position will incorporate it into the growing polymer. For every single probe to increase in length by one base, four cycles of light directed synthesis are needed, since one of four nucleotides may be incorporated. A different mask is used for each cycle to facilitate which oligonucleotide will incorporate the next incoming base.

Roche-Nimblegen also manufactures high density microarrays using maskless array synthesizer technology (MAS). The concept of *in situ* oligonucleotide synthesis using MAS is similar to the process described above except that a digital micromirror device (DMD) is used instead of masks to direct site specific photodeprotection (89).

Light is projected onto a glass slide from the DMD where thousands of miniature digital mirrors may be turned on or off in combinations to form "virtual masks". When the micromirrors are on, light is reflected in specific locations for selective photodeprotection.

Roche-Nimblegen has several designs for chips that vary in probe size and density which are described at www.nimblegen.com. The *S.* Typhimurium LT2 microarray from Roche-Nimblegen used in this project consists of 385 000 probes that are 60 bases long representing 4527 ORFs. Each ORF is represented by 17 probes, and five complete sets of whole genome probes are found on one microarray to minimize experimental error.

### 1.6 Thesis Objective

MDR DT104 harbouring SGI1 has emerged and disseminated globally where increased morbidity and mortality in humans and livestock have been observed. Failure of antibiotic treatments may be a factor since the strain is resistant to frontline antimicrobials used to treat invasive infections. Another possible explanation is that the virulence of *S.* Typhimurium DT104 is enhanced due to elements of SGI1 other than antimicrobial resistance genes. SGI1 contains 15 ORFs with an unknown function or no homology to any known genes. Also other serovars of *Salmonella* that have acquired SGI1 (or variants) have rapidly emerged, illustrated nicely by the outbreak of ACSSuT *S.* Paratyphi B $dT^+$ in Canada where those bearing SGI1disseminated rapidly where ACSSuT strains without SGI1 did not (85).

Genomic islands likely acquired by horizontal transfer have previously been shown to modulate expression of bacterial chromosomal genes (58, 70, 105). My hypothesis is that SGI1 influences expression of *S.* Typhimurium DT104 chromosomal

genes. Genes differentially expressed in the presence of SGI1 may elucidate a mechanism to explain the association between the genomic island and hypervirulence due to up-regulation of bona fide virulence genes or alteration of gene expression resulting in increased bacterial fitness. Global expression analysis using microarrays with a *S.* Typhimurium LT2 isogenic strain pair (with and without SGI1) demonstrated that SGI1 influences chromosomal gene expression (57). Since *S.* Typhimurium LT2 is avirulent because of a non-functional regulatory gene *rpoS*, an isogenic strain pair of virulent *S.* Typhimurium DT104 (with and without SGI1) was constructed by our collaborators from Institut National de la Recherche Agronomique (INRA) in France (35, 113). Song *et al.* (2004) have reported that *S.* Typhimurium grown to early stationary phase were 10-20 fold more invasive than those grown to mid-log and late stationary phase *in vitro* (112). The purpose of this study was to employ microarray analysis to detect any changes in the *S.* Typhimurium DT104 transcriptome influenced by SGI1 in mid-log and early stationary growth phase.

## 2. Materials and Methods

### 2.1 Strains and Culturing

The isogenic strain pair *S.* Typhimurium DT104 1948SA96 and DT104 1948SA96ΔSGI1:$Km^r$ was used to identify differences in global gene expression influenced by SGI1 (herein referred to as *S.* Typhimurium DT104 and DT104ΔSGI1, respectively). These strains were obtained from National de la Recherche Agronomique (INRA) (Tours, France) and susceptibilities and relevant genetic information are summarized in Table 1. The deletion mutant was constructed using the one-step chromosomal gene inactivation technique displacing SGI1 with a kanamycin (Km) resistance cassette through site specific homologous recombination (31, 35). Stock cultures of the isogenic strain pair were stored in Microbank™ Vials on porous beads (Pro-lab Diagnostics, Richmond Hill, Ontario) at -80°C until use. Overnight cultures were prepared by inoculating 2 ml of Brain Heart Infusion broth (BHI) (Becton Dickenson, Franklin Lakes, NJ) with a bead from the Microbank™ stock culture and incubated 18-24 hours at 37 °C with aeration (150-200 rpm).

**Table 1: The isogenic strain pair used in expression studies**

| Strain | Resistance phenotype* | Relevant genetic information |
|---|---|---|
| *S.* Typhimurium DT104 1948Sa96 | ACSSuT | harbours SGI1 |
| *S.* Typhimurium DT104 1948Sa96ΔSGI1 | Km | SGI1 deleted and replaced with a $Km^r$ cassette |

*A, ampicillin; C, chloramphenicol; S, streptomycin; Su, sulfomamides; T, tetracycline; Km, kanamycin

## 2.2 Growth Curves

Growth curves for each isogenic strain were averaged from 3 biological replicate cultures. Overnight cultures were used to inoculate 20 ml of BHI broth in 50 ml Falcon tubes (Becton Dickenson, Franklin Lakes, NJ) at a dilution factor of 1 in 100. These cultures were incubated at 37°C with aeration (150-200 rpm) and growth was monitored by optical density at a wavelength of 600 nm ($OD_{600}$) at 30 minute intervals. $OD_{600}$ readings were obtained by aliquoting 200 µl of each culture into UVette disposable cuvettes (Eppendorf, Germany) and then measuring absorbance using the Biophotometer spectrophotometer (Eppendorf, Germany). When cultures became increasingly turbid, aliquots for $OD_{600}$ readings were diluted 1 in 4 with BHI broth for accurate readings for $OD_{600}$ greater than 1.0. Mid-log phase was defined at an $OD_{600}$ of $0.317 \pm .018$ and early stationary phase at an $OD_{600}$ of $2.720 \pm 0.169$ observed shortly after cultures have transitioned from late-log phase.

## 2.3 DNA Extraction

BHI agar plates were streaked with beads from stock Microbank™ cultures and incubated at 37°C for 18-24 hours. Colonies lifted from plates were suspended in 1.5 ml microfuge tubes (DiaMed Lab Supplies, Mississauga, ON) containing 50 µl of 0.1 mm silica beads (Scientific Industries, Ottawa, ON) and 600 µl of neutralization buffer [30 mM Tris pH 8.4, 2 mM EDTA pH 9] and vortexed. Samples were heated at 95°C for 2 minutes using a heat block then lysed by mechanical disruption with the Vortex Genie 2 (Scientific Industries, Ottawa, ON) for 2 minutes. Samples were then centrifuged at 1000 x g for 1 minute to collect the beads. Supernatants were transferred to new microfuge

tubes and centrifuged for 10 minutes at 10000 x g to collect cell debris. DNA preparations were stored at -20°C until further use.

### 2.4 DNA Amplification

Polymerase chain reaction (PCR) was carried out in a reaction volume of 25 µl buffered by a 1X concentration of AmpliTaq Gold PCR Buffer (Applied Biosystems, Foster City, CA). Other ingredients include 3 mM $MgCl_2$, 0.2 mM of each dATP, dCTP, dGTP, and dTTP, 0.5 mmol of both forward and reverse primers; 0.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA) and 2.5 µl of template DNA. PCR was performed with a 96 well GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA). Cycling conditions were as follows: 94°C for 5 minutes, then 25 cycles of 94°C for 30 seconds, 55°C for 30 seconds (60°C for integron amplification), and 72°C for 1 minute and followed by 72°C for 7 minutes then a 4°C hold. Primers used in PCR reactions are found on Table 2.

Agarose gel electrophoresis was done to visualize PCR products using a 1% agarose gel in 0.5X Tris-Borate-EDTA (TBE) buffer [0.045M Tris-Borate, 0.001M EDTA pH 8.3] (Fisher Scientific, Ottawa, ON). Five µl aliquots of PCR reactions were mixed with 2 µl of bromophenol blue loading dye solution [0.25% w/v bromophenol blue, 40% w/v sucrose], loaded onto the agarose gel and electrophoresed at 5 – 10 V/cm for 80 -100 minutes. A 100 base pair (bp) ladder (Invitrogen, Carlsbad, CA) was used as a standard. The gel was stained in a 0.5 mg/L ethidium bromide solution and visualized under ultraviolet light by the AlphaImager®HP (Alpha Innotech. San Leandro, CA).

**Table 2: List of PCR primers used in this project**

| Region | Primer Name | Direction | Primer (5'-3') | Product Size (bp) | Reference |
|---|---|---|---|---|---|
| SGI1 left junction | U7-L12 | F | ACACCTTGAGCAGGGCAAAG | 500 | (21) |
| | LJ-R1 | R | AGTTCTAAAGGTTCGTAGTCG | | |
| SGI1 right junction | 104-RJ | F | CTGACGAGCTGAAGCGAATTG | 500 | (21) |
| | C9-l2 | R | AGCAAGTGTCGTAATTTGG | | |
| Integron | 5'CS | F | GGCATCCAAGCAGCAAG | 1000 and 1200 | (76) |
| | 3'CS | R | AAGCAGACTTGACCTGA | | |
| Retron-yidY junction | DB-T7 | F | ACCAGTGTTTTGTTGATTATGC | 800 | (21) |
| | 104-D | R | ACCAGGCAAAACTACACAG | | |

## 2.5 Cultures for Microarray Analysis

### 2.5.1 Mid-Log Growth Phase Cultures

All samples used for microarray analysis were cultured on the same day. Step-up cultures for each strain that served as inoculation sources were made by diluting an overnight culture 1 in 100 into 20 ml of BHI broth in 50 ml Falcon tubes. Four biological replicate cultures for each strain were made by inoculating 300 ml of BHI broth in a 1 L Erlenmeyer flask with 3 ml of the step-up culture and incubated at 37°C with aeration (150-200 rpm). $OD_{600}$ readings were taken as described in section 2.2 and cultures were harvested at mid-log phase predetermined from the growth curve, and stabilized in RNAprotect™ Bacterial Reagent (Qiagen, Mississauga, ON) as described below. Ten ml of the bacterial cultures were pipetted into previously prepared 50 ml Falcon tubes containing 20 ml RNAprotect™ Reagent (2 volumes), vortexed, then incubated at room temperature for 10 minutes. The samples were pelleted by centrifugation at 5000 x g for 10 minutes and supernatant was discarded. Stabilized pellets were stored at -20°C until RNA extraction.

### 2.5.2 Early Stationary Growth Phase Cultures

All samples used for microarray analysis were cultured on the same day. Three biological replicate cultures for each strain were made by inoculating 10 ml of BHI broth in a 50 ml Falcon tube with 200 µl of an overnight culture and incubated at 37°C with aeration (150-200 rpm). $OD_{600}$ was monitored every 30 minutes until late-log phase was approached where $OD_{600}$ was monitored every 5 to 10 minutes. Cultures were harvested

in early stationary phase predetermined by the growth curve and 2 ml culture aliquots were stabilized in 4 ml RNAprotect™ Reagent and stored as described in section 2.5.1.

## 2.6 Preparation of RNA Samples

### 2.6.1 RNA Extraction

Total RNA was extracted using the RNeasy Midi Kit (Qiagen, Mississauga, ON) according to manufacturer's instructions with minor adjustments. The steps are described below where all centrifugations were done at room temperature (RT) at 4000 x g. Stabilized mid-log phase culture pellets were resuspended in 0.5 ml TE buffer pH 8 (10 mM Tris-Cl, pH 8, 1 mM EDTA, pH 8) with 1 mg/ml of lysozyme (Sigma-Aldrich, Oakville, ON) and incubated at RT for 5 to 10 min. Two ml of lysis buffer RLT containing 143 mM β-mercaptoethanol was added to samples, and then vortexed followed by a 5 minute centrifugation. Supernatants were transferred to 15 ml Falcon tubes where 1.4 ml of 100% ethanol was added then vortexed. The solutions were transferred into RNeasy Midi columns placed inside 15 ml tubes then centrifuged for 5 minutes and flow through was discarded. For denser stationary phase cultures, 2 volumes of Lysosyme-TE buffer (1 ml), RLT buffer (4 ml), and ethanol (2.8 ml) were used. All samples were processed the same from this point unless specified.

Sample RNA bound to the filter membrane of RNeasy columns were treated with 4 ml wash buffer RW1 and centrifuged for 5 minutes, then two 2.5 ml of buffer RPE and centrifuged for 2 minutes. Centrifugation after the second RPE treatment was increased to 5 minutes to ensure filter membranes were free of ethanol. RNeasy filter cartridges were transferred to new 15 ml collection tubes. Total RNA was eluted with 300 µl of

RNase free water, except for mid-log RT-qPCR samples that were eluted in 150 µl of RNase free water. Total RNA samples were quantified using Nanodrop® (ND-1000 V3.1.0, Nano-drop Technologies, Wilmington, DE).

RNA extracts from early stationary phase were concentrated into a smaller volume of RNase free water. The RNA extracts were transferred into Microcon™ Ym-30 spin columns (Millipore Corporation, Etobicoke, ON) and centrifuged at 14000 x g for 12 minutes to collect RNA on the filter. RNA for microarray analysis was reconstituted with 50 µl of RNase free water and RNA prepared for RT-qPCR with 100 µl of RNase free water. The spin columns were carefully inverted and transferred to new 1.5 ml Microcon tubes then centrifuged at 1000 x g for 3 minutes to collect RNA samples.

### 2.6.2 DNase Treatment

Residual genomic DNA (gDNA) was eliminated from RNA extracts by DNase treatment using the Turbo DNAfree™ kit (Ambion, Austin, TX). RNA extracts for mid-log samples were prepared in a buffered DNase solution [1X TURBO DNase buffer, 0.026 units/µl TURBO DNase]. The concentration of DNase for early stationary samples was increased to 0.052 units/µl. RNA extracts were incubated in a 37°C water bath for 30 minutes to allow digestion of gDNA, followed by a 5 second centrifugation to collect the reaction. Stationary phase RNA extracts were treated for an additional 30 minutes with 0.026 units of DNase. Digestion was terminated by adding 0.2 volumes of TURBO DNase inactivation reagent to the RNA extract and incubated at RT for 2 minutes with occasional agitation. The DNase Inactivation Ingredient was pelleted by centrifugation then supernatants were transferred to new RNase free 1.5 ml tubes. RNA was quantified

with Nanodrop® (ND-1000 V3.1.0, Nano-drop Technologies, Wilmington, DE), and then stored at -80°C

### 2.6.3 PCR Verification of DNase Treatment

Elimination of gDNA was verified with PCR of an 800 bp region spanning the 3′ end of the retron sequence and 5′ end of *yidY* located downstream of the SGI1 insertion site in both strains. Primers for PCR of this region (104-D and DB-T7) are found in Table 2 and reactions and analysis were carried as described in section 2.4 with the exception that 30 and 40 cycles were used instead of 25 cycles for mid-log and stationary phase samples, respectively. If no PCR product was observed after agarose gel electrophoresis (described in section 2.4), the sample was deemed free of DNA.

### 2.6.4 Assessment of RNA Integrity

Samples were analyzed using the Nanodrop® spectrophotometer (ND-1000 V3.1.0, Nano-drop Technologies, Wilmington, DE) to ensure a sufficient quantity and purity of RNA was obtained. Spectrophotometry cannot distinguish between intact and degraded RNA. To ensure RNA integrity, 1 µl of RNA was loaded onto the Agilent 2100 Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, Mississauga, ON) according to manufacturer's directions briefly described below. A 1 µl aliquot of RNA 6000 Nano Dye was added to 65 µl of filtered RNA Nano 6000 gel and vortexed, centrifuged at 13000 x g for 10 minutes, then 9 µl aliquots were added to the RNA 6000 Nano Chip in the designated wells. RNA Nano Marker (5 µl) was aliquoted to all sample wells followed by 1 µl of the RNA samples and RNA 6000 Nano ladder that were

denatured by heating for 70°C for 2 minutes on a thermocycler. The chip was vortexed to mix the sample and run on the Agilent 2100 Bioanalyzer to generate electropherograms used for qualitative RNA integrity assessment. Electropherograms for *E. coli* RNA extractions illustrating high and low quality RNA can be seen in Jahn *et al.* (2008) (68).

### 2.7 Microarray Analysis

Commercially available microarrays were used to assess the effect of SGI1 on global gene expression in mid-log and early stationary growth phase. Microarray analysis for mid-log samples was carried out using NimbleExpress™ arrays (Roche-NimbleGen, Madison, WI) supported by the Affymetrix platform (Affymetrix, Santa Clara, CA). Nimblegen had discontinued the NimbleExpress™ chips before stationary phase expression experiments began. Early stationary growth phase RNA samples were sent to Roche-Nimblegen (Reykjavik, Iceland) for microarray processing.

#### 2.7.1 Microarray Analysis for Mid-Log Phase Samples

Ten μg of total RNA for the 4 biological replicates per strain (n= 8) was submitted to the Genomics Core Facility (National Micrboiology Laboratory, Winnipeg, MB) for microarray processing with the Affymetrix platform. Affymetrix supported NimbleExpress™ *S.* Typhimurium LT2 chips (S_typhimur530142) were used and sample processing and hybridization carried out in the core facility following the Affymetrix protocol for Prokaryotic array processing. The detailed protocol can be found at affymetrix.com where a brief description is as follows.

Ten ug of total RNA was reverse transcribed into single stranded (ss) cDNA using the random primer method. RNA was combined with 25 ng/µl of random hexamers (Invitrogen, Carlsbad, CA) in 30 µl of nuclease free water and incubated at 70°C for 10 minutes, and then 25°C for 10 minutes, and cooled to 4°C. The RNA/primer mix was diluted to 60 µl by the addition of cDNA synthesis ingredients [1X 1st Strand Buffer, 10 mM DTT, 10 mM dNTPs, 0.5 u/µl SUPERase-In (Ambion), 25 u/µl SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA)] and incubated at 25°C for 10 minutes, 37°C for 60 minutes, and 42°C for 60 minutes. The synthesis reaction was terminated by incubation at 70°C for 10 minutes then cooled to 4°C. RNA was removed by adding 1N NaOH (20 µl) with incubation at 65°C, followed by addition of 1 N HCl to neutralize the solution.

The cDNA was column purified using MinElute PCR Purification Columns (Qiagen, Mississauga, ON). Five volumes of Buffer PB was added to the cDNA sample which was then placed in a MinElute filter column inside a collection tube and centrifuged at 10000 x g for 1 minute. The filter bound cDNA was washed with 750 µl Buffer PE and centrifugation as described above. The MinElute filter was transferred to a new 1.5 ml tube and eluted in 12 µl of EB buffer. cDNA was quantified using the Nanodrop® spectrophotometer (ND-1000 V3.1.0, Nano-drop Technologies, Wilmington, DE).

The purified cDNA sample (10 µl) was fragmented into 50 -200 bp products. DNase I (0.6 u/µl) (Invitrogen, Carlsbad, CA) was diluted into 1X One-Phor-All Buffer (Amersham Biosciences, Piscataway, CA) and 10 µl was added to the cDNA sample and incubated at 37°C for 10 minutes followed by a 98°C incubation for 10 minutes to

inactivate DNase I. Fragmented cDNA (400 ng) was aliquoted and stored for the gel-shift assay prior to hybridization (see below).

The GeneChip® DNA Labelling Reagent (Affymetrix, Santa Clara, CA) was used to label the 3′ termini of fragmented cDNA with biotin. This reaction was carried out in a 50 µl volume containing 0.3 mM GeneChip® DNA Labelling Reagent, 0.3 u/µl Terminal Deoxynucleotidyl Transferase (TDT) (Promega, Nepean, ON), TDT reaction buffer [1X, Cacodylate K 140 mM pH 7.2, $CoCl_2$ 1 mM, $ZnSO_4$ 0.33 mM, BSA 10 mg/ml] and the entire fragmented cDNA sample. The labelling reaction was incubated at 37°C for 60 minutes and terminated by adding 2.5 µl of 0.5 M EDTA, pH 8.

A gel-shift assay was used to verify cDNA labelling efficiency, where 2 labelled cDNA aliquots and fragmented cDNA (200 ng) were used. Five µl of Neutravidin solution [1X PBS pH 7.2, 2mg/ml NeutrAvidn] was added to one 200 ng aliquot of each fragmented and labelled cDNA and incubated at RT for 5 minutes. Sucrose gel loading dye (5X) (Amresco, Burlington, ON) was diluted to 1X in cDNA samples and loaded on a 4-20% TBE gel (Invitrogen, Carlsbad, CA) placed in a Novex XCell SureLock™ Mini-Cell (Invitrogen, Carlsbad, CA) in 1X TBE buffer. Ten bp and 100 bp ladders (Invitrogen, Carlsbad, CA) were used as standards. The gel was run at 150 V/cm for 1 hour then stained in a 1X SYBR Gold solution (Molecular Probes, Eugene, OR) and visualized under ultraviolet light by the AlphaImager®HP (Alpha Innotech, San Leandro, CA).

Labelled cDNA ( 3.6 µg ± 0.75 µg) was diluted into 200 µl of a hybridization cocktail containing Hybridization Buffer [1X, 100 mM MES buffer, 1 M NaCl, 20 mM EDTA, 0.01% Tween-20], B2 Control Oligo (50 pM), Herring Sperm DNA (0.1 mg/ml),

bovine serum albumin (BSA) (0.5 mg/ml), and DMSO (7.8 %). This solution was injected into a room temperature equilibrated microarray cartridge (GeneChip®) by pipette. The cartridge was incubated for 16 hours at 45°C in a hybridization oven with rotation at 60 rpm.

The microarray cartridge was then placed in the GeneChip® Fluidics Station where subsequent washing and staining steps were automated using the ProkGE_WS2 fluidics protocol script. Buffers are sequentially infused into the microarray cartridge in 250 µl volumes termed mixes. The post hybridization wash sequence is as follows; post hybridization wash 1 with Non-Stringent Wash Buffer A [6X SSPE, 0.01% Tween-20] for 6 cycles of 2 mixes/cycle at 30°C, followed by post hybridization wash 2 with Stringent Wash Buffer B [100 mM MES buffer, 0,1 M NaCl, 0.01% Tween-20] for 6 cycles of 15 mixes/cycle at 50°C. The staining sequence is as follows; 1st stain with Streptavidin Phycoerythrin (SAPE) solution [1X Stain Buffer, 2 mg/ml BSA, 10 µg/ml Streptavidin] infused continuously for 300 seconds at 35 °C, post stain wash with Non-Stringent Wash Buffer A for 10 cycles of 4 mixes/cycle at 30 °C, 2nd stain with the Antibody Solution [100mM MES buffer, 2mg/ml BSA, 0.1 mg/ml Normal Goat IgG, 5 µg/ml biotinylated Anti-streptavidin Antibody] infused for 300 seconds at 35°C, repeat stain with SAPE solution infused continuously for 300 seconds at 35°C, followed by a final wash with Non-Stringent Buffer A for 15 cycles of 4 mixes/cycle at 25°C.

Array scanning was done using the GeneChip® Scanner 3000 at the wavelength of 570 nm where 1 pixel = 3 $\mu m^2$. GeneChip® Operating Software (GCOS) software was used for data extraction where 1 value is calculated for 1 probe averaged from intensity values from all pixels within a spot. Intensity values for each ORF were derived from the

intensity values within the ORF probeset. Data was quantile and $\log_2$ normalized using the Partek Genomics Suite (Partek, St. Louis MO) and analysis was done using Significance Analysis Microarray (SAM) software (Stanford University, CA) (117). A delta threshold of 0.843 and a 2.33% false discovery rate (FDR) were parameters used to identify ORFs with significantly altered expression. Differential expression of ORFs influenced by SGI1 in *S.* Typhimurium DT104 was defined at a minimum level of 1.8 fold.

### 2.7.2 Microarray Analysis for Early Stationary Phase Samples

Total RNA for the 3 biological replicates per strain (n= 6) were sent to Roche-NimbleGen for microarray processing with their expression platform using the TI99287 60mer design for *S.* Typhimurium LT2 chip for hybridization. The detailed protocol is found at NimbleGen.com. A brief description is found below.

Total RNA was reverse transcribed into double stranded (ds) cDNA using the SuperScript™ Double Stranded cDNA Synthesis kit (Invitrogen, Carlsbad, CA). Synthesis of single stranded DNA was done as per protocol for SuperScript™ Single Stranded cDNA Synthesis kit described in section 2.7 with modifications. In an 11 µl volume, 10 µg of total RNA was combined with 50 ng/µl of random hexamers and incubated at 70°C for 10 minutes. The RNA nucleotide mixture was then diluted into 18 µl of cDNA Synthesis Mix (without Superscript™ III) as described in section 2.7 with 1.1 mM dNTPs. The reaction mixture was preheated to 42°C before Superscript™ III was added, and then it was incubated at 42°C for 60 minutes. For second strand synthesis, the sample was diluted to 150 µl in the Second Strand Synthesis Mix [1X

Second Strand Buffer, 0.2 nM dNTP mix, 0.067 u/µl DNA Ligase, 0.26 u/µl DNA Polymerase I, 0.013 u/µl RNaseH], incubated at 16°C for 2 hours, then reaction was terminated by the addition of 10 µl of 0.5 M EDTA, pH 8. To eliminate RNA, 1 µl of 4 mg/ml RNase A was added to the reaction and incubated at 37°C for 10 minutes.

For sample clean up, the cDNA preparation was transferred to a 1.5 ml Phase Lock tube (Fisher Scientific, Ottawa, ON) containing 163 µl of phenol:chloroform:isoamyl alcohol (25:24:1) (Ambion, Austin, TX), vortexed, followed by a RT centrifugation at 12000 x g for 5 minutes. The aqueous layer was transferred to a new 1.5 ml tube. The cDNA was precipitated by the addition of 0.1 volumes of 7.5 M ammonium acetate, 7 µl of 5 mg/ml glycogen, and 2 volumes of ice cold absolute ethanol, mixed thoroughly by repeated inversions, RT centrifugation at 12000 x g for 20 minutes, then the supernatant was discarded. The pellet was washed by the addition of 500 µl of ice-cold 80% ethanol, centrifuged at 12000 x g for 5 minutes, then the supernatant was discarded. This wash was repeated a second time and then the pellet was dried in a SpeedVac (Thermo Savant, Asheville, NC) and reconstituted with 20 µl of VWR water (VWR International, Mississauga, ON). The cDNA samples were quantified using the NanoDrop® spectrophotometer (ND-1000 V3.1.0, Nano-drop Technologies, Wilmington, DE) and quality was verified using the Agilent Bioanalyzer.

The cDNA was labelled using the NimbleGen One Colour DNA Labelling Kit. Supplied Cy3 labelled Random-Nonamers (6 µl) was diluted into 1000 µl of Random Primer Buffer with 25 mM β-mercaptoethanol. One µg of cDNA was combined with 40 µl of prepared Random Primer Buffer and nuclease-free water in an 80 µl volume and heat denatured at 98°C for 10 minutes, then placed on an ice water bath for 10 minutes.

A 20 µl dNTP/Klenow Master Mix solution was prepared by combining 10 µl of 10 mM dNTP Mix, 2 µl of 50 u/µl Klenow Fragment (3'-> 5' exo-), and 8 µl of nuclease-free water. This solution was combined with the denatured sample and incubated at 37°C for 2 hours. The 100 µl labelling reaction was terminated by the addition of 10 µl of 0.5 M EDTA pH 8. An 11.5 µl aliquot of 5 M NaCl was added to the reaction mixture and transferred to a new 1.5 ml tube. Labelled cDNA was precipitated by the addition of 110 µl of isopropanol (1.1 volumes), vortexed, and incubated at RT away from light for 10 minutes. The cDNA was pelleted by RT centrifugation at 12000 x g for 10 minutes. The cDNA was then washed by adding 500 µl of ice cold 80% ethanol and centrifuging at RT for 12000 x g for 2 minutes then the supernatant was discarded. The cDNA pellets were dried in a SpeedVac on low heat for 5 minutes and then reconstituted in 25 µl of Nuclease-free water. The labelled cDNA was quantified using the NanoDrop® spectrophotometer (ND-1000 V3.1.0, Nano-drop Technologies, Wilmington, DE). Three µg of labelled cDNA was aliquoted to a new 1.5 ml tube and dried using the SpeedVac on low heat.

Hybridization was carried out using the NimbleGen Hybridization Kit. The labelled cDNA was reconstituted in 5 µl of VWR water. A Hybridization Master Mix was prepared by combining 11.8 µl of 2X Hybridization Buffer, 4.7 µl of Hybridization Component A, and 0.5 µl of Alignment Oligo, and then 13 µl was added to the labelled cDNA sample, followed by incubation at 42°C until hybridization. The samples were then preheated to 95°C and injected into a NimbleChip X1 mixer cartridge with a microarray chip previously preheated to 42°C. The prepared samples were placed in the NimbleGen Hybridization System and incubated at 42°C for 16-20 hours. The

microarray slides were removed from the X1 mixer and washed with 3 successive buffers; Wash Buffer 1 [1X, 0.1 M DTT] at 42 °C for 2 minutes, Wash Buffer II [1X , 0.1 M DTT] at RT for 1 minute, and then Wash Buffer III [1X , 0.1 M DTT] for 15 seconds.

Microarrays were scanned using a GenePix 3000B Scanner at a wavelength of 532 nm where 1 pixel = 5 $\mu m^2$. Raw data was extracted from the scanned image using NimbleScan 2.3 software and processed to generate quartile normalized intensity values for the 5 replicates of each ORF per array. These data were provided by NimbleGen for data analysis, which was done using Arraystar software (DNAstar, Madison, WI). Intensity data were log base 2 ($log_2$) normalized, where differential expression of ORFs was evaluated using an unpaired T test. Genes with altered expression in *S.* Typhimurium harbouring SGI1were defined at a minimum level of 2 fold and significant to a *p*-value less than 0.05

**2.8 RT-qPCR Validation**

Validation of differentially expressed genes observed from microarray analysis was done with Real Time Quantitative PCR (RT-qPCR). This assay involves three steps for quantifying gene expression; the conversion of RNA to cDNA through reverse transcription, PCR amplification of cDNA, and quantification of PCR amplification in real time with the use of a fluorescent dsDNA reporter (88). Three biological replicates per isogenic strain were used where all samples were grown on the same day. Samples were inoculated (1 in 100 dilution ratio), cultured, and stabilized with RNAprotect™ as

previously described in section 2.5 with the exception that 20 ml BHI broth cultures were used. RNA was extracted and processed as described in section 2.6.

Primers used in this study were designed from *S.* Typhimurium LT2 sequences using Primer Express® Software v2.0 (Applied Biosystems, Foster City, CA) with the exception of those for *gapA* from Golding *et al.* (2007) and *gmk* and *rpoD* found in Bottledorn *et al.* (2006) (17, 57). All primers were synthesized in house (Genomics Core Facility, NML, MB). GeNorm v3.5, a Microsoft Excel applet, was applied to evaluate the stability of housekeeping genes and it also generates normalization factors for expression analysis (120). The 2 most stable housekeeping genes for each growth phase were selected using GeNorm after running an RT-qPCR assay (described below) with several housekeeping genes listed in Table 3. Normalization factors based on two housekeeping genes instead of one are more accurate (120). Primers used for RT-qPCR confirmation of differentially expressed genes observed from microarray analysis in mid-log and stationary growth phase are found in Table 4 and Table 5, respectively.

RNA was reverse transcribed into first-strand complimentary DNA (cDNA) prior to RT-qPCR using the Superscript™ III First Strand Synthesis System for RT-qPCR (Invitrogen, Carlsbad, CA). Reverse transcription of cDNA from RNA using random hexamer primed reactions was done in triplicate for each sample (biological replicate). Two µg of total RNA was combined with 50 ng/µl of random hexamers, and 1 mM dNTPs in 10 µl of DEPC-treated water, then incubated at 65°C for 5 minutes, and placed on ice. The RNA nucleotide mixture was then brought to a 20 µl volume in cDNA Synthesis Mix [1X RT buffer, 5mM $MgCl_2$, 0.01M dithiothreitol, 40 units/µl RNaseOUT, 200 units/µl SuperScript III reverse transcriptase], centrifuged for 1 minute

at 1000 rpm, followed by heating at 25°C for 10 minutes and 50°C for 50 minutes using a thermocycler. The reaction was terminated by heating at 85°C for 5 minutes and then the reaction tubes were placed on ice. The mixture was treated with 0.1 units of RNase H at 37°C for 30 minutes. The three cDNA preps for each sample (biological replicate) were pooled, diluted 1:10 and stored at -80°C for RT-qPCR.

**Table 3: List of primers for housekeeping genes**

| Gene locus | Gene name | Primer name | Primer (5'-3') | Reference | |
|---|---|---|---|---|---|
| STM1290 | *gapA* | STM1290F | GGCGCTAACTTTGACAAATACGA | (57) | glyceraldehyde-3-phosphate dehydrogenase |
| | | STM1290R | GCAGTTGGTGGTGCAGGAA | | |
| STM3740 | *gmk* | STgmkF | TTGGCAGGGAGGCGTTT | (17) | guanylate kinase |
| | | STgmkR | GCGCGAAGTGCCGTAGTAAT | | |
| STM3835 | *gyrB* | STgyrBF | TCTCCTCACAGACCAAAGATAAGCT | this study | B subunit of DNA gyrase |
| | | STgyrBR | CGCTCAGCAGTTCGTTCATC | | |
| STM0386 | *proC* | STproCF | TCAGGTCGCGGATATCGTTT | this study | pyrroline-5-carboxylate reductase |
| | | STproCR | TTCAGGCTGGAGGAGATTTCA | | |
| STM3211.S | *rpoD* | STrpoDF | ACATGGGTATTCAGGTAATGGAAGA | (17) | RNA polymerase sigma factor |
| | | STrpoDR | CGGTGCTGGTGGTATTTTCA | | |
| STM0249 | *rrsH (16S)* | ST16SF | CCGGATTGGAGTCTGCAACT | this study | 16S ribosomal RNA |
| | | ST16SR | GAACGTATTCACCGTGGCATT | | |

**Table 4: List of primers for RT-qPCR validation of mid-log phase microarray data**

| Gene locus | Gene name | Primer name | Primer (5'-3') |
|---|---|---|---|
| STM0557 | - | STM0557F | GAGATGCAAGGAACGCAACA |
| | | STM0557R | TTTTATTCATGCTGGGTGCAAT |
| STM0558 | *yfdH* | STM0558F | AATTGAACGCCACCCAGAAA |
| | | STM0558R | TGGCTACCCGTCTCTTCTTGTT |
| STM1568 | *fdnI* | STM1568F | CTTCCGCCTTCTCGATTTCA |
| | | STM1568R | GGGCGAAGAAACATCATCCA |
| STM1959 | *fliC* | STM1959F | GTTACAGAAGCCGTACCATTCGT |
| | | STM1959R | CGGGTCTTGATGATGCAGCTA |
| STM2770 | *fljA* | STM2770F | GTAGAAATTGAACTCGACGAGCAA |
| | | STM2770R | AAATATGGCCGCGGGATTAT |
| STM2771 | *fljB* | STM2771F | TGCAACTTGTACATTTTTCACATCCT |
| | | STM2771R | GGTGGACTACCTGCGACAGC |
| STM3812 | *ccmH* | STM3812F | CCTCCTGCATCAGGTCATACAC |
| | | STM3812R | CGATGATAGCCACCGACATG |
| STM3813 | *ccmG* | STM3813F | CCGATAAACTCAGCGCATACG |
| | | STM3813R | CGGTGGCCTGGTTAAAGGA |

**Table 5: List of primers for RT-qPCR validation of early stationary phase microarray data**

| Gene locus | Gene name | Primer name | Primer (5'-3') |
|---|---|---|---|
| STM0544 | *fimI* | STM0544F | ATTTGCGCGTATTGATGGGA |
| | | STM0544R | CAGGACCGGTAAACGCATTC |
| STM0853 | *bssR* | STM0853F | GCCGTTTGTTTAATGAGCGG |
| | | STM0853R | GACGGCAATAAAGGTCGGAC |
| STM1132 | - | STM1132F | TTGCCGAAGCGTATGGTTG |
| | | STM1132R | CGGGTAGCAAACCGACAAAA |
| STM1382 | *orf408* | STM1382F | CCGCACGTTGTATATTCCTGC |
| | | STM1382R | CCAGCGGCATCTTTTACCAC |
| STM1482 | *ydgF* | STM1482F | AGCTCTGGCTATCGCGACTG |
| | | STM1482R | GCCCATTTCATTGAGAGCGT |
| STM2066 | *sopA* | STM2066F | TCCCCGGTGGAGTCTCTGTA |
| | | STM2066R | CTTCAGAAACCGTGGGCCT |
| STM2781 | *virK* | STM2781F | GCCCAGTAAACATATCGGCC |
| | | STM2781R | CAATCTTTGGTGGCGTTACGT |
| STM2770 | *fljA* | STM2770F | GTAGAAATTGAACTCGACGAGCAA |
| | | STM2770R | AAATATGGCCGCGGGATTAT |
| STM2771 | *fljB* | STM2771F | TGCAACTTGTACATTTTTCACATCCT |
| | | STM2771R | GGTGGACTACCTGCGACAGC |
| STM2876 | *hilA* | STM2876F | TGTACGGACAGGGCTATCGG |
| | | STM2876R | GCGGAGACACCACTACGACC |
| STM2897 | *invE* | STM2897F | AAACCGATCCGAAGACCCTC |
| | | STM2897R | GGGCCTTCAACGCACAATTA |
| STM3245 | *tcdA* | STM3245F | ACCCGCCGTCAGTAAAATCA |
| | | STM3245R | TCAACGCCAAAATAGGCCTC |
| STM3339 | *nanA* | STM3339F | ACTGGAGATTGTCGCCGAAG |
| | | STM3339R | TGGGCGATCAACGTGATTTT |
| STM3432 | *rpmC* | STM3432F | AGTGGCCAGCTGCAACAGT |
| | | STM3432R | GACATCACGACGCACTTGCTT |
| STM3764 | *mgtC* | STM3764F | CGGCGCAGCGTATAAATCA |
| | | STM3764R | AACGTTTTTCTCCCTCAGCG |
| STM4074 | *ego* | STM4074F | CTTGTTTGGCTTGCAGGGAC |
| | | STM4074R | AGCTGCTGCATTTTTTCCGT |
| STM4258 | *siiB* | STM4258F | TGACAATGGTAGCGTCGCTTT |
| | | STM4258R | ATGCGGTATTCATCCCTTTCA |
| STM4262 | *siiF* | STM4262F | TCGCGTTAAAACCGCAAAAC |
| | | STM4262R | GCTGAAGCGCCTTGAGTACC |
| STM4534 | - | STM4534F | GGCATGATCCATGATACGCA |
| | | STM4534R | TACCAGCTCGTACAGCGCC |

Amplification assays were carried out on MicroAmp® Optical 96-Well reaction plates (Applied Biosystems, Foster City, CA) where 50 µl reactions contained 25 µl of Power Sybr® Green Master Mix for RT-qPCR (Applied Biosystems, Foster City, CA), 200 nM of gene specific primers, 5 µl of the 1 in 10 dilution of pooled cDNA sample, and dd$H_2O$. For each sample, two technical replicate RT-qPCR reactions were done for each ORF expression assay. Non-template control (NTC) reactions with no template cDNA were included in assays for all tested ORFs as negative controls. Standard curves for growth phase specific housekeeping genes were used for quantification of relative expression and were included on every plate. Serial 1 in 10 dilutions of a cDNA sample to $10^{-5}$ were used as template for standard curve RT-qPCR reactions with primers for housekeeping genes. These dilutions were set-up in duplicate. RT-qPCR reactions were carried out on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Cycling conditions used were as follows: 50°C for 2 minutes; 95°C for 10 minutes; and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. This was followed by a dissociation curve: 95°C for 15 seconds; 60°C for 15 seconds; and 95°C for 15 seconds at a 2% ramp rate.

Critical threshold (Ct) values were converted into relative quantities based on the standard curve. For each sample, measured expression for ORFs was standardized with the normalization factor calculated from GeNorm in order to compare relative expression between samples. Data analysis was done using Microsoft Excel where averaged expression was calculated for each strain along with the fold change in ORF expression for *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1.

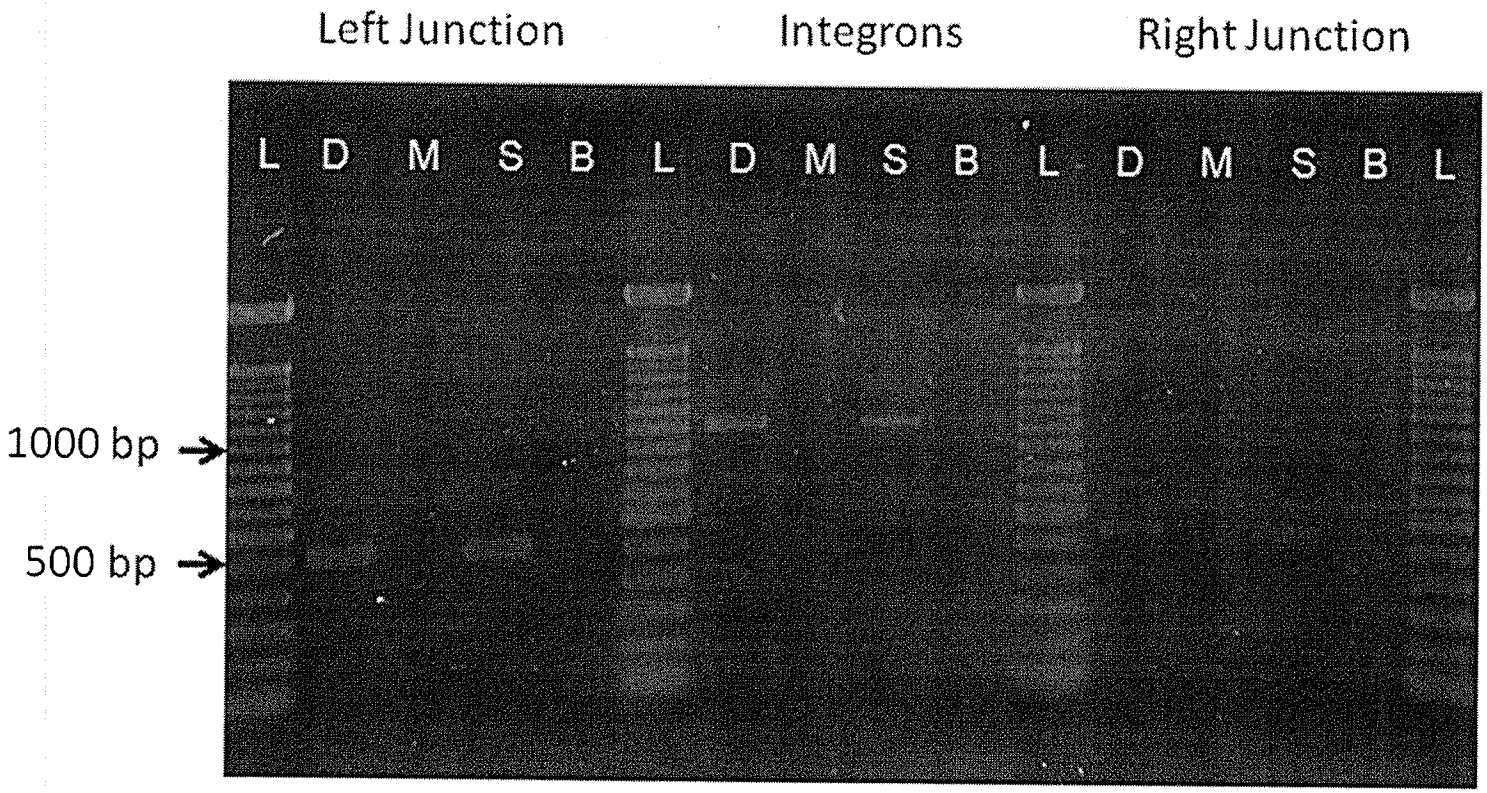
## 3. Results

### 3.1 Strains

*S.* Typhimurium DT104 SGI1 grew in BHI broth with streptomycin (Sm) (50 μg/ml), tetracycline (Tc) (10 μg/ml) and not with kanamycin (Km) (50 μg/ml). *S.* Typhimurium DT104 1948Sa96ΔSGI1:$Km^r$ (herein referred to as *S.* Typhimurium DT104ΔSGI1) grew in BHI broth with Km and not in Sm or Tc, as expected. PCR reactions using SGI1 detecting primer sets for the left junction (500 bp), right junction (500 bp), and integron (1000 bp and 1200 bp) regions yielded properly sized bands for *S.* Typhimurium DT104. Amplicons for SGI1 regions and junctions were absent for *S.* Typhimurium DT104ΔSGI1. Figure 2 shows the SGI1 PCR results for this isogenic strain acquired from Institut National de la Recherche Agronomique (INRA) (Tours, France) compared to the DT104 96-5227 standard strain which harbours SGI1. The presence and absence of SGI1 in *S.* Typhimurium DT104 and *S.* Typhimurium DT104ΔSGI1 were confirmed by PCR, respectively.

**Figure 2: PCR amplification of the SGI1 left junction (500 bp), right junction (500 bp), integron regions (1000 bp and 1200 bp) for the isogenic strain pair.**

Lanes marked (D) denotes *S.* Typhimurium DT104ΔSGI1 and (M) denotes *S.* Typhimurium DT104ΔSGI1. Lanes labelled (L) denote the 100 bp ladder (Invitrogen) (S) denotes the *S.* Typhimurium DT104 96-5227 positive control, and (B) is blank.


Left Junction
Integrons
Right Junction
L D M S B L D M S B L D M S B L
1000 bp
500 bp

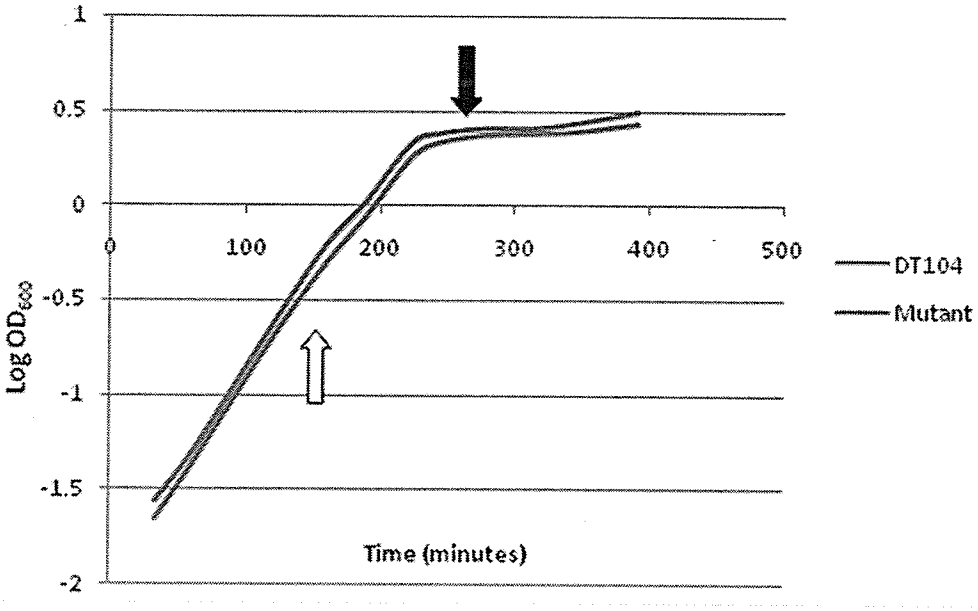
### 3.2 Growth of the Isogenic Strain Pair

A growth curve for the SGI1 isogenic strain pair is illustrated in Figure 3. The generation time was estimated at 30 minutes since $OD_{600}$ values doubled in every 30 minute measurement in log-phase. Mid-log was approached at an average $OD_{600}$ of $0.317 \pm 0.018$ typically achieved between 1 and 2 hours. The mean $OD_{600}$ mid-log for samples harvested for microarray and RT-qPCR samples were $0.309 \pm 0.015$ and $0.328 \pm 0.018$, respectively.

Early stationary phase was determined to be at an $OD_{600}$ of $2.720 \pm 0.169$ when readings values ceased to double and reached a maximum $OD_{600}$ $OD_{600}$ readings were observed to fluctuate after approaching stationary phase. Samples for microarray analysis and RT-qPCR assays were harvested at $2.672 \pm 0.105$ and $2.768 \pm 0.215$, respectively.

**Figure 3: A semi-log growth curve for *S.* Typhimurium DT104 and SGI1 deletion mutant *S.* Typhimurium DT104ΔSGI1.**

The lines represent the average $OD_{600}$ of triplicate cultures of each isogenic strain grown in BHI broth. The white arrow indicates mid-log growth phase and the black arrow indicates early stationary growth phase.


1
0.5
0
-0.5
-1
-1.5
-2
0
100
200
300
400
500
Log OD600
Time (minutes)
DT104
Mutant

### 3.3 RNA Extraction and Quality Assessment

The quantity and purity of RNA was assessed using a NanoDrop 2000 spectrophotometer (Nano-drop Technologies, Wilmington, DE). Spectrophotometry ensured sufficient amounts of pure RNA for the expression assays, however, cannot assess RNA integrity. In this project, the Agilent 2100 Bioanalyzer (Agilent Technologies, Missasauga, ON) was used for qualitative assessment of RNA integrity. This technology employs electrophoresis, which separates nucleotides by size (108). Electropherogram traces generated are plot of nucleotide size (nt) increasing on the x axis and fluorescence units (FU) that measure amount of RNA of a given size, on the y axis. Electropherograms for RNA of varying quality are discussed in Schroeder *et al.* (2006), and in Jahn *et al.* (2008) (68, 108).

Traces for selected samples are shown in Figure 4. The trace for *Bacillus cereus* RNA (Figure 4A) illustrates a typical bacterial RNA profile with sharp prominent peaks for 16S and 23S rRNA, and low signal relative to the rRNA peaks over the range of nucleotide sizes. Electropherograms of RNA with decreasing integrity correlates with the loss of 23S and 16S peaks and concentration of signal at low nucleotide sizes (108). Representative traces for *S.* Typhimurium DT104 RNA are illustrated in Figure (4B) and (4C) for mid-log and early stationary phase, respectively. RNA traces for the SGI1 mutant were indistinguishable from those for *S.* Typhimurium DT104 in mid-log and early stationary gowth phases.

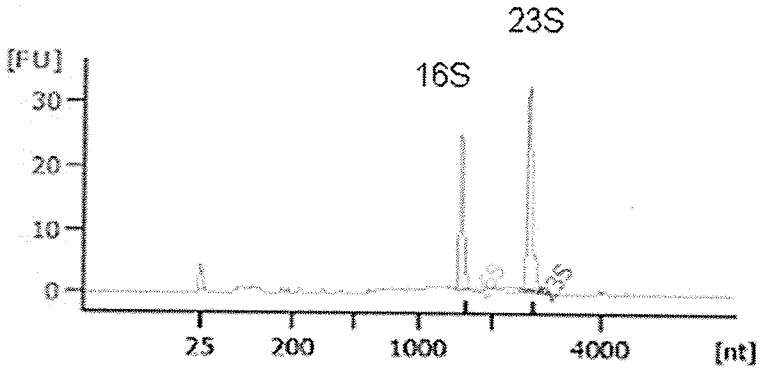
The RNA profile for *S.* Typhimurium DT104 consisted of a 16S peak (1.5 kb), however, the 23S peak (2.9 kb) was absent. Furthermore, additional peaks were observed in the approximate 500-600 nt, 1200 nt, 1700 nt, and 2400 nt regions. These peaks

remained after heat denaturing which should remove any secondary structure remaining in the RNA. RNA extraction using an independent kit (RiboPure™-Bacteria Kit, Ambion) showed the same result as Figure 4B, indicating these extra peaks were not dependant on the extraction method. Winkler *et al.* (1979) reported that 23S rRNA is fragmented in *S.* Typhimurium strains during maturation (127). Later it was found that the 23S RNA (2.9 kb) peak in *Salmonella* spp. undergoes site specific digestion by RNase III, and rapidly degrades in stationary phase (66, 67, 91). The absence of the 23S peak and presence of the extra peaks is consistent with those observed for 23S rRNA degredation products for *S.* Typhimurium in the aforementioned studies.

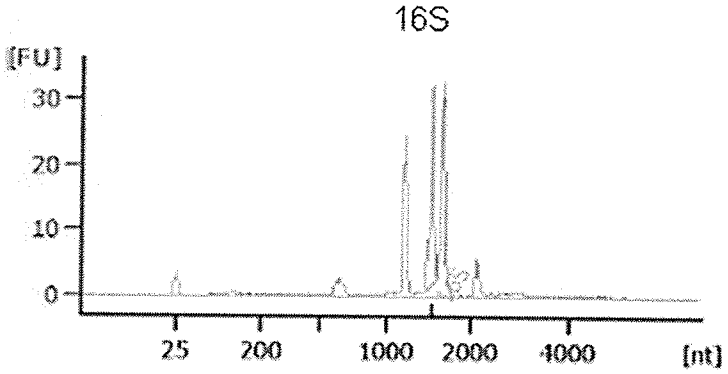
**Figure 4: Sample electropherograms for RNA extracted from the isogenic strains at mid-log and early stationary growth phases.**

The X and Y axis denotes nucleotide size (nt) and flourescencence units (FU), respectively. Electropherograms are representative of RNA integrity of *Bacillus cerius* (A) *S.* Typhimurium DT104 and *S.* Typhimutium DT104ΔSGI1 harvested in mid-log phase (B) and early stationary phase (C). The absence of a prominent 23S peak and presence of additional peaks were consistent features in all RNA samples extracted from the isogenic strain pair.

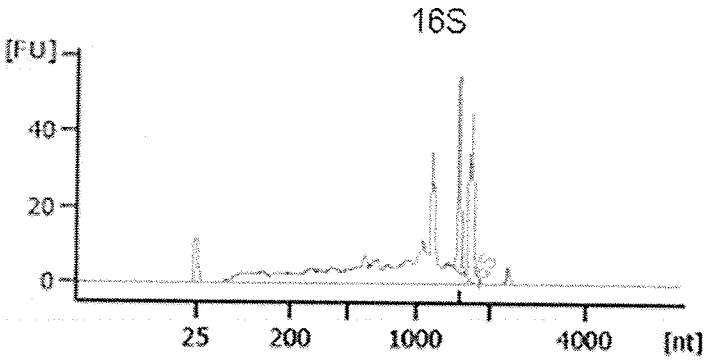
A


23S
16S
[FU]
30
20
10
0
25
200
1000
4000
[nt]

B


16S
[FU]
30
20
10
0
25
200
1000
2000
4000
[nt]

C


16S
[FU]
40
20
0
25
200
1000
4000
[nt]

### 3.4 Results from Microarray Analysis

#### 3.4.1 Mid-Log Phase Expression Data

Significant Analysis of Microarrays (SAM) was used to identify differentially expressed genes that were significant for mid-log phase microarray data using the unpaired two class *T*-test (117). In SAM, *q*-values calculated for pair-wise tests were a measure of significance, and can be considered as *p*-values modified for multiple comparisons. *Q*-values of differentially expressed genes identified as significant fall below a critical value known as the False Discovery Rate (FDR). This adjustable parameter is based on an estimate of genes falsely identified as differentially expressed. SAM is described in detail in Tusher *et al.* (2001) (117).

Each feature (ORF, intron sequence) on the NimbleExpress microarray is represented by several short probes that constitute a probeset (See section 1.5.4.3). The NimbleExpress LT2 microarray has probesets for 6577 features including 4459 for ORFs, 111 for *S.* Typhimurium LT2 plasmid SLT (pSLT) ORFs, and 2016 for intron regions. The latter were excluded from the analysis since (1) this study focused on SGI1-influenced expression of defined ORFs and (2) they were not represented on microarrays used for early stationary phase experiments. Analysis of the mid-log array data with a 2 class unpaired analysis in SAM revealed 58 significant genes with a threshold (delta) of 0.843 and a FDR of 2.33%. However, the difference in expression between *S.* Typhimurium DT104 and *S.* Typhimurium DT104ΔSGI1 was minimal (~1.2 to 1.7-fold).

No features representing pSLT genes were significant or differentially expressed in mid-log growth phase. Lists of significantly up-regulated and down-regulated genes as

determined by SAM (delta = 0.843 and FDR = 2.33%) before a 1.8-fold change cut-off was applied, are shown in Table 6 and Table 7, respectively. For all significant genes, the differentially expressed genes of the isogenic strains had a fold change range of 1.17-15.0-fold where the majority are less than 2-fold.

## Table 6: List of up-regulated genes in mid-log phase

| Gene Locus | Name | Description | Fold Change | [a]*q*-value (%) |
|---|---|---|---|---|
| STM0230 | *rnhB* | ribonuclease HII | 1.4 | 0 |
| STM0634 | *ybeF* | putative transcriptional regulator LysR-type | 1.3 | 0 |
| STM1152 | *yceK* | hypothetical protein | 1.3 | 2.33 |
| STM1188 | | putative inner membrane lipoprotein | 1.3 | 1.66 |
| STM1482 | *ydgF* | putative cationic transporter | 1.6 | 0 |
| STM1483 | *ydgE* | putative cationic transporter | 1.3 | 0 |
| STM1568 | *fdnI* | formate dehydrogenase-N gamma subunit | 1.8 | 2.33 |
| STM2208 | | putative inner membrane protein | 1.3 | 2.33 |
| STM2247 | *ccmH* | putative heme lyase subunit | 1.6 | 1.66 |
| STM2248 | *ccmG* | disulfide oxidoreductase; biogenesis of cytochrome c | 3.1 | 2.33 |
| STM2770 | *fljA* | repressor of *fliC* | 4 | 0 |
| STM2771 | *fljB* | phase-2 flagellin | 15.2 | 0 |
| STM3293 | *secG* | protein-export membrane protein | 1.3 | 0 |
| STM3706 | *yigQ* | putative periplasmic protein | 1.4 | 0 |
| STM3812 | *ccmH* | putative heme lyase subunit | 2.7 | 0 |
| STM3813 | *ccmG* | heme lyase disulfide oxidoreductase | 3.1 | 1.66 |
| STM4335 | *ecnA* | putative entericidin A precursor | 1.5 | 2.33 |
| STM4369 | *yjfH* | putative tRNA rRNA methyltransferase | 1.2 | 1.66 |

[a]q-value indicate significant genes at a FDR $\leq 2.33\%$

## Table 7: List of down-regulated genes in mid-log phase

| Gene Locus | Name | Description | Fold Change | [a]*q*-value (%) |
|---|---|---|---|---|
| STM0080 | | putative outer membrane lipoprotein | 1.3 | 0 |
| STM0543 | *fimA* | fimbrin | 1.6 | 0 |
| STM0544 | *fimI* | fimbrial protein | 1.5 | 0 |
| STM0545 | *fimC* | periplasmic chaperone | 1.5 | 0 |
| STM0546 | *fimD* | outer membrane usher protein precursor | 1.5 | 0 |
| STM0549 | *fimZ* | fimbrial protein Z | 1.6 | 0 |
| STM0550 | *fimY* | putative regulatory protein | 1.3 | 0 |
| STM0551 | | putative diguanylate cyclase/phosphodiesterase domain 0 | 1.7 | 0 |
| STM0557 | | inner membrane protein | 3.2 | 0 |
| STM0558 | *yfdH* | glucosyl transferase | 1.9 | 0 |
| STM0559 | *rfbI* | glucosyl translocase | 1.7 | 0 |
| STM0608 | *ahpC* | alkyl hydroperoxide reductase C22 protein | 1.3 | 1.66 |
| STM1054 | | hypothetical protein | 1.4 | 0 |
| STM1490 | *clcB* | putative voltage-gated ClC-type chloride channel ClcB | 1.2 | 2.33 |
| STM1564 | *yddX* | biofilm-dependent modulation protein | 1.4 | 1.66 |
| STM1586 | | putative periplasmic protein | 1.5 | 2.33 |
| STM1769 | *ychN* | putative sulfur reduction protein | 1.2 | 1.66 |
| STM1959 | *fliC* | phase-1-I flagellin | 3.2 | 0 |
| STM2169 | *yohC* | putative transport protein | 1.3 | 2.33 |
| STM2550 | *asrC* | anaerobic sulfite reductase subunit C | 1.3 | 0 |
| STM2786 | | tricarboxylic transport | 1.2 | 0 |
| STM3031 | | Ail OmpX-like protein | 1.5 | 0 |
| STM3202 | *ygiF* | putative cytoplasmic protein | 1.3 | 0 |
| STM3245 | *tdcA* | transcriptional activator | 1.5 | 0 |
| STM3521 | | putative ribonucleoprotein related protein | 1.3 | 0 |
| STM3658 | *yiaH* | putative Inner membrane protein | 1.2 | 2.33 |
| STM3688 | | putative cytoplasmic protein | 1.3 | 0 |
| STM3882 | *rbsA* | high-affinity D-ribose transport protein | 1.3 | 1.66 |
| STM4052 | | putative C4-dicarboxylate transport system | 1.2 | 0 |
| STM4230 | *malK* | maltose transport protein repressor | 1.3 | 2.33 |

[a]q-value indicate significant genes at a FDR of 2.33%

### 3.4.1.1 SGI1-influenced up-regulated genes in mid-log growth phase

Significant genes that are differentially expressed in mid-log growth phase at 1.8-fold or greater included 8 genes. Of these genes, 5 were up-regulated and 3 were down-regulated in the presence of SGI1. These differentially expressed genes are summarized in Table 8. Those up-regulated include *fdnI*, *fljA*, *fljB*, *ccmG*, and *ccmH* and those down regulated include STM0557, *yfdH*, and *fliC*.

Microarray analysis of the isogenic strains in mid-log growth phase revealed that *fljA* and *fljB* were up-regulated 4-fold and 15.2-fold, respectively, whereas *fliC* is down-regulated 3.2-fold in *S.* Typhimurium DT104 with SGI1. The expression of other *S.* Typhimurium DT104 genes involved in flagella biosynthesis and motility were not observed to be influenced by SGI1 in mid-log growth phase. FliB (phase 2 flagellin) is alternately expressed with FliC (phase 1 flagellin) on the flagellum shaft. Inversion of a DNA segment harbouring the *fljBA* promoter governs if the phase 1 or phase 2 flagellin is expressed. Orientation of the promoter in the transcriptional direction of *fljBA* induces expression of this operon. The *fljA* gene is coexpressed with *fljB*, and is the repressor of the phase 1 antigen (*fliC*) (55). The expression pattern of flagellar antigen genes observed from the microarray data indicate that the phase 2 FliB antigen is expressed over phase 1 FliC on flagella in *S.* Typhimurium DT104 harbouring SGI1.

## Table 8: Summary of SGI1-influenced genes more than 1.8-fold

| Gene | Locus | Function | [a]Fold change |
|---|---|---|---|
| *fdnI* | STM1568 | formate dehydrogenase-N gamma subunit | **1.8** |
| *fljA* | STM2770 | phase-1 flagellin repressor | **4** |
| *fljB* | STM2771 | phase 2 flagellin | **15.2** |
| *ccmH* | STM3812 | putative heme lyase subunit | **2.7** |
| *ccmG* | [b]STM3813/2248 | heme lyase disulfide oxidoreductase | **3.1** |
| *gtrC1* | STM0557 | inner membrane protein | **-3.2** |
| *yfdH /gtrB1* | STM0558 | glycosyl transferase | **-1.9** |
| *fliC* | STM1959 | phase 1 flagellar protein | **-3.2** |

[a]negative numbers denote down-regulation

[b]the *ccm* operon is duplicated on the LT2 genome where up-regulation of 3.1-fold was observed for both *ccmG* loci (STM3813 and STM2248 )

[c]names *gtrB1* and *gtrC1* proposed for STM0558 and STM0557, respectively by Bogomolnaya *et al.* (2008) (15)

Cytochrome c plays a role in both aerobic and anaerobic respiration in bacteria (29). The cytochrome c maturation operon in the *S.* Typhimurium genome consists of genes *ccmABCDEFGH* which are involved in the covalent attachment of haem to apocytochrome c (29). Mutations of genes in this operon resulted in reduced growth in iron limited environments and loss of siderophore production in other bacterial spp. (9, 29, 29, 130). Two copies of this 7.5 kb operon are found in the *S.* Typhimurium chromosome with 99% homology in the LT2 strain (29, 82). It is not known if S. Typhimurium DT104 harbours 2 copies of *ccm* genes since the genome is not currently annotated. Up-regulation of the haem lyase disulfide oxidoreductase gene *ccmG* and a putative haem lyase subunit *ccmH* in the presence of SGI1 was observed for both *ccm* loci. A 3.1-fold up-regulation for *ccmG* was detected on probesets for both operons, whereas 2.7-fold and 1.6-fold up-regulation was noted for STM3812 and STM2247, respectively. All other *ccm* genes were slightly up-regulated (1.2 to 1.8-fold) but this increase was not significant.

Formate serves as a primary electron donor during anaerobic respiration in the presence of nitrate. The formate dehydrogenase complex, encoded by *fdnGHI,* is involved in the electron transfer from formate to the electron acceptor nitrate (8). The gamma subunit of this complex (*fdnI*) was significantly up-regulated 1.8-fold. Slight up-regulation for *fdnGH* and nitrate reductase gene expression was noted, however it did not meet the statistical criteria.

### 3.4.1.2 SGI1-influenced down-regulated genes in mid-log growth phase

*fliC* (STM1959), STM0557, and *yfdH* (STM0558) were down-regulated in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. The gene *fliC* was discussed in section 3.4.1.1. The latter two are involved in the glucosylation of the O12 surface antigen of *S.* Typhimurium

Genes STM0557 – STM0559 are part of an operon recently termed *Salmonella* pathogenicity island 16 identified using Interpolated Variable Order Motifs (IVOMs), a computational method for detection of horizontally acquired DNA (123). Gene STM0557, a putative inner membrane protein and *yfdH* (STM0558), a putative glucosyl transferase were down-regulated 3.2-fold and 1.9-fold in *S.* Typhimurium DT104 harbouring SGI1, respectively. STM0559 (*rfbI*) of this operon, a glucosyl translocase, was down-regulated below the cut off at 1.7-fold. This operon is involved in form variation of the O12 antigen where it is converted into the serologically different O12-2 form by glucosylation of the galactose residue at the carbon 4 (C4) position (15). Since this loci shares homology and has similar function as *gtrAB* and *gtr (type)* found in *Shigella flexneri,* the names *gtrC1*, *gtrB1*, and *gtrC1* have proposed for STM0557, STM0558 (*yfdH*), and STM0559 (*rfbI*) as *gtrC1*, *gtrB1*, and *gtrC1*, respectively (5).

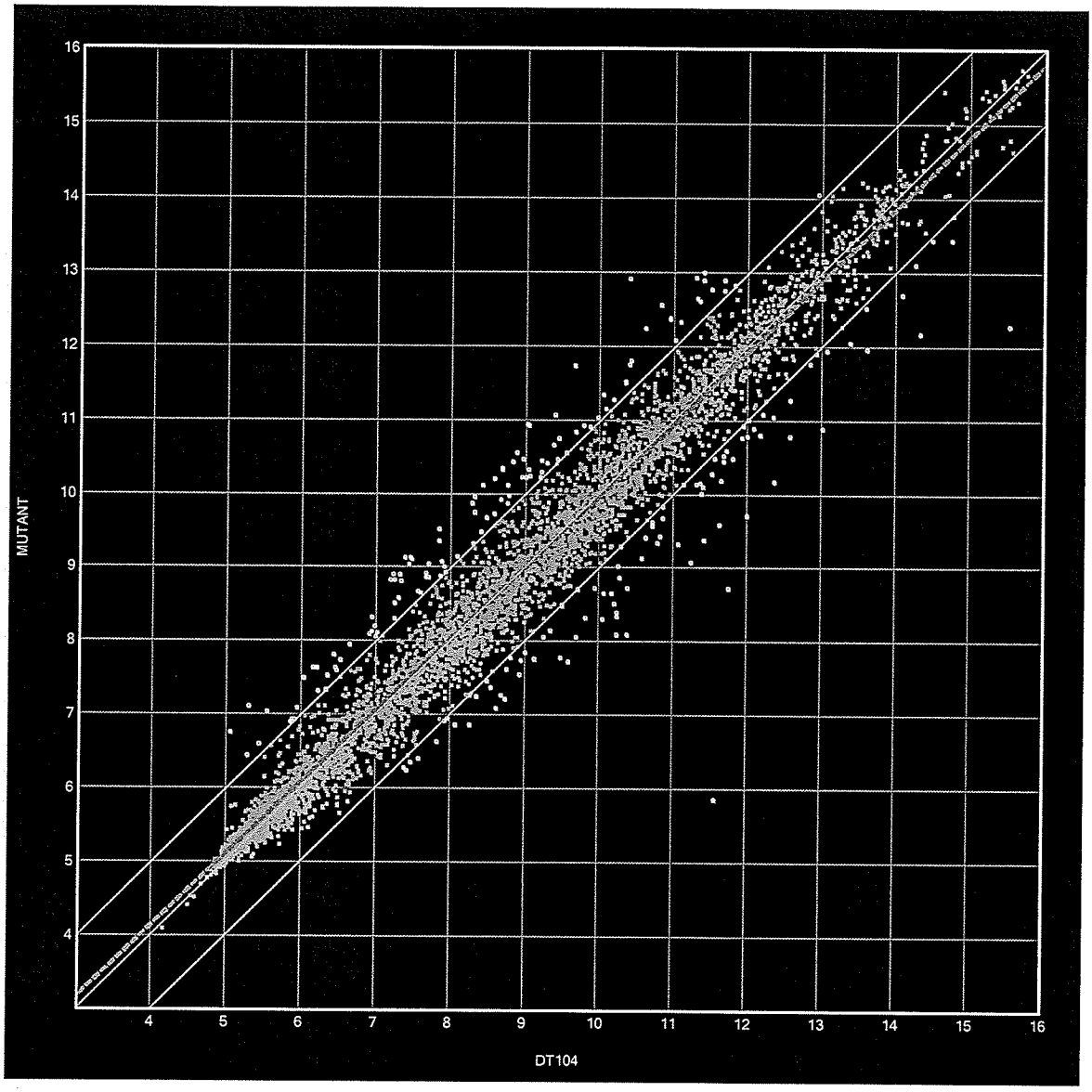
### 3.4.2 Early Stationary Phase Microarray Expression Data

The microarray used for early stationary phase expression assays contained probes for 4525 *S.* Typhimurium LT2 genes including 111 for pSLT. Microarray analysis with ArrayStar revealed that 206 genes were differentially expressed 2-fold or greater with 95% confidence in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. Of these genes, 94 were up-regulated and 112 were down-regulated with significance of $p \leq 0.05$. Of the 206 genes, 189 were significant at a level of $p \leq 0.005$. None of the pSLT genes represented on the microarray were differentially expressed. A plot generated in ArrayStar comparing gene expression of *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 is shown on Figure 5.

Genes with a variety of functions, including several that are hypothetical or of unknown function, were found to be differentially expressed in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. Up-regulated genes include those for uptake and usage of sugars, amino acid metabolism, and virulence genes. Several down-regulated genes were associated with phage, translation, and synthesis of carboxysome-like structures.

**Figure 5: A comparison of global gene expression between *S.* Typhimurium DT104 and the SGI1 isogenic mutant generated from $log_2$-transformed data using ArrayStar.**

*S.* Typhimurium DT104 is represented on the x-axis and *S.* Typhimurium DT104ΔSGI1 on the y-axis. The central line denotes a 1:1 expression ratio where the dashed line represents a best fit line for the microarray data ($R^2 = 0.9656$). The solid lines flanking the central line represent the 2-fold threshold boundaries.


MUTANT
DT104
16
15
14
13
12
11
10
9
8
7
6
5
4
4
5
6
7
8
9
10
11
12
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14
15
16

### 3.4.2.1 SGI1-Influenced Up-Regulated Genes in Early Stationary Phase

Aside from those associated with virulence, genes up-regulated in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 are involved in a broad range of functions. Several up-regulated genes were distributed around the *S.* Typhimurium DT104 chromosome and not concentrated in operons. Ten of these genes have putative functions, or are hypothetical ORFs (STM0551, STM1131, STM1132, STM1320, STM1328, STM1588, STM1701, STM1941, STM2915, STM2932, STM3772, STM4447, and STM4448). Other up-regulated genes are associated in the uptake of sugars like galactose, mannose, ribose and maltose as well as amino acid metabolism (2.00 to 8.08-fold). Multidrug efflux proteins including *ydgE* and *ydgF* encoding genes were also up-regulated in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1.

Microarray analysis in early stationary growth phase for the isogenic strains revealed that *fljA* and *fljB* were up-regulated 52.8-fold and 9.73-fold, respectively in *S.* Typhimurium DT104. These genes were also up-regulated in mid-log growth phase, however down-regulation of *fliC* expression (1.77-fold, $p < 0.05$) in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 was less than 2-fold in early stationary phase. In addition to the *fljBA* operon, *hin* and *fliE* were also up-regulated 2.37 and 2.27-fold, respectively. The *hin* gene encodes a recombinase involved in the inversion of the DNA segment harbouring the *fljBA* promoter (55). The gene *fliE* is involved in flagellar synthesis and forms a junction between the M-ring and *flgB*. Differential expression of other flagellar genes was not detected with the statistical criteria used. Up-regulated genes in *S.* Typhimurium DT104 harbouring SGI1 are summarized in Table 9.

**Table 9: List of SGI1-influenced up-regulated genes in early stationary phase**

| Gene Locus | Name | Gene Function | Fold | p val ue |
|---|---|---|---|---|
| STM0317 | *gpt* | xanthine phosphoribosyltransferase | 2.57 | 8.37E-08 |
| STM0439 | *cyoE* | protoheme IX farnesyltransferase | 2.16 | 0.0137 |
| STM0440 | *cyoD* | cytochrome o ubiquinol oxidase subunit IV | 2.28 | 0.0103 |
| STM0441 | *cyoC* | cytochrome o ubiquinol oxidase subunit III | 2.22 | 0.00514 |
| STM0442 | *cyoB* | cytochrome o ubiquinol oxidase subunit I | 2.11 | 0.00712 |
| STM0544 | *fimI* | fimbrial protein | 2.32 | NaN* |
| STM0545 | *fimC* | periplasmic chaperone | 2.62 | 4.19E-14 |
| STM0551 | | hypothetical protein | 2.26 | 9.13E-12 |
| STM0600 | *cstA* | carbon starvation protein | 2.02 | 0.0104 |
| STM0634 | *ybeF* | putative transcriptional regulator | 2.01 | 3.84E-08 |
| STM0662 | *gltL* | glutamate aspartate transporter | 3.36 | 0.000956 |
| STM0663 | *gltK* | glutamate aspartate transporter | 2.66 | 0.009 |
| STM0736 | *kgd/sucA* | 2-oxoglutarate dehydrogenase | 2.08 | 0.00836 |
| STM1131 | | putative outer membrane protein | 2.84 | 0.00154 |
| STM1132 | | putative sialic acid transporter | 4.87 | 0.0018 |
| STM1304 | *astA* | arginine succinyltransferase | 2.3 | 0.0116 |
| STM1305 | *astD* | aldehyde dehyrogenase | 2.24 | 0.00156 |
| STM1306 | *astB* | succinylarginine dihydrolase | 2.31 | 0.000145 |
| STM1320 | *ydjN* | kinase transporter-like protein | 2.45 | 3.72E-09 |
| STM1328 | | putative outer membrane protein | 3.1 | 4.03E-14 |
| STM1482 | *ydgF* | putative cationic transporter | 2.67 | 3.19E-10 |
| STM1483 | *ydgE* | putative cationic transporter | 2.28 | 3.87E-14 |
| STM1588 | *yncC* | putative regulatory protein | 2.14 | 1.06E-05 |
| STM1701 | *yciW* | putative cytoplasmic protein | 3.26 | 8.27E-10 |
| STM1802 | *dadX/alr* | alanine racemase | 2.45 | 0.000573 |
| STM1803 | *dadA* | D-amino acid dehydrogenase small subunit | 2.1 | 0.00512 |
| STM1909 | *argS* | arginyl-tRNA synthetase | 2.03 | 7.18E-05 |
| STM1941 | | putative inner membrane protein | 2.15 | 6.02E-08 |
| STM1968 | *fliE* | flagellar M-ring protein | 2.27 | 0.000137 |
| STM2027 | *cbiH* | precorrin-3B C17-methyltransferase | 2 | 0.0138 |
| STM2028 | *cbiG* | cobalamin biosynthesis protein CbiG | 2.01 | 0.00763 |
| STM2030 | *cbiT* | precorrin-8w decarboxylase | 2.06 | 0.00311 |
| STM2066 | *sopA* | secreted effector protein | 2 | 7.03E-10 |
| STM2080 | *udg* | 6-phosphogluconate dehydrogenase | 2.02 | 6.08E-06 |
| STM2188 | *mglC* | beta-methylgalactoside transporter inner membrane component | 3.5 | 0.00117 |
| STM2189 | *mglA* | methyl-galactoside transport protein | 2.59 | 0.0191 |
| STM2443 | *cysU* | thiosulfate transport protein | 2.28 | 0.000939 |
| STM2444 | *cysP* | thiosulfate transport protein | 2.18 | 1.71E-05 |
| STM2512 | *xseA* | exodeoxyribonuclease VII large subunit | 2.06 | 5.08E-07 |
| STM2770 | *fljA* | phase-1 flagellin repressor | 52.77 | 6.49E-14 |

| | | | | |
|---|---|---|---|---|
| STM2771 | *fljB* | flagellar biosynthesis protein | 9.73 | NaN* |
| STM2772 | *hin* | DNA-invertase Hin | 2.37 | 1.16E-09 |
| STM2781 | *virK* | virulence protein | 3.13 | 4.02E-09 |
| STM2782 | *mig-14* | putative transcriptional activator | 2.34 | 1.06E-07 |
| STM2792 | *gabT* | 4-aminobutyrate aminotransferase | 2.17 | 0.000573 |
| STM2868 | *orgC* | putative cytoplasmic protein | 2.21 | 7.18E-14 |
| STM2869 | *orgB* | needle complex export protein | 2.36 | 6.29E-14 |
| STM2870 | *orgA* | needle complex assembly protein | 2.18 | 2.19E-12 |
| STM2874 | *prgH* | needle complex inner membrane protein | 2.23 | 3.73E-14 |
| STM2876 | *hilA* | invasion protein transcriptional activator | 2.29 | 2.15E-12 |
| STM2887 | *spaS* | type III secretion protein | 2.17 | 2.50E-09 |
| STM2888 | *spaR* | needle complex export protein | 2.87 | 1.21E-08 |
| STM2889 | *spaQ* | needle complex export protein | 2.61 | 8.47E-09 |
| STM2890 | *spaP* | needle complex export protein | 2.03 | 2.26E-09 |
| STM2893 | *invI* | needle complex assembly protein | 2.32 | 8.71E-10 |
| STM2894 | *invC* | type III secretion system ATPase | 2.48 | 1.64E-09 |
| STM2897 | *invE* | invasion protein | 2.32 | 1.24E-11 |
| STM2898 | *invG* | outer membrane secretin precursor | 2.46 | 6.94E-14 |
| STM2899 | *invF* | invasion regulatory protein | 2.29 | 1.59E-13 |
| STM2915 | *ygbM* | putative endonuclease | 2.02 | 0.000599 |
| STM2932 | *ygbE* | putative inner membrane protein | 2.23 | 0.000024 |
| STM2933 | *cysC* | adenylylsulfate kinase | 2.61 | 8.47E-07 |
| STM2934 | *cysN* | sulfate adenylyltransferase subunit 1 | 2.52 | 8.63E-08 |
| STM2935 | *cysD* | sulfate adenylyltransferase subunit 2 | 2.62 | 6.94E-09 |
| STM2948 | *cysJ* | sulfite reductase beta subunit | 2.25 | 4.60E-07 |
| STM3513 | *malQ* | 4-alpha-glucanotransferase | 2.12 | 3.78E-09 |
| STM3628 | *dppC* | dipeptide transport protein 2 | 2.05 | 9.55E-08 |
| STM3693 | *lldR* | putative transcriptional regulator | 2.89 | 0.00737 |
| STM3694 | *lldD* | L-lactate dehydrogenase | 3.78 | 0.000502 |
| STM3767 | | putative cytoplasmic protein | 4.4 | 2.87E-08 |
| STM3768 | | putative selenocysteine synthase | 4.12 | 1.32E-08 |
| STM3769.S | | putative phosphotransferase system enzyme II | 2.76 | 2.72E-05 |
| STM3770 | | putative phosphotransferase system enzyme IIC | 3.36 | 0.000406 |
| STM3771 | | putative phosphotransferase system enzyme IIB | 2.73 | 0.00934 |
| STM3772 | | putative phosphotransferase system enzyme IIA | 2.39 | 0.0294 |
| STM3885 | *rbsK* | ribokinase | 3.16 | 1.71E-06 |
| STM3886 | *rbsR* | rbs operon transcriptional repressor | 4.39 | 3.80E-09 |
| STM3982 | *fadA* | acetyl-CoA acetyltransferase | 2.68 | 0.000048 |
| STM4063 | *sbp* | sulfate transport protein | 2.68 | 0.000179 |
| STM4227 | *malG* | maltose transport protein | 4.88 | 4.38E-14 |
| STM4228 | *malF* | maltose transport protein | 4.15 | 4.12E-07 |
| STM4229 | *malE* | periplasmic maltose-binding protein | 2.52 | 2.05E-11 |
| STM4230 | *malK* | maltose transport protein repressor | 4.57 | 7.05E-09 |

| | | | | |
|---|---|---|---|---|
| STM4231 | *lamB* | maltoporin precursor | 4.5 | 4.57E-14 |
| STM4232 | *malM* | periplasmic protein precursor | 8.08 | NaN* |
| STM4257 | *siiA* | hypothetical protein | 3.22 | 1.26E-06 |
| STM4258 | *siiB* | putative methyl-accepting chemotaxis protein | 3.66 | 1.71E-07 |
| STM4259 | *siiC* | putative ABC exporter outer membrane component | 2.03 | 0.000861 |
| STM4261 | *siiE* | putative inner membrane protein | 2.03 | 9.41E-07 |
| STM4262 | *siiF* | putative ABC-type bacteriocin lantibiotic exporter | 2.21 | 2.59E-07 |
| STM4445 | | dihydroorotase | 2.3 | 0.000986 |
| STM4446 | | putative selenocysteine synthase | 3.6 | 7.32E-08 |
| STM4447 | | putative periplasmic protein | 4.52 | 3.12E-10 |
| STM4448 | | putative phosphotransferase system mannitol fructose-specific IIA domain | 2.72 | 1.63E-10 |

* NaN denotes that Arraystar could not generate a p-value however an unpaired T-test using Microsoft Excel determined these genes to be significant ($p < 0.05$)

#### 3.4.2.1.1 SGI1-Influenced Virulence Gene Expression

In this project virulence genes were defined as those that were directly involved in any stage in *Salmonella* pathogenesis, and/or located on a PAI. In early stationary growth phase, 24 genes associated with *Salmonella* pathogenesis were up-regulated in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI and 3 were down-regulated. The differentially expressed virulence genes in *S.* Typhimurium DT104 are listed in Table 10. Up-regulated virulence genes included those from SPI1, SPI4 and some not found on PAIs. Down-regulated virulence genes include putative regulatory gene *orf408* (3.00-fold, $p = 3.6 \times 10^{-7}$), *mgtB* (2.23-fold, $p = 3.7 \times 10^{-8}$), and Mg2+ transporter *mgtC* (2.25-fold, $p = 1.0 \times 10^{-7}$). Gene *orf208* is found on SPI2 and *mgtBC* are found on SPI3 where both PAIs are associated with systemic infection and intracellular survival (62). Down-regulation of other genes associated with SPI2 or SPI3 was not observed.

SPI1 is involved in epithelial cell invasion and encodes 39 genes including regulatory genes, type three secretion system (TTSS) genes, translocon genes, and effector genes (42). The TTSS structural genes encode a membrane bound needle structure that secretes effector proteins into target cells. Fourteen SPI1 genes were up-regulated by a factor of 2-fold or greater (2.03 to 2.87-fold, $p \leq 10^{-8}$) in *S.* Typhimurium DT104 harbouring SGI1. These included 10 TTSS structural genes (*orgA*,*prgH*,*spaPQRS*, and *invICEG*), 2 virulence regulatory genes (*hilA* and *invF*), and those of unknown function (*orgBC*) (42). Low level up-regulation (1.5 to 2-fold) for 17 additional SPI1 genes was observed.

SopA is a virulence effector encoded elsewhere on the *Salmonella* genome and is translocated with SPI1 encoded effectors through the TTSS of SPI1. SopA plays a role in mucosal fluid accumulation and the PMN influx in *S.* Dublin and was found to colocalize with mitochondria of infected target cells (73, 128). The gene *sopA* is up-regulated 2-fold in *S.*Typhimurium DT104 harbouring SGI1.

SPI4 is comprised of 6 genes (*siiABCDEF*) where 5 were up-regulated 2-fold or more ($p \leq .0009$) in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. This PAI encodes a type one secretion system (TOSS) involved in the secretion of a non-fimbral adhesin (SiiE), which is up-regulated 2.03-fold in *S.* Typhimurium DT104. This adhesin was found to be induced by mucosal cell contact (MDCK polarized epithelial cell line) and is essential for adhesion in the polarized epithelial cells (MDCK cell line) (54). The TOSS is encoded by 3 genes (*siiCDF*) in which 2 of them, *siiC* and *siiF*, were observed up-regulated 2.03-fold, 2.21-fold, respectively. Genes, *siiA* that encodes a hypothetical protein, and *siiB* a putative methyl-accepting chemotaxis protein, were up-regulated 3.22-fold and 3.66-fold, respectively (54). The functions of these genes are unclear.

Up-regulated gene, *fimI* (2.32-fold) and *fimC* (2.62-fold) encode a fimbral protein and a periplasmic chaperone protein, respectively on the fimbriae operon (*fim*). Also up-regulated were the virulence protein *virK* which is homologous to virulence determinant of the same name in *Shigella* (3.13-fold) and *mig-14* (2.34-fold). *mig-14* is involved in *Salmonella* persistence in the spleen and mesenteric lymph nodes in mice. Both *mig-14* and *virK* are associated with resistance to cationic peptides and long term survival in murine macrophages *in vitro* (24).

## Table 10: Summary of SGI1-influenced up-regulated virulence genes

| | Gene Locus | Name | Gene Function | Fold Change | *p*-value |
|---|---|---|---|---|---|
| SPI1 | STM2868 | *orgC* | putative cytoplasmic protein | 2.21 | 7.18E-14 |
| | STM2869 | *orgB* | needle complex export protein | 2.36 | 6.29E-14 |
| | STM2870 | *orgA* | needle complex assembly protein | 2.18 | 2.19E-12 |
| | STM2874 | *prgH* | needle complex inner membrane protein | 2.23 | 3.73E-14 |
| | STM2876 | *hilA* | invasion protein transcriptional activator | 2.29 | 2.15E-12 |
| | STM2887 | *spaS* | type III secretion protein | 2.17 | 2.50E-09 |
| | STM2888 | *spaR* | needle complex export protein | 2.87 | 1.21E-08 |
| | STM2889 | *spaQ* | needle complex export protein | 2.61 | 8.47E-09 |
| | STM2890 | *spaP* | needle complex export protein | 2.03 | 2.26E-09 |
| | STM2893 | *invI* | needle complex assembly protein | 2.32 | 8.71E-10 |
| | STM2894 | *invC* | type III secretion system ATPase | 2.48 | 1.64E-09 |
| | STM2897 | *invE* | invasion protein | 2.32 | 1.24E-11 |
| | STM2898 | *invG* | outer membrane secretin precursor | 2.46 | 6.94E-14 |
| | STM2899 | *invF* | invasion regulatory protein | 2.29 | 1.59E-13 |
| [a]SPI4 | STM4257 | *siiA* | hypothetical protein | 3.22 | 1.26E-06 |
| | STM4258 | *siiB* | putative methyl-accepting chemotaxis protein | 3.66 | 1.71E-07 |
| | STM4259 | *siiC* | type one secretion system | 2.03 | 0.000861 |
| | STM4261 | *siiE* | adhesin | 2.03 | 9.41E-07 |
| | STM4262 | *siiF* | type onee scretion system | 2.21 | 2.59E-07 |
| other | STM0544 | *fimI* | fimbral protein | 2.32 | 0.0007 |
| | STM0545 | *fimC* | periplasmic chaperone | 2.62 | 4.19E-14 |
| | STM2066 | *sopA* | secreted effector protein | 2 | 7.03E-10 |
| | STM2781 | *virK* | virulence protein | 3.13 | 4.02E-09 |
| | STM2782 | *mig-14* | putative transcriptional regulator | 2.34 | 1.06E-07 |

[a]SPI4 names and functions of SPI4 genres proposed by Gerlach et al. (2007) (54)

### 3.4.2.2 SGI1-Influenced Down-Regulated Genes in Early Stationary Phase

There were 112 down-regulated genes *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 in early stationary phase. With the exception of 2 genes (*nanA* and *nanT*), which were down-regulated greater than 4-fold, all other genes were down-regulated 2 to 4-fold in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. Several down-regulated genes were clustered together in groups of similar function. Twenty-eight of these genes were associated with prophage elements. The *S.* Typhimurium LT2 chromosome contains 2 homologous Gifsy phage elements known as Gifsy-1 and Gifsy-2 (82). Down-regulation of 15 ORFs and 13 ORFs was observed for Gifsy-1 and Gifsy-2, respectively (2.02-fold to 3.34-fold), that are homologous to genes encoding phage tail assembly proteins, excisionase, exodeoxyribonuclease VIII, along with several hypothetical ORFs (82).

Genes involved in the formation of polyhedral bodies (PHB) or carboxysome like structures were also down-regulated. PHBs are organelles that are not well understood and are hypothesized to store toxic aldehydes or volatile nutrients are hypothesized functions (95). Propanediol and ethanolamine utilization operons are involved in formation of these structures (14, 95). Six propanediol usage genes (*pduACDEU*) including the propanediol usage regulator gene *pocR* were down-regulated in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 (2.06-fold to 2.45-fold). Three genes (*eutKLC*) on the ethanolamine operon were also down-regulated (2.02-fold to 2.29-fold). Propanediol usage as a carbon source or in PHB synthesis is dependant on cobalamin (vitamin D), where the biosynthesis operon is also positively regulated by PocR (13, 14). Genes (*cobTU* and *cbiP*) from the cobalamin biosynthesis operon were

observed to be down-regulated (2.08-fold to-2.47-fold) in *S.* Typhimurium DT104 harbouring SGI1influence and in contrast cobalamin genes *cbiHGT* were up-regulated 2-fold.

Other notable down-regulated genes included 3 virulence genes (described in section 3.4.2.1), 10 genes that encode ribosomal proteins, nitrate reductase genes *narJHGK* (2.0-fold to 3.24-fold), as well as *nanA* (5.67-fold) and *nanT* (4.23-fold) involved in sialic acid utilization. Excluding genes associated with Gifsy phage, down-regulation of 13 ORFs with hypothetical or putative functions was also observed (STM0907, STM1324, STM2240, STM2400, STM2740, STM2740.1N, STM2741, STM3237, STM3343, STM3512, STM3519, STM3814, STM4378, and STM4379).

Genes down-regulated in mid-log growth phase (*gtrC1*, *gtrB1* and *fliC* ) were not differentially expressed in early stationary phase in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 based on statistical criteria. However, slight down-regulation in STM0557 was observed. Down-regulated genes in *S.* Typhimurium DT104 harbouring SGI1 are summarized in Table 11.

**Table 11: List of SGI1-influenced down-regulated genes in early stationary phase**

| Gene Locus | Name | Gene function | Fold | *p* value |
|---|---|---|---|---|
| STM0066 | *carA* | carbamoyl-phosphate synthase small subunit | 2.44 | 7.59E-13 |
| STM0067 | *carB* | carbamoyl-phosphate synthase large subunit | 2.27 | 2.25E-13 |
| STM0178 | *yadI* | putative PTS enzyme | 2.55 | 5.09E-13 |
| STM0210 | *cdaR* | putative inner membrane protein | 2.11 | 3.68E-05 |
| STM0367 | *prpR* | prp operon regulator | 2.22 | 0.00189 |
| STM0853 | *bssR, yliH* | putative cytoplasmic protein | 2.46 | 2.07E-09 |
| STM0907 | | putative chitinase | 2.24 | 3.29E-10 |
| STM1006 | | Gifsy-2 prophage - excisionase | 2.81 | 1.19E-13 |
| STM1007 | | Gifsy-2 prophage - hypothetical protein | 2.57 | 9.14E-15 |
| STM1008.S | | Gifsy-2 prophage - hypothetical protein | 2.68 | 2.67E-15 |
| STM1009 | | Gifsy-2 prophage - exodeoxyribonuclease | 3.05 | 3.47E-19 |
| STM1010 | | Gifsy-2 prophage - hypothetical protein | 3.02 | 3.33E-15 |
| STM1010.1n | | Gifsy-2 prophage - hypothetical protein | 3.08 | 1.06E-17 |
| STM1011 | | Gifsy-2 prophage - hypothetical protein | 2.44 | 8.68E-11 |
| STM1013 | | Gifsy-2 prophage - probable regulatory protein | 3.18 | 2.47E-12 |
| STM1014 | | Gifsy-2 prophage - probable regulatory protein | 3.12 | 3.89E-09 |
| STM1015 | | Gifsy-2 prophage - probable regulatory protein | 3.18 | 8.72E-10 |
| STM1016 | | Gifsy-2 prophage - hypothetical protein | 2.78 | 8.27E-09 |
| STM1017 | | Gifsy-2 prophage - hypothetical protein | 2.33 | 7.06E-08 |
| STM1048.1N | | Gifsy-2 prophage - hypothetical protein | 2.06 | 1.17E-07 |
| STM1071 | *sulA* | cell division inhibitor | 2.38 | 6.60E-17 |
| STM1251 | | putative molecular chaperone | 2.4 | 0.000403 |
| STM1324 | | putative cytoplasmic protein | 2.36 | 9.12E-13 |
| STM1349 | *pps* | phosphoenolpyruvate synthase | 3.76 | 0.00345 |
| STM1382 | *orf408* | SPI2 - putative regulatory protein | 2.99 | 3.63E-07 |
| STM1612 | | putative cellulase protein | 2.05 | 8.50E-08 |
| STM1613 | | putative PTS system enzymeIIB component | 2.13 | 1.81E-05 |
| STM1633 | | putative periplasmic binding protein | 2.02 | 3.96E-10 |
| STM1762 | *narJ* | nitrate reductase 1 delta subunit | 2.01 | 2.31E-05 |
| STM1763 | *narH* | nitrate reductase 1 beta subunit | 2.26 | 4.55E-08 |
| STM1764 | *narG* | nitrate reductase 1 alpha subunit | 2.4 | 2.69E-11 |
| STM1765 | *narK* | nitrite extrusion protein | 3.24 | 3.02E-11 |
| STM2016 | *cobT* | nicotinate-nucleotide--dimethylbenzimidazolephosphoribosyltransferase | 2.47 | 4.21E-06 |
| STM2018 | *cobU* | adenosylcobinamide kinase | 2.3 | 0.000142 |
| STM2019 | *cbiP* | cobyric acid synthase | 2.08 | 0.0021 |
| STM2036 | *pocR* | transcriptional regulator | 2.13 | 2.08E-06 |
| STM2038 | *pduA* | polyhedral body protein | 2.45 | 0.000598 |
| STM2040 | *pduC* | propanediol dehydratase large subunit | 2.29 | 0.0078 |

| | | | | |
|---|---|---|---|---|
| STM2041 | *pduD* | propanediol dehydratase medium subunit | 2.33 | 0.00781 |
| STM2042 | *pduE* | propanediol dehydratase small subunit | 2.15 | 0.00364 |
| STM2055 | *pduU* | polyhedral body protein | 2.06 | 3.35E-06 |
| STM2088 | *rfbX* | putative O-antigen transferase | 2.06 | 1.10E-09 |
| STM2240 | | putative cytoplasmic protein | 2.08 | 2.63E-12 |
| STM2257 | *napH* | quinol dehydrogenase membrane component | 2.18 | 8.64E-08 |
| STM2316.S | *nuoN* | NADH dehydrogenase subunit N | 2.11 | 6.31E-10 |
| STM2398 | *pgtC* | phosphoglycerate transport regulatory protein precursor | 2.15 | 2.17E-10 |
| STM2399 | *pgtP* | transporter | 3 | 0.00292 |
| STM2400 | | putative inner membrane protein | 2.08 | 3.60E-10 |
| STM2455 | *eutK* | putative carboxysome structural protein | 2.03 | 1.19E-12 |
| STM2456 | *eutL* | putative carboxysome structural protein | 2.24 | 1.92E-07 |
| STM2457 | *eutC* | ethanolamine ammonia-lyase small subunit | 2.02 | 1.73E-05 |
| STM2560 | *yjdL* | putative di- tripeptide transport protein | 2.29 | 0.0235 |
| STM2586 | | Gifsy-1 prophage - phage tail assembly-like protein | 2.19 | 1.62E-09 |
| STM2587 | | Gifsy-1 prophage - phage tail assembly-like protein | 2.37 | 3.62E-10 |
| STM2588 | | Gifsy-1 prophage - tail fiber-like protein | 2.02 | 7.06E-09 |
| STM2623 | | Gifsy-1 prophage - hypothetical protein | 2.61 | 2.44E-08 |
| STM2624 | | Gifsy-1 prophage - hypothetical protein | 2.67 | 4.24E-09 |
| STM2625 | | Gifsy-1 prophage - DNA replication protein DnaC | 3.12 | 2.17E-10 |
| STM2626 | | Gifsy-1 prophage - replication protein 15-like | 3.05 | 5.02E-09 |
| STM2627 | | Gifsy-1 prophage - cI-like protein | 3.34 | 1.12E-11 |
| STM2629 | | Gifsy-1 prophage - hypothetical protein | 2.28 | 2.18E-10 |
| STM2630 | | Gifsy-1 prophage - hypothetical protein | 3.16 | 1.68E-17 |
| STM2631 | | Gifsy-1 prophage - hypothetical protein | 3.14 | 8.20E-17 |
| STM2632 | | Gifsy-1 prophage - exodeoxyribonuclease VIII-like protein | 3.11 | 9.49E-19 |
| STM2633.S | | Gifsy-1 prophage - enterohemolysin 1-like protein | 2.72 | 4.63E-17 |
| STM2634 | | Gifsy-1 prophage - putative cytoplasmic protein | 2.63 | 1.18E-14 |
| STM2635 | | Gifsy-1 prophage - excisionase-like protein | 2.33 | 5.55E-13 |
| STM2740 | | integrase-like protein | 2 | 5.74E-12 |
| STM2740.1N | | hypothetical protein | 2 | 2.62E-10 |
| STM2741 | *nadB* | putative periplasmic protein | 2.1 | 1.87E-08 |
| STM2759 | | putative dipeptide oligopeptide nickel ABC-type transport system periplasmic component | 2.15 | 3.05E-07 |
| STM3013 | *lysA* | diaminopimelate decarboxylase | 2.09 | 0.0872 |
| STM3031 | | Ail OmpX-like protein | 2.82 | 3.40E-12 |
| STM3134 | | putative permease | 3.17 | 3.06E-09 |
| STM3197 | *glgS* | glycogen synthesis protein GlgS | 3.15 | 6.11E-09 |
| STM3237 | *yhaL* | putative cytoplasmic protein | 2.17 | 9.40E-08 |
| STM3244 | *tcdB* | threonine dehydratase | 2.29 | 0.0119 |
| STM3245 | *tcdA* | transcriptional activator | 3.23 | 8.48E-06 |
| STM3338 | *nanT* | putative sialic acid transporter | 4.23 | 2.05E-05 |

| | | | | |
|---|---|---|---|---|
| STM3339 | *nanA* | N-acetylneuraminate lyase | 5.69 | 3.14E-07 |
| STM3343 | | putative cytoplasmic protein | 2.24 | 2.87E-09 |
| STM3431 | *rpsQ* | 30S ribosomal protein S17 | 2.8 | 2.78E-10 |
| STM3432 | *rpmC* | 50S ribosomal protein L29 | 3.66 | 1.11E-11 |
| STM3433 | *rplP* | 50S ribosomal protein L16 | 3.13 | 4.31E-12 |
| STM3434 | *rpsC* | 30S ribosomal protein S3 | 3.35 | 4.70E-10 |
| STM3435 | *rplV* | 50S ribosomal protein L22 | 3.02 | 1.16E-09 |
| STM3436 | *rpsS* | 30S ribosomal protein S19 | 2.82 | 6.23E-09 |
| STM3437 | *rplB* | 50S ribosomal protein L2 | 3.11 | 6.04E-10 |
| STM3438 | *rplW* | 50S ribosomal protein L23 | 2.41 | 1.78E-08 |
| STM3440 | *rplC* | 50S ribosomal protein L3 | 2.12 | 4.45E-08 |
| STM3441 | *rpsJ,nusE* | 30S ribosomal protein S10 | 2.23 | 3.72E-06 |
| STM3505 | *feoA* | ferrous iron transport protein A | 2.02 | 1.48E-10 |
| STM3519 | *rtcB* | putative cytoplasmic protein | 2.68 | 4.42E-19 |
| STM3521 | | putative ribonucleoprotein related-protein | 3.51 | 1.49E-18 |
| STM3614 | *dctA* | putative diguanylate cyclase phosphodiesterase | 2.53 | 0.0036 |
| STM3763 | *mgtB* | SPI3 - Mg2+ transporter | 2.23 | 3.70E-08 |
| STM3764 | *mgtC* | SPI3 - Mg2+ transport protein | 2.25 | 1.01E-07 |
| STM3834 | | putative transcriptional regulator | 2.23 | 5.61E-08 |
| STM3877 | *asnA* | asparagine synthetase AsnA | 2.77 | 0.0068 |
| STM3965 | *metE* | 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase | 2.4 | 4.30E-09 |
| STM4073 | *ydeW* | putative transcriptional repressor | 2.42 | 5.58E-06 |
| STM4074 | *ego* | putative ABC-type aldose transport system ATPase component | 3.2 | 4.13E-06 |
| STM4075 | *ydeY* | putative sugar transport protein | 2.22 | 0.00377 |
| STM4112 | *frwC* | PTS system fructose-like IIC component | 2.84 | 2.21E-07 |
| STM4172 | *zraP* | zinc-resistance associated protein | 2.6 | 6.90E-08 |
| STM4277 | *nrfA* | nitrite reductase periplasmic cytochrome c552 | 2.28 | 3.74E-10 |
| STM4297 | *melR* | melibiose operon regulator | 2.05 | 3.46E-07 |
| STM4378 | *yjfN* | putative inner membrane protein | 2.17 | 8.07E-09 |
| STM4379 | *yjfO* | putative lipoprotein | 2.31 | 8.37E-09 |
| STM4423 | | putative DNA-binding protein | 2.29 | 1.45E-10 |
| STM4534 | | putative transcriptional regulator | 2.1 | 1.38E-09 |
| STM4535 | | putative PTS permease | 2.27 | 0.00825 |
| STM4540.S | | putative glucosamine-fructose-6-phosphate aminotransferase | 2.57 | 0.0396 |

### 3.5 Reverse Transcriptase Quantitative PCR (RT-qPCR) Validation

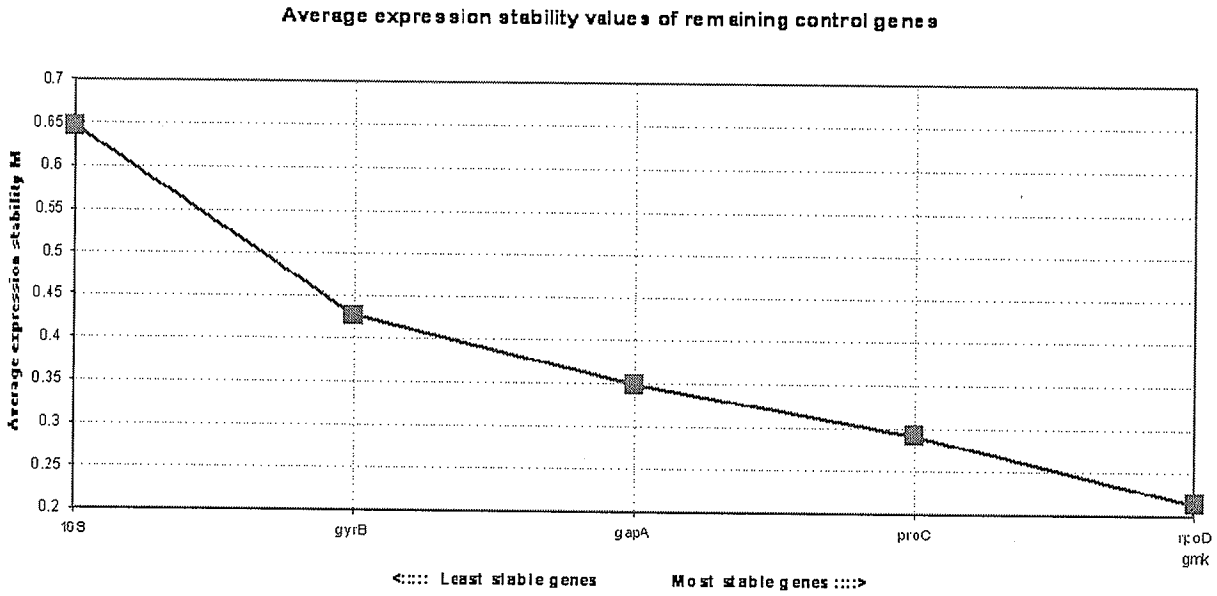
In mid-log phase and early stationary phase, 8 genes and 206 genes were found to be differentially expressed using microarray analysis, respectively. RT-qPCR analysis for validation of SGI1 influenced genes was done with an independently grown culture than those used for microarray analysis. Since very few genes were found differentially expressed by microarray analysis in mid-log phase, RT-qPCR expression assays were run for all 8 genes for confirmation. A sample of 20 of the 206 differentially expressed early stationary phase genes was selected for RT-qPCR validation, with a special focus on those associated with *Salmonella* virulence

#### 3.5.1 Housekeeping Genes

Quantified expression for a target gene for each sample is typically normalized by that of a housekeeping gene before comparing relative expression between samples. In this project the GeNorm software was used to calculate normalization factors for each sample based on expression quantified for 2 housekeeping genes (120). Normalization to 2 housekeeping genes is less susceptible to error since absolute expression of test genes are not standardized solely to the expression of a single gene. The stability of 6 housekeeping genes was assessed in RT-qPCR assays using this program in mid-log and early stationary growth phase. Out of the six tested housekeeping genes guanylate kinase (*gmk*) and DNA gyrase subunit B (*gyrB*) expression was found most stable in mid-log growth phase. RNA polymerase sigma factor (*rpoD)* and *gmk* were found most stable in early stationary phase, as shown in Figure 6.

**Figure 6: GeNorm output for the selection of housekeeping genes used in RT-qPCR assays for early stationary phase samples.**

Relative expression stability of the 6 tested housekeeping genes as determined using GeNorm. Smaller average expression stability values (M) (y axis) indicate that housekeeping genes (*gmk* and *rpoD*) have the most stable expression, and were used for calculation of a normalization factor for each sample. Housekeeping genes in order of decreasing in stability are located on the x axis. The same analysis was used to determine that *gmk* and *gyrB* were the most stable housekeeping genes in mid-log phase.


Average expression stability values of remaining control genes
Average expression stability M
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
16S
gyrB
gapA
proC
rpoD
gmk
<:::: Least stable genes
Most stable genes ::::>

### 3.5.2 Mid-Log Phase RT-qPCR Validation

Confirmatory RT-qPCR assays were done for all 8 genes found to be differentially expressed at least 1.8-fold according to microarray data using samples cultured and processed independently of those for the microarray experiments. The results for the RT-qPCR assay in comparison to the microarray data are summarized in Table 12. Of the 8 genes tested, differential expression of 3 genes was not validated in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. Genes *ccmG*, *ccmH* and *fdnI,* all shown to be up-regulated 3.1, 2.7, and 1.8-fold, respectively by microarray analysis were only found to be up-regulated 1.3, 1.4, and 1.2-fold, respectively using RT-qPCR and the changes were insignificant ($p > 0.1$).

The up-regulation of *fljA* and *fljB* and the down-regulation of *fliC*, *gtrB1* and *gtrC1* were validated in *S.* Typhimurium DT104 harbouring SGI1 using RT-qPCR. Down-regulation of *fljC* by a factor of 3-fold in *S.* Typhimurium DT014 relative to *S.* Typhimurium DT104ΔSGI1 was confirmed. The difference in expression between the isogenic strains (fold change) was observed with a greater magnitude for all genes confirmed by RT-qPCR than the microarrays, with the exception of *fliC*. The magnitude of up-regulation for *fljA* and *fljB* observed using RT-qPCR was considerably higher than up-regulation observed using microarray analysis. Genes, *fljA* and *fljB* were found to be 62.3 and 103.6-fold with RT-qPCR compared to 4 and 15.2-fold from microarray analysis, respectively.

## Table 12: RT-qPCR confirmation for mid-log phase

| Gene | Locus | Function | [a]Fold Change | *p*-value | Array Data (fold change) |
|---|---|---|---|---|---|
| *fdnI* | STM1568 | formate dehydrogenase-N gamma subunit | **1.2** | 0.100881 | **1.8** |
| *fljA* | STM2770 | phase-1 flagellin repressor | **62.3** | 0.004758 | **4** |
| *fljB* | STM2771 | phase 2 flagellin | **103.6** | 0.000118 | **15.2** |
| *ccmH* | STM3812 | putative heme lyase subunit | **1.4** | 0.474328 | **2.7** |
| *ccmG* | STM3813/2248 | heme lyase disulfide oxidoreductase | **1.3** | 0.462274 | **3.1** |
| *gtrCI* | STM0557 | inner membrane protein | **-5.7** | 0.012803 | **-3.2** |
| *yfdH /gtrBI* | STM0558 | glycosyl transferase | **-3** | 0.000501 | **-1.9** |
| *fliC* | STM1959 | phase 1 flagellar protein | **-3** | 0.000647 | **-3.2** |

[a]negative numbers denote down-regulation

### 3.5.3 Early Stationary Phase RT-qPCR Validation

Microarray analysis revealed that 206 ORFs were found differentially expressed 2-fold or greater *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 in early stationary phase. Of these ORFs, 20 were selected for RT-qPCR validation with a special focus on but not limited to genes associated with virulence.

Up-regulated virulence genes selected for RT-qPCR confirmation were the fimbriae operon genes *fimI*, *fimC*, secreted effector *sopA*, and virulence protein *virK*. Virulence regulator *hilA* and invasion protein encoding *invE* were selected for RT-qPCR for validation of SPI1 up-regulation. Genes, *siiB* and *siiF* were also evaluated for validation of SPI4 up-regulation. Selected genes that are not classical virulence factors included STM1132, *ydgF*, *fljA* and *fljB*. Significant ($p < 0.05$) up-regulation of 8 of these genes was confirmed by RT-qPCR. Data for RT-qPCR and microarray expression analyses for comparison are summarized on Table 13 for the 12 up-regulated genes selected for confirmation.

**Table 13: RT-qPCR confirmation for up-regulated genes from early stationary phase microarray data**

| Gene Locus | Name | Function | Fold change | *p*- value | Microarray Result |
|---|---|---|---|---|---|
| STM1482 | *ydgF* | Multi-drug efflux | 2.3 | 0.0071 | 2.7 |
| STM2066 | *sopA* | Virulence | 3.2 | 1.37E-04 | 2 |
| STM2770 | *fljA* | FliC repressor | 37.5 | 3.64E-07 | 52.8 |
| STM2771 | *fljB* | Phase 2 flagellin | 28.4 | 8.94E-06 | 9.7 |
| STM2781 | *virK* | Virulence | 3.1 | 6.79E-04 | 3.1 |
| STM2876 | *hilA* | Virulence (SPI1) | 2.6 | 3.74E-05 | 2.3 |
| STM2897 | *invE* | Virulence (SPI1) | 1.9 | 1.91E-04 | 2.3 |
| STM4258 | *siiB* | Virulence (SPI4) | 2.8 | 1.60E-08 | 3.7 |
| STM0544 | *fimI* | Virulence | -1.6 | 0.0023 | 2.3 |
| STM0545 | *fimC* | Virulence | -1.5 | 1.84E-04 | 2.6 |
| STM1132 | | Putative | -5.5 | 0.1347 | 4.9 |
| STM4262 | *siiF* | Virulence (SPI4) | -1.2 | 0.5129 | 2.2 |

Up-regulation of PAI independent virulence genes *sopA* and *virK* was confirmed with fold change values of 3.18 and 3.13, respectively. Since up-regulation of *virK* was validated, the adjacent *mig-14* gene is most likely up-regulated as well. Selected SPI1 genes *hilA* and *invE* were up-regulated 2.6-fold and 1.93-fold, respectively. Up-regulation of the latter gene observed with RT-qPCR is less than 2-fold, however was significant ($p < 0.0002$) and close to 2.32-fold observed in microarray analysis. These results suggest the up-regulation of all other SPI1 genes observed with microarray analysis is likely real. The SPI4 gene *siiB* was observed to be up-regulated 2.76-fold, however RT-qPCR detected no difference in expression for *siiF* (-1.2-fold, $p = 0.51$). Up-regulation of multidrug efflux gene *ydgF* (2.67-fold) was validated. With the exception of *siiF* and *fljB*, the magnitude of differential expression (fold change) for the above genes in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 measured by microarray analysis and RT-qPCR were relatively similar.

The up-regulation of the *fljA* repressor of phase 1 flagellin, and phase 2 flagellar antigen *fljB* were confirmed where expression was increased 37.50-fold and 28.43-fold in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1, respectively. Up-regulation of *fimI*, *fimC*, *siiF*, and STM1132 observed in microarray analysis was not confirmed with RT-qPCR. In contrast, down-regulation of these genes 1.63-fold, 1.55-fold and 5.5-fold, respectively was observed in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1. Down-regulation of the *fim* genes was minimal, however significant ($p < 0.002$).

Genes down-regulated in microarray analysis totaled 112. The 8 genes selected for independent validation using RT-qPCR were the SPI2 gene *ORF208*, SPI3 gene *mgtC*, sialic acid usage gene *nanA*, biofilm suppressor regulator *bssR*, the *tcd* operon transcriptional activator *tcdA*, 50s ribosomal protein L29 encoding gene *(rpmC)*, putative ATP binding protein encoding gene (*ego*), and putative transcriptional regulator STM4534. Data for RT-qPCR and microarray expression analyses for comparison are summarized on Table 14 for the 8 down-regulated genes selected for confirmation.

Down-regulation of 6 of the 8 selected genes was confirmed in *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1with RT-qPCR. These genes were *bssR* (3.23-fold), *orf208* (7.01-fold), *tcdA* (4.33-fold), *mgtC* (2.7-fold), *ego* (6.74-fold), and STM4534 (4.8-fold). The fold change magnitude was observed to be greater for RT-qPCR compared to that observed from microarray analysis for these genes. A 4.60-fold down-regulation was observed for *nanA* with RT-qPCR, however was not significant ($p = 0.06$). Down-regulation of *rpmC* was not confirmed (fold change ~1, $p = 0.50$) with RT-qPCR.

Low-level up-regulation (< 1.8-fold) of *ydgEF*, and *tcdA* and down-regulation of *fim* genes was noted for *S.* Typhimurium DT104 harbouring SGI1 in mid-log phase as observed in Tables 6 and 7. RT-qPCR for *ydgF*, *tcdA*, and *fimC* was also conducted on mid-log samples since they were observed with differential expression in early stationary phase. Differential expression of these genes *S.* Typhimurium DT104 relative to *S.* Typhimurium DT104ΔSGI1 in mid-log phase was not confirmed.

**Table 14: RT-qPCR confirmation for down-regulated genes from early stationary phase microarray data**

| Gene Locus | Name | Function | Fold change | *p*-value | Microarray Result |
|---|---|---|---|---|---|
| STM0853 | *bssR (yliH)* | Biofilm repressor | -3.2 | 0.0436 | -2.5 |
| STM1382 | *orf408* | Virulence (SPI2) | -7 | 3.79E-04 | -3 |
| STM3245 | *tcdA* | Transcriptional Activator | -4.3 | 8.36E-05 | -3.2 |
| STM3764 | *mgtC* | Virulence | -2.7 | 0.008 | -2.2 |
| STM4074 | *ego* | ATP binding protein | -6.7 | 0.0017 | -3.2 |
| STM4534 | | Transcriptional Activator | -4.8 | 0.0012 | -2.1 |
| STM3339 | *nanA* | Sialic acid usage | -4.6 | 0.0633 | -5.7 |
| STM3432 | *rpmC* | Ribosomal protein | 1.1 | 0.5046 | -3.7 |

## 4 Discussion

DT104 was a once rare strain of *S.* Typhimurium which emerged in the 1980's with an MDR (ACSSuT R-type) phenotype and is now disseminated worldwide. MDR DT104 are suspected to be hypervirulent since infections were associated with increased morbidity and mortality as compared to those with susceptible *Salmonella* in both humans and cattle (56, 61, 81, 98, 121). Ineffective antibiotic treatments may explain increased morbidity of MDR DT104, however, hypervirulence of this phagetype may be attributed to the presence of SGI1 (20, 85, 86).

Typical SGI1 contains 44 ORFs including those conferring the ACSSuT R-type in addition to ORFs with unknown or regulatory functions, that may influence virulence or fitness genes on the *S.* Typhimurium DT104 chromosome (20). SGI1 is suspected to be spread by horizontal transfer, where there is concern that other organisms such as *Shigella* spp., *Vibrio* spp. that acquire SGI1 may display hypervirulent properties attributed to MDR DT104 (33, 37).

Studies have been conducted to assess if MDR DT104 are hypervirulent relative to susceptible strains with inconclusive results (4, 25, 28). Recent studies have shown that MDR DT104 harbouring SGI1 were transiently hyperinvasive *in vitro* and *in vivo* after exposure to RPz that are commonly found in cattle (101). To determine if SGI1 effects global gene expression, microarray analysis using an isogenic strain pair of *S.* Typhimurium LT2 revealed that 36 genes were found differentially expressed in the presence of SGI1 in mid-log growth phase (57). No classical virulence genes were observed influenced by SGI1 in *S.* Typhimurium LT2 which may have resulted from the

growth phase used, or the fact that this strain is avirulent due to a non-functional *rpoS* gene (57, 113).

Virulence genes involved in invasion have been reported to be induced upon entrance into early stationary phase in *S.* Typhimurium (112). In this project the influence of SGI1 on fully virulent *S.* Typhimurium DT104 was assessed in both mid-log and early stationary phases using an isogenic strain pair. SGI1 was indeed observed to influence gene expression of *S.* Typhimurium DT104 in planktonic cultures grown in rich BHI broth. In early stationary growth phase we found that virulence genes located on SPI1 and SPI4 were up-regulated in the presence of SGI1, which is evidence in support of the hypothesis that the MDR DT104 hypervirulence is attributed to strains harbouring SGI1.

### 4.1 Growth Curves

Growth rates are often used as a measure of bacterial fitness. Bacteria that become drug resistance by mutation or horizontal acquisition typically exhibit decreased growth rates and are often at a competitive disadvantage relative to susceptible strains in culture media and persistance in hosts (11, 132). Organisms have been found to 'adapt' or undergo compensatory mutations in order to maintain drug resistance elements (11, 18, 132). Growth rates and host persistance for several adapted strains have been reported to be partially or completely restored to competitive levels with susceptible strains, even in the absence of antibiotic selective pressure (11, 18, 107, 132).

Growth rates were similar between *S.* Typhiumurium DT104 harbouring SGI1 and *S.* Typhimurium DT104ΔSGI1 in BHI broth with no antibiotics. Golding *et al.*

(2007) have also reported similar growth rates for SGI1 isogenic strains of *S.* Typhimurium LT2 (57). Based on these findings using isogenic strains, the presence of the 43 kb chromosomal element SGI1 was not observed to influence growth rates. This indicates that organisms that acquire SGI1 may incur little or no fitness cost and therefore may be maintained in the absence of antibiotic pressure.

### 4.2 Expression in Mid-Log Growth Phase

The transcriptome of *S.* Typhimurium DT104 was not observed to be largely influenced by SGI1in mid-log growth phase. Only 5 genes were found to be differentially expressed in mid-log growth phase in the presence of SGI1. These genes are involved in the alteration of the surface (O) and flagellar (H) antigens.

A phase variation mechanism involving the orientation of an invertible DNA segment harbouring the *fliBA* promoter controls the expression of either FliC (phase 1) or FliB (phase 2) antigen on the flagella of *Salmonella*. When the *fljBA* promoter is oriented in the opposite direction of *fljBA* transcription (*fljAB*-OFF), phase 1 (FliC) flagellin is expressed. If the *fljBA* promoter is in orientation of the *fljBA* operon (*fljAB*-ON), *fljB* is expressed and *fljA* which is coexpressed with *fljB* represses the phase 1 flagellin. Repression of phase 1 flagellin (*fliC*) by FliA was observed to be from a post transcriptional mechanism in addition to gene expression. With the use of transcriptional and translational *fliC-lacZ* fusions, Bonifield *et al.* (2003) reported that in *S. enterica* FliA represses *fliC* gene expression and translation 5-fold and 200-fold, respectively (16). In mid-log growth phase the *fljBA* operon was up-regulated (60 and 100-fold) and *fliC* was down-regulated 3-fold in DT104.

The magnitude of *fljBA* up-regulation suggests that the FliB antigen is dominant over FliC on flagella of *S.* Typhimurium DT104 harbouring SGI1 in mid-log and early stationary growth phase. The moderate down-regulation of *fliC* gene expression in mid-log phase in the presence of SGI1 was similar to the level of gene repression by *fljA* observed in *fljBA*-ON cells by Bonifield *et al.* (2003) (16). This can be explained by post translational repression in addition to transcriptional repression by FliA as reported by Bonifield *et al.* (2003) (16).

Flagellar (H) antigens are potent immune system sensitizers and are recognized by flagella specific TLR-5 receptors found on host mucosal immune cells (65). The expression of the alternate phase 2 flagellar antigen may provide temporary immune evasion during infection. Expression of flagella synthesis genes on the *flg*, *flh*, *fli*, and *flj* operon were not modulated by SGI1 with the exception up-regulation of the flagellar hook-basal body encoding *fliE* and DNA invertase *hin* involved in inversion of the *fljBA* promoter in early stationary phase. Based on gene expression studies conducted in this thesis, SGI1 influenced the type of antigen expressed but not synthesis of flagella.

The LPS is a component of the outer membrane of Gram-negative bacteria such as *Salmonella* and contains the surface O antigen. The O antigen consists of repeating oligosaccharide units of variable length. In addition to flagella, the O surface antigen is also immunologically important and may be altered to evade recognition by the host (75). The O12 antigen found in *S.* Typhimurium consists of a chain of repeating trisaccharide units [→2)-D-mannose-(1→4)-L-rhabdanose-(1→3)-D-galactose-(1→] that can vary in length. The *gtr* locus (STM0557-0559) of *S.* Typhimurium was implicated in the modification or 'form variation' of this antigen by facilitating the addition of glucose to

specific sites on the O12 oligosaccharide chain (15). The addition of a glucose residue or 'glucosylation' of O12 units at the carbon 4 position of the galactose residue gives rise to the form variant O12-2 (15, 87, 103). Glucosylation of this antigen occurs non-stoichiometrically, where the LPS of *S.* Typhimurium may vary in O12 and O12-2 content (15).

Genes *gtrC1* (STM0557), *gtrB1* (STM0558) and *gtrA1* (STM0559) form an operon on the recently identified pathogenicity island 16 (SPI16) (123). These genes are involved in the conversion of O12 antigen into the serologically different form variant O12-2. Bogomolnaya *et al.* (2008) recently described that generation of the O12-2 variant is essential in long term persistence of *Salmonella* in mice (15). SPI16 genes, *gtrC1* (5.7-fold) encoding an inner membrane protein, and *gtrB1* (3.2-fold) encoding a glucosyl transferase were down-regulated in *S.* Typhimurium DT104 harbouring SGI1. The gene *gtrA1* did not exhibit decreased expression based on selection criteria, however, it did show a trend to be down-regulated. In future studies, the expression of *gtrA1* in the *S.* Typhimurium DT104 isogenic strain should be evaluated using RT-qPCR. Down-regulation of this operon was not observed for *S.* Typhimurium DT104 harbouring SGI1 in early stationary growth phase or in previous experiments with *S.* Typhimurium LT2 harbouring SGI1 (57).

An O antigen glucosylation operon *gtrABV* orthologous to the *gtr* loci in *S.* Typhimurium was found in *Shigella flexneri* (5). *S. flexneri* which were unable to glucosylate the O antigen were found attenuated in epithelial cell invasion *in vitro* whereas this was not the case for *S.* Typhimurium (15, 126). The O12 antigen of *S.* Typhimurium deleted in *gtrA1* (STM0559) was found to be heavily glucosylated and thus

contained a higher percentage of O12-2. This mutant had a decreased ability to invade epithelial cells and murine macrophages *in vitro*. In addition, these highly glucosylated mutants displayed reduced intracellular replication in murine macrophages relative to the wild type *in vitro*. The *gtrC1* gene was found to be essential for long term persistence of *S.* Typhimurium in mice. *S.* Typhimurium deleted in *gtrC1* (STM0557) were unable to glucosylate and therefore were completely comprised of O12 antigen. The unglucosylated *gtrC1* mutant was indistinguishable from the wild type in epithelial cell invasion *in vitro*. Bogomolnaya *et al.* (2008) reported that a *S.* Typhimurium ΔSTM0557 (*gtrC1*) mutant replicated to a higher level in J774 murine macrophages in a short term infection (24 hours) relative to the wild type *in vitro* (15). They also reported that the ΔSTM0557 (*gtrC1*) mutant and the wild type were equally invasive in J774 macrophages. However, upon closer examination of the invasion data, the mutant appears to adhere to and invade J774 cells approximately 2-fold higher relative to the wild type (15).

Down-regulation of the O antigen form variation operon infer less glucosylation of the O12 antigen and thus lower O12-2 content in *S.* Typhimurium DT104 harbouring SGI1. As described above, *S.* Typhimurium unable to glucosylate the O12 antigen were found with a higher intracellular count after 24 hours in murine macrophages and potentially more invasive and than the wild type. Perhaps SGI1 modulated down-regulation of O12 antigen glucosylation genes results in a *S.* Typhimurium DT104 phenotype better able to invade macrophages in environmental conditions resembling those in rich culture media.

### 4.3 Expression in Early Stationary Phase

In early stationary phase, SGI1 was observed to have a larger influence on *S.* Typhimurium DT104 global gene expression relative to mid-log growth phase. The SGI1 influenced up-regulation of *fljBA* was the only consistent observation between mid-log and early stationary growth phases which indicates that the FliB antigen is favoured over FliC independent of growth phase. The expression of a diverse collection of genes was influenced by SGI1 in early stationary phase. SGI1 encodes 4 ORFs with putative regulatory functions (S004, S006-7, and S035) (20, 57). In particular, ORF S035 encodes a putative regulator with homology to the DNA binding LysR-type family (20). LysR-type regulators are activators involved in modulation of complex regulons and typically require a coinducer molecule (104). They are known as activators for diverse operons including those involved in oxidative stress, nitrogen fixation, and virulence (104). Future studies should investigate the role of SGI1 encoded putative regulators/hypothetical ORFs, especially S035 in the regulation of *S.* Typhimurium DT104 genes in early stationary phase. Perhaps cofactors of LysR and other regulators are encoded by the hypothetical ORFs. Interestingly, classical virulence genes were observed to be up-regulated in the presence of SGI1, which is evidence in support of the idea that MDR DT104 hypervirulence is attributed to SGI1. Up-regulated virulence genes include those found on SPI1 and SPI4 that are involved in attachment and invasion of mucosal cells during *Salmonella* pathogenesis (42, 54).

*Salmonella* genes involved in host cell invasion are induced in high osmolarity and low oxygen conditions *in vitro*, which are reminiscent of the mucosal environment (7). The invasion regulator HilA and genes under its control were reported to be

expressed upon entry into stationary phase *in vitro* (112). The aforementioned study also reports that *S.* Typhimurium grown to early stationary phase were 10 to 20-fold more invasive than those grown to mid-log or late stationary phase *in vitro*.

SPI1 is involved in host cell invasion and encodes transcriptional regulators that include secreted effector proteins that alter host cell structure to enable *Salmonella* invasion and proteins that constitute a TTSS, which is involved in the translocation of effectors into mucosal cells (42). SPI1 encoded an invasion regulator protein HilA that is expressed upon entry into stationary phase dependant on stringent signal molecule ppGpp but not the stationary phase sigma factor RpoS (112). The genes *relA* and *spoT* involved in ppGpp synthesis as well as *rpoS* were not found influenced by SGI1. The two-component regulatory systems OmpR/EnvZ, SirA/BarA, PhoP/Q, and PhoB/R and other genes with suspected involvement in SPI1 regulation (*hha, lon, fis, fadD, fimZ, fimY, fur, and fliZ*) (reviewed in Ellermeier *et al.* (2007)) were not found to be influenced by SGI1 as well (42). This indicates factor(s) from SGI1 influenced the up-regulation of *hilA*, the SPI1 encoded invasion regulator, in a manner independent of the known mechanisms of SPI1 regulation.

HilA plays a central role in the activation of TTSS biosynthesis genes and secreted effector genes involved in invasion. *S.* Typhimurium lacking *hilA* are phenotypically similar to this strain deleted of SPI1, which are attenuated in invasion of mucosal cells (41). HilA binds the promoters and directly induces the *prg/org* and *inv/spa* operons. The *sic/sip* operon, indirectly induced by HilA, was either transcribed by read through from the *inv/spa* operon, or from a secondary regulator, InvF, encoded

on the *inv/sic* operon (30). These 3 operons regulated by HilA encode all necessary proteins for biosynthesis of a functional TTSS.

SGI1 influenced a 2 to 3-fold up-regulation of 14 SPI1 genes including the invasion regulators *hilA* and *invF*, 10 TTSS structural genes, and 2 genes with unknown function in *S.* Typhimurium DT104 in early stationary phase. Up-regulated genes were found on the *org/prg* and *inv/spa* operons. An additional 17 SPI1 genes were observed to be up-regulated under the 2-fold cut off limit (1.5 to 2-fold) which included genes found on the *sic/sip* operon. Even though up-regulation of these genes was below the 2-fold threshold, they are in agreement with *hilA* up-regulation of SPI1 virulence genes associated with invasion.

The expression level of *hilA* varies proportionally with the invasiveness of *S.* Typhimurium (74). The gene *hilA* was found up-regulated 2.6-fold in *S.* Typhimurium DT104 harbouring SGI1 which may not be enough to produce a hyperinvasive phenotype relative to strains that lack SGI1. However, Weir *et al.* (2008) observed that a non-SGI1 harbouring DT104 strain with an ACSSuT phenotype exposed to a subinhibitory concentration of tetracycline (Tc) was 2.5-fold more invasive in HeLa cells and that *hilA* was up-regulated 3.5-fold relative to those not exposed (125). Therefore an increase in *hilA* expression of only 3.5-fold, was correlated with a hyperinvasive phenotype. Perhaps a 2.6-fold induction of *hilA* may indicate that *S.* Typhimurium DT104 harbouring SGI1 is more invasive in early stationary phase or similar environmental conditions. Additional experiments examining invasiveness in the *S.* Typhimurium DT104 SGI1 isogenic strain pair will need to be conducted to confirm the suggested increased invasiveness.

HilA has been reported to regulate virulence genes located outside of SPI1 including *sopA* and *siiA* found on SPI4 (discussed in a later section) (115). SopA is a virulence effector protein implicated in mucosal fluid accumulation during salmonellosis (128). This effector, which is secreted through the TTSS of SPI1 during invasion, and its expression has been observed to be up-regulated in *S.* Typhimurium harbouring SGI1 (128). This further supports that the induction of the *hilA* regulon is enhanced in the presence of SGI1. Further studies should be conducted on the influence of SGI1 on *hilA* expression, perhaps in high osmolarity and low oxygen conditions reminiscent of the mucosal environment that have been reported to induce this gene and an invasive phenotype in *S.* Typhimurium (7).

SPI4, a 23.5 kb element, contains 6 genes (*siiABCDEF*) and encodes a TOSS which is a membrane bound complex involved in protein export and a large non-fimbral adhesin (54). SPI4 genes *siiAB* have unknown functions, and *siiCDF* encode the TOSS involved in secretion of the adhesin encoded by *siiE*. Five out of 6 SPI4 genes (*siiABCEF*) were observed up-regulated in *S.* Typhimurium DT104 harbouring SGI1 with microarray analysis of the isogenic strain pair. Gerlach *et al.* (2007a) demonstrated that SiiE was essential for adhesion to polarized epithelial cells such as the MDCK line, with closer resemblance to those in the mucosa, but not for HEp-2 cells (54). Polarized epithelial cells contain the apical and basolateral sides and are able to form tight junctions.

All SPI4 genes are suspected to be expressed on a single large mRNA transcript from a promoter upstream of *siiA* (53). Based on luciferase transcriptional fusion assays, expression levels of *siiE* and *siiF* were reported to be 4-fold and 10-fold lower than *siiA*,

respectively (53). This indicates that SPI4 genes were expressed in a decreasing gradient from the promoter. Genes, *siiB* and *siiF* were selected for RT-qPCR confirmation using independent samples where up-regulation was only confirmed for *siiB* in the presence of SGI1. If *siiF* is indeed expressed 10-fold less than *siiA* as described by Gerlach *et al.* (2007b) perhaps differential expression of this gene was undetectable between the isogenic strains with RT-qPCR (53). However, up-regulation of *siiF* was detected with microarray analysis. Despite the different observations for *siiF* from microarray analysis and RT-qPCR, SGI1 factor(s) may initiate the transcriptional cascade of SPI4 genes.

SPI4 has been shown to be involved in adhesion and co-regulated with SPI1 during invasion through the global regulator SirA in a HilA-dependant manner (2, 53). SirA gene expression was not observed to be influenced by SGI1 in *S.* Typhimurium DT104. This indicates that SGI1 factor(s) may induce SPI4 independently of SirA. HilA has been reported to bind the promoter upstream of *siiA* using electrophoretic mobility shift assay, and expression of *siiA* was increased relative to a *ΔhilA* mutant in *S.* Typhimurium using microarray analysis (115). The induction of SPI4 in *S.* Typhimurium DT104 harbouring SGI1 may be indirect through its influence on the *hilA* regulon.

Up-regulation of *virK* from early stationary phase cells was confirmed with RT-qPCR on independent samples. Confirmation of *virK*, a homologue of the virulence gene of the same name in *S. flexneri*, infers that the adjacent *mig-14* is also up-regulated. In *S.* Typhimurium, *phoP* regulated genes *virK* and *mig-14* have been linked to cationic peptide resistance, survival in macrophages, and in systemic infection sites (eg. liver and spleen) in mice (24). Expression of *phoP* was not influenced by SGI1 indicating that *virK* and *mig-14* are modulated by this genomic island independently of this regulator.

SPI2 and SPI3 are involved in intracellular replication and survival within macrophages (12, 62). SPI3 encoded $Mg^{2+}$ transport genes *mgtCB* and SPI2 encoded putative regulator *orf408* were observed down-regulated in *S.* Typhimurium DT104 harbouring SGI1. Other genes encoded on these PAIs were not influenced by SGI1 in early stationary phase. The gene *orf408* located within the boundaries of SPI2 as well as most SPI3 genes are not well characterized (63, 102). The gene *mgtC* co-transcribed with *mgtB* is required for growth in $Mg^{2+}$ limited conditions and intra-macrophage survival *in vitro.* SPI1 and SPI2/SPI3 are induced upon different conditions where the two-component regulator PhoP/PhoQ was found to positively regulate the expression of SPI2 as well as *mgtC,* and repress invasion associated SPI1 (12, 42, 45). This regulator was not found influenced by SGI1 in early stationary phase. The purpose for down-regulation of these genes specifically (and not other SPI2 and SPI3 genes) in *S.* Typhimurium DT104 harbouring SGI1 in early stationary phase is unclear. The down-regulation of these genes may be direct or indirect consequence of SGI1 influenced gene expression in early stationary phase cultures.

In addition to virulence genes, microarray analysis revealed that the expression of a diverse collection of *S.* Typhimurium DT104 genes was found modulated by SGI1 in early stationary phase. It is unlikely that SGI1 directly influences expression of all these genes, however, the influence may be indirect through chromosomal regulatory genes. The expression of 6 genes with known or putative transcriptional regulatory functions were up-regulated [*ybeF* (STM0634), *yncC* (STM1588) , *hilA* (STM2876), *invF* (STM2899), *lldR* (STM3693), and *rbsR* (STM3886)] and 12 were down-regulated [*prpR* (STM0367), STM1013-1014 (Gifsy-2 prophage), *orf408* (STM1382), *pocR* (STM2036),

STM2627 (gifsy-1 prophage), *tcdA* (STM3245), STM3834, *ydeW* (STM4073), *melR* (STM4297), and STM4534]. One could speculate that altered expression of the majority of these genes in the presence of SGI1 may be beneficial to the overall fitness of *S.* Typhimurium DT104. Notable up-regulated genes include those involved in drug efflux and carbohydrate uptake. Notable down-regulated genes include those for ethanolamine and propanediol usage involved in the synthesis of polyhedral body structures that store volatile aldehydes (14, 95); Gifsy-1 and Gifsy-2 prophage; nitrate reductase; and regulatory protein *bssR* (*yliH*) attributed to repression of biofilm production in *E. coli* (32). The BssR homolog in *Salmonella* was not found to modulate biofilm production and a function has not been elucidated (92).

### 4.4 Comparison of Expression between Growth Phases

The SGI1 induced up-regulation of the *fljBA* operon was the only consistent finding in *S.* Typhimurium DT104 grown to mid-log and early stationary phase (section 4.3). Based on microarray analysis using an isogenic strain pair of *S.* Typhimurium DT104, SGI1 was observed to influence a larger portion of the transcriptome in early stationary phase (~200 genes) compared to mid-log growth phase (5 genes).

SGI1 encodes an ORF (S006), a putative regulatory protein with homology to flagellar transcriptional activator Fr1B (FlhC) in *Bordetella bronchiseptica* (20). FlhC and FlhD constitute a master regulator involved in the activation of the flagellar and chemotaxis genes in *S.* Typhimurium (55). The regulation of structural genes, including flagellar phase switching, was found to be independent of FlhC, however, the homolog encoded on SGI1 may play a role in the suspected preference of flagellar antigen FliB

over FliC. Future studies should investigate the role of SGI1 ORF S006 on flagellar antigen expression. In addition, protein quantification assays should be used to verify gene expression data that *S.* Typhimurium DT104 harbouring SGI1 indeed favours FliB over FliC.

SGI1 influenced the expression of classical virulence genes in early stationary phase, which supports the hypothesis that SGI1 plays a role in MDR DT104 hypervirulence. It was previously reported that *hilA* is induced upon entry into early stationary phase and that *S.* Typhimurium grown to this phase are 10 to 20-fold more invasive to HEp-2 cells than grown to mid-log or late stationary phase (112). SGI1 may enhance this invasive phenotype induced upon entering stationary phase. Future studies should investigate the invasiveness of *S.* Typhimurium DT104 harbouring SGI1 on cultured mucosal cells after grown to early stationary phase. SGI1 induced hyperinvasiveness was evident after exposure to RPz, however, it may also be observed after exposure to different environmental stress conditions (101).

### 4.5 Comparison of Expression between *S.* Typhimurium DT104 and LT2

Microarray analysis revealed that SGI1 influenced the expression of 36 genes in *S.* Typhimurium LT2 grown to mid-log growth phase in LB broth (57). The SGI1 influenced transcription profile of *S.* Typhimurium DT104 differed greatly from that observed for *S.* Typhimurium LT2. Genes influenced by SGI1 in *S.* Typhimurium DT104 are inconsistent with those for *S.* Typhimurium LT2 in mid-log growth phase, with the exception of *fliC* down-regulation (57). SGI1 influenced up-regulation of *yjfN*, encoding a putative inner membrane protein, was the only consistent observation in both

*S.* Typhimurium DT104 grown to early stationary phase and *S.* Typhimurium LT2 (mid-log phase) over 2-fold. The sialic acid usage gene *nanT* was observed up-regulated in *S.* Typhimurium LT2 harbouring SGI1. In contrast, SGI1 was observed to have the opposite influence on this gene in *S.* Typhimurium DT104, however, RT-qPCR did not confirm the down-regulation of *nanA* (indicating that *nanT* expression may not be reproducible as well). The global gene expression profiles of *S.* Typhimurium LT2 and *S.* typhimurium DT104 with SGI1 may be explained as follows: (1) the *S.* Typhimurium LT2 strain is avirulent because *rpoS* is non-functionial (113); (2) expression analysis for *S.* Typhiumurium cultures grown in LB instead of BHI broth; (3) the microarray gene expression systems used in the two studies were different (see section 1.5.4). Potential differences in the phagetype DT104 sequence could limit hybridization of DT104 samples to LT2 probes. Genes that are truly differentially expressed in *S.* Typhimurium DT104 with SGI1 may have been potentially missed due to poor hybridization.

Golding *et al.* (2007) characterized the expression profile of all SGI1 ORFs relative to *gapA* in *S.* Typhimurium LT2 using RT-qPCR (57). Similar experiments should be done using the *S.* Typhimurium DT104 strain harbouring SGI1 grown to mid-log and early stationary phase. In particular, the expression of ORFs with putative regulatory functions and unknown functions should be characterized in order to correlate their expression with that of chromosomal genes influenced by SGI1. This may increase our understanding of the role of SGI1 influence of global gene expression, and potentially elucidate a mechanism for the observed enhanced expression of *hilA* regulon genes in the presence of this genomic element.

### 4.6 Limitations

Expression can be evaluated for all known ORFs simultaneously using microarrays, however, they are susceptible to variation where false positive and negative results are commonly reported. An independent method of expression quantification such as RT-qPCR is useful in the validation of microarray results. For example, microarray analysis revealed that eight genes were influenced by SGI1 in mid log growth phase 1.8-fold or greater. RT-qPCR on independently grown samples confirmed 5 of the 8 genes were differentially expressed. Samples were cultured in 20 ml for RT-qPCR instead of 300 ml as for microarray sample, however, they were harvested at the same $OD_{600}$ and thus in identical growth environments. Also in this project, different commercially available microarray platforms were used for mid-log and early stationary phase global gene expression analyses. This was because the NimbleExpress chips used for mid-log microarray analysis were discontinued during the time frame of this project. In addition NimbleExpress arrays like those from Affymetrix, have short 25 bp probe sequences. Genomes of *S.* Typhimurium strains may or may not be highly conserved. At the time of this project, the genome for *S.* Typhimurium DT104 was not annotated and potential differences in sequence from probes designed from the *S.* Typhimurium LT2. False negative results may have incurred due to poor hybridization. Perhaps the SGI1 influence of *S.* Typhimurium DT104 gene expression should be evaluated with Roche-Nimblegen arrays. Even though these arrays are also LT2 genome based, they have longer probes (70 bp) and array data between growth phases can be directly compared.

In early stationary phase, 206 genes were found differentially expressed in the presence of SGI1 using microarray analysis. Since RT-qPCR validation of all 206 genes

was impractical, a sample of 20 SGI1 influenced genes was selected. Independent expression analysis validated 14 (70%) of the 20 selected genes. The least significant gene would then be ORF STM4073 encoding putative transcriptional repressor YdeW ($p = 5.6 \times 10^{-6}$) when genes were sorted by p values. Error and variation are expected for multiple testing assays such as microarray analysis. However, variation may have been introduced from stationary phase cultures due to a mixed population including non-replicating and dying cells (131).

## 5.0 Conclusions

SGI1 has been hypothesized to enhance the virulence of MDR *Salmonella* strains in which it is contained. This genomic island harbours ORFs with putative regulatory functions that may influence transcription of genes located outside this element. Previously, SGI1 was observed to influence gene expression in avirulent *S.* Typhimurium LT2 (57). In this project, microarray analysis using an isogenic strain pair revealed that SGI1 also influenced gene expression in an *S.* Typhimurium DT104. SGI1 did not have a large effect on global gene expression of this organism grown to mid-log phase, where only 5 genes involved in the alteration of flagellar and O surface antigens were observed to be influenced. In early stationary phase, SGI1 was found to be influenced in the expression of a larger portion of the *S.* Typhimurium DT104 transcriptome (~200 genes) than in mid-log growth phase, and included the up-regulation of classical virulence genes. *S.* Typhimurium was reported 10 to 20-fold more invasive after grown to early stationary phase relative to those in mid-log and late stationary phase (112). The invasion regulator *hilA* was found up-regulated and genes activated under the HilA regulon were also up-regulated. This suggests that the invasiveness may be enhanced in *S.* Typhimurium harbouring SGI1 grown to early stationary phase. The SGI1 influenced up-regulation of genes attributed to virulence supports the hypothesis that the hypervirulence of MDR DT104 is attributed to SGI1 mediated factors. Future studies should investigate which ORFS found on SGI1, particularly those with putative regulatory functions, influence the expression of *hilA*. Since the level of *hilA* expression reflects the invasiveness of *S.* Typhimurium, epithelial cell invasion assays should be conducted using the SGI1 isogenic strain pair grown to early stationary phase in order to verify if *S.* Typhimurium

DT104 with SGI1 displays a hyperinvasive phenotype reflecting the gene expression profile.

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