Regulation of O-antigen modification in

Salmonella

Nicholas James Donohue

PhD

University of York

Biology

March 2016
Abstract

The extracellular surface of the pathogen *Salmonella* contains the lipopolysaccharide (LPS) molecule, which provides structural support and defence against predators. A polysaccharide component of the LPS, the O-antigen has a highly variable composition and is a primary target for the antibody mediated immune response. The O-antigen displays vast structural diversity across the *Salmonella* genus, and this variation is further enhanced through the action of genes such as glycosyltransferase (*gtr*) operons, which add carbohydrates to the O-antigen and affect phage resistance and antibody recognition. Importantly, certain *gtr* operons are also capable of phase variation, meaning their expression can be switched ON or OFF in a heritable, but reversible manner. A switched ON, *gtr* expressing cell, can produce daughter cells with switched OFF *gtr* expression and vice versa. *gtr* phase variation produces modified and unmodified O-antigen molecules, thereby creating population heterogeneity.

Previous work identified the mechanism of *gtr* phase variation: methylation of GATC sequences by Dam and binding of the oxidative stress response regulator OxyR. GATC sequences overlap with OxyR half sites in the *gtr* regulatory region. Depending on which GATC sequences are methylated and which half-sites OxyR occupies, *gtr* expression switches between the ON and OFF phase.

This research aimed to identify influences on *gtr* phase variation and expression. Naturally occurring sequence variations of *Salmonella* serovars were found to alter the phase variation switch frequency, or even abrogate the process. Stationary phase growth caused reduced *gtr* expression. The role of OxyR in *gtr* regulation was also further defined. The OxyR regulator can switch between an oxidised and a reduced state. Although the reduced state was previously found to be sufficient for phase variation to occur, the role of oxidised OxyR in this process was not understood. In this work, a possible role for oxidised OxyR was found in regulating expression of a short RNA (STnc1870), which was divergently transcribed from the *gtr* regulatory region.

Finally, a potential target for STnc1870 was found: the *ssaO* mRNA which encodes a component of the *Salmonella* Pathogenicity Island 2 secretion needle. Artificial overexpression of STnc1870 reduced expression of *ssaO*. This connection indicates a possible further role for the *gtr* regulatory region beyond controlling O-antigen modification.
List of Contents

Abstract ........................................................................................................................................ 2
List of Figures ................................................................................................................................ 7
List of Tables .................................................................................................................................. 9
Acknowledgements ......................................................................................................................... 10
Author’s declaration ......................................................................................................................... 11
Chapter 1 Introduction .................................................................................................................... 12
  1.1 Salmonella biology ..................................................................................................................... 13
    1.1.1 General biology and burden of disease ............................................................................. 13
    1.1.2 Gastrointestinal Salmonella infections ........................................................................... 14
    1.1.3 Typhoidal Salmonella infections ...................................................................................... 18
    1.1.4 Prokaryotic gene regulation ............................................................................................. 21
  1.2 The LPS Molecule ..................................................................................................................... 23
    1.2.1 Structure of LPS ................................................................................................................ 23
    1.2.2 Modifications of the LPS molecule .................................................................................. 24
  1.3 The gtr operon .......................................................................................................................... 25
    1.3.1 Function of the gtr operon ............................................................................................... 25
    1.3.2 Phase variation and gtr operons ....................................................................................... 27
    1.3.3 Relevance of gtr operons ............................................................................................... 30
  1.4 Questions .................................................................................................................................. 32
Chapter 2 Materials and Methods .................................................................................................... 34
  2.1.1 Bacterial strains, plasmids, primers and genome sequences .............................................. 35
  2.2 Media ....................................................................................................................................... 50
    2.2.1 LB media ......................................................................................................................... 50
    2.2.2 SOC media ...................................................................................................................... 50
    2.2.3 tet^5 media ...................................................................................................................... 50
    2.2.4 M9 media ....................................................................................................................... 51
    2.2.5 InSPI2 media ................................................................................................................ 51
    2.2.6 NonSPI2 media .............................................................................................................. 51
    2.2.7 Antibiotics ..................................................................................................................... 52
    2.2.8 Catalase solution ............................................................................................................ 52
  2.3 PCR techniques ......................................................................................................................... 52
    2.3.1 Colony PCR ..................................................................................................................... 52
    2.3.2 Q5 PCR .......................................................................................................................... 52
    2.3.3 GoTaq PCR .................................................................................................................... 53
    2.3.4 qRT-PCR ....................................................................................................................... 53
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4</td>
<td>Nucleic acid techniques</td>
<td>54</td>
</tr>
<tr>
<td>2.4.1</td>
<td>Genomic DNA extraction</td>
<td>54</td>
</tr>
<tr>
<td>2.4.2</td>
<td>DNA gel separation</td>
<td>54</td>
</tr>
<tr>
<td>2.4.3</td>
<td>DNA/RNA quantification</td>
<td>54</td>
</tr>
<tr>
<td>2.4.4</td>
<td>DNA gel extraction</td>
<td>55</td>
</tr>
<tr>
<td>2.4.5</td>
<td>DNA restriction digests</td>
<td>55</td>
</tr>
<tr>
<td>2.4.6</td>
<td>DNA precipitation</td>
<td>55</td>
</tr>
<tr>
<td>2.4.7</td>
<td>RNA extraction</td>
<td>56</td>
</tr>
<tr>
<td>2.4.8</td>
<td>RNA testing for genomic DNA presence</td>
<td>56</td>
</tr>
<tr>
<td>2.4.9</td>
<td>cDNA synthesis</td>
<td>57</td>
</tr>
<tr>
<td>2.4.10</td>
<td>Lambda RED recombination</td>
<td>57</td>
</tr>
<tr>
<td>2.4.11</td>
<td>Lambda RED counterselection</td>
<td>58</td>
</tr>
<tr>
<td>2.4.12</td>
<td>CRIM vector integration</td>
<td>58</td>
</tr>
<tr>
<td>2.4.13</td>
<td>Plasmid DNA extraction</td>
<td>59</td>
</tr>
<tr>
<td>2.4.14</td>
<td>PCR purification</td>
<td>59</td>
</tr>
<tr>
<td>2.4.15</td>
<td>DNA Ligation</td>
<td>59</td>
</tr>
<tr>
<td>2.4.16</td>
<td>Sequencing</td>
<td>60</td>
</tr>
<tr>
<td>2.4.17</td>
<td>PCR site directed mutagenesis (SOE-PCR)</td>
<td>60</td>
</tr>
<tr>
<td>2.5</td>
<td>Reporter assay techniques</td>
<td>60</td>
</tr>
<tr>
<td>2.5.1</td>
<td>β-galactosidase assays</td>
<td>60</td>
</tr>
<tr>
<td>2.5.2</td>
<td>Fluorescence measurement</td>
<td>61</td>
</tr>
<tr>
<td>2.6</td>
<td>LPS analysis techniques</td>
<td>61</td>
</tr>
<tr>
<td>2.6.1</td>
<td>LPS sample preparation</td>
<td>61</td>
</tr>
<tr>
<td>2.6.2</td>
<td>TSDS-PAGE</td>
<td>62</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Silver staining</td>
<td>63</td>
</tr>
<tr>
<td>2.7</td>
<td>Miscellaneous techniques</td>
<td>63</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Switch frequency determination</td>
<td>63</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Preparing electrocompetent cells</td>
<td>64</td>
</tr>
<tr>
<td>2.7.3</td>
<td>Electroporation</td>
<td>64</td>
</tr>
<tr>
<td>2.7.4</td>
<td>Freezer stock preparation</td>
<td>64</td>
</tr>
<tr>
<td>2.8</td>
<td>Bioinformatic techniques</td>
<td>65</td>
</tr>
<tr>
<td>2.8.1</td>
<td>Primer design</td>
<td>65</td>
</tr>
<tr>
<td>2.8.2</td>
<td>Sequence alignments</td>
<td>65</td>
</tr>
<tr>
<td>2.8.3</td>
<td>Phylogenetic trees</td>
<td>65</td>
</tr>
<tr>
<td>2.8.4</td>
<td>sRNA target prediction</td>
<td>65</td>
</tr>
<tr>
<td>3.1</td>
<td>Influence of oxidative stress on <em>gtr</em> expression</td>
<td>67</td>
</tr>
<tr>
<td>3.1.1</td>
<td>The OxyR regulator</td>
<td>67</td>
</tr>
</tbody>
</table>

Chapter 3 Influence of growth conditions on *gtr* regulation .......................... 66
Chapter 5

5.1.2 Inducing persistently oxidised OxyR ................................................. 68
5.1.3 Influence of oxidised OxyR on gtr phase variation .............................. 71
5.1.4 Influence of oxidised OxyR on gtr transcription ................................ 72
3.2 Influence of stationary phase growth on gtr expression .......................... 76
3.2.1 Expression of LT2_I gtr:lacZ increases between exponential and stationary phase .................................................................................................................. 76
3.2.2 The promoter region is sufficient for LT2_I gtr:lacZ expression increases ...... 76
3.2.3 gtr operons have a conserved -10 site that is characteristic of σ38 dependent promoters ........................................................................................................ 80
3.2.4 Altering the -13 position of LT2_I gtr reduces proportional expression increases. ...................................................................................................................... 82
3.2.5 Analysis of LT2_I gtr expression using qRT-PCR ...................................... 82
3.3 Influence of low pH on LT2_I gtr ............................................................... 87
3.4 Discussion .................................................................................................. 89

Chapter 4 Investigation of a short RNA molecule produced by the gtr regulatory region .... 93
4.1 Introduction ............................................................................................... 94
4.2 The gtr regulatory region produces a short RNA molecule under macrophage-like conditions ........................................................................................................ 95
4.3 Using a locked OFF mutant gtr to test STnc1870 transcription ..................... 101
4.4 The OxyR C199S mutant represses STnc1870 transcription after H2O2 treatment ... 109
4.5 A potential alternative OxyR site could be required to activate STnc1870 transcription ........................................................................................................ 112
4.6 The effect of STnc1870 overexpression on gtrA ........................................... 116
4.7 The effect of STnc1870 overexpression on ssaO .......................................... 122
4.8 A 13 bp sequence in STnc1870 is required for ssaO degradation .................. 124
4.9 Discussion .................................................................................................. 127

Chapter 5 Influence of DNA sequence on gtr phase variation ............................ 134
5.1 Introduction ............................................................................................... 135
5.2 The S. Typhi 2_II gtr ............................................................................... 136
5.2.1 Diversity of gtr regulatory regions ........................................................... 136
5.2.2 Phase variation does not require four GATC sites ..................................... 142
5.2.3 Switch frequency differences between three and four GATC gtr regulatory regions ........................................................................................................ 143
5.3 The S. Infantis_I gtr .................................................................................. 143
5.3.1 The S. Infantis_I gtr also contains three GATC sites .................................... 143
5.3.2 The TATC sequence variation affects phase variation ............................... 146
5.4 Differences between gtr:lacZ and gtrABC::lacZ reporter fusions .................... 148
5.5 Discussion .................................................................................................. 150
5.5.1 gtr sequence variation in naturally occurring serovars ............................ 150
5.5.2 Effect of sequence variation on gtr phase variation .................................. 152
5.5.3 Effect of chromosomal location and orientation on gtr phase variation........ 155
5.5.4 Potential future experiments ................................................................. 158
Chapter 6 Discussion ......................................................................................... 160
6.1 Introduction ............................................................................................... 161
6.2 Influences on gtr expression ...................................................................... 161
6.3 The gtr regulatory region produces a short RNA molecule ...................... 163
6.4 The S. Typhi 2_II gtr contains a sequence similar to STnc1870 ................. 167
6.5 OxyR regulation of gtr and STnc1870 ........................................................ 168
6.6 Influence of DNA sequence on gtr phase variation .................................... 172
6.7 Conclusions .............................................................................................. 175
Abbreviations ................................................................................................. 177
References ....................................................................................................... 180
List of Figures

Fig.1.1 gtr phase variation is controlled by GATC methylation and OxyR binding .................. 31
Fig.3.1. Fluorescence levels of Path 760 containing pFH255 (Erv1p + DsbC) and pLA40 (ahpC::gfp) with and without arabinose addition ................................................... 70
Fig.3.2a. β-galactosidase assay of ‘full length’ P22 gtr::lacZ fusion (Path 728) with expression of Erv1p and DsbC......................................................................................... 73
Fig.3.2b. β-galactosidase assay of ‘full length’ LT2_I gtr::lacZ fusion (Path 730) with expression of Erv1p and DsbC................................................................. 74
Fig.3.2c. β-galactosidase assay of ‘truncated’ P22 gtr::lacZ fusion (Path 732) with expression of Erv1p and DsbC................................................................. 75
Fig.3.3. Graph of gtr regulatory regions used in this chapter as part of reporter fusions with the lacZ gene contained in the CRIM vector ................................................. 77
Fig.3.4. β-galactosidase levels of gtr::lacZ fusion strains grown from exponential to stationary phase ............................................................. 78
Fig.3.5. Alignment of the regulatory regions of 46 gtr operons (dataset derived from Davies et al., 2013) ...................................................................................... 81
Fig.3.6. Colony phenotype of Path 859 (gtrC::lacZ fusion) .................................................................................................................. 84
Fig.3.7. TSDS-PAGE gel separation of Path 859 (gtrC::lacZ fusion) LPS samples visualised by silver stain ............................................................. 85
Fig.3.8. qRT-PCR results of gtrC::lacZ fusion strains grown in M9 minimal media from exponential to stationary phase ................................................................. 86
Fig.3.9. β-galactosidase assay of gtr::lacZ fusion strains grown in NonSPI2 (pH = 7.4) and InSPI2 media (pH = 5.8) ............................................................................... 90
Fig.4.1. Overview of the gtr regulatory region ...................................................................... 96
Fig.4.2. Detailed sequences of the gtr regulatory region .................................................. 96
Fig.4.3. Colony phenotypes of S. Typhimurium ST4/74_I gtrC::lacZ strains ......................... 98
Fig.4.4. TSDS-PAGE gel separation of gtrABC::lacZ fusion strains Path 891 and Path 931 LPS samples visualised by silver stain ................................................. 99
Fig.4.5. Growth curve of Path 891 in InSPI2 media +/- H2O2 treatment ............................. 100
Fig.4.6. Absolute gtrA and STnc1870 expression of Path 891 (gtrABC::lacZ) +/- 1 mM H2O2 treatment .................................................................................. 102
Fig.4.7. β-galactosidase results of gtr::lacZ fusion strains to test functionality of Path 931 gtr promoter region ........................................................................... 106
Fig.4.8. Absolute gtrA and STnc1870 expression of Path 931 +/- 1 mM H2O2 treatment .... 107
Fig.4.9. Relative gtrA and STnc1870 expression of Path 931 and Path 959 +/- 1 mM H2O2 treatment .................................................................................. 111
Fig.4.10. Sequence of the predicted alternate OxyR site in the gtr regulatory region ......... 113
Fig.4.11. Overview of strains used to investigate alternate OxyR site hypothesis .......... 114
Fig.4.12. Colony phenotypes of strains with mutations of the predicted alternate OxyR site .............................................................. 115
Fig.4.13. Relative STnc1870 expression of Path 891, Path 946 and Path 947 +/- 1 mM H2O2 treatment .................................................................................. 117
Fig. 4.14. Diagram of plasmid pMV449 and description of the predicted ssaO binding site in STnc1870.

Fig. 4.15. Relative gtrA and STnc1870 expression of Path 923 +/- 1 mM IPTG treatment.

Fig. 4.16. TSDS-PAGE gel separation of Path 923 (gtrC: lacZ, pMV449) LPS samples visualised by silver stain.

Fig. 4.17. Relative ssaO and STnc1870 expression of Path 923 and Path 953 + 1 mM IPTG treatment.

Fig. 4.18. Relative ssaO and STnc1870 expression of Path 923, Path 953 and Path 961 +/- 1 mM IPTG treatment.

Fig. 5.1. Cladogram of gtr regulatory regions.

Fig. 5.2. Alignment of gtr regulatory regions.

Fig. 5.3. Diagram of the CRIM vector.

Fig. 5.4. Colony phenotypes of S. Typhi gtr: lacZ CRIM vector strains.

Fig. 5.5. Colony phenotypes of S. Infantis_I gtr: lacZ fusion CRIM vector strains.

Fig. 6.1. Model for regulation of the gtr operon.

Fig. 6.2. Alignment of the S. Typhi 2_II and S. Typhimurium LT2_1 gtr regulatory sequences.

Fig. 6.3. Predicted secondary structures of STnc1870 sRNA (A) and the corresponding sequence in S. Typhi 2_II gtr (B).
List of Tables

Table 2.1. *Salmonella* Typhimurium and *Salmonella* Typhi strains .................................................. 35
Table 2.2. *E. coli* strains ..................................................................................................................... 39
Table 2.3. Plasmids ................................................................................................................................. 40
Table 2.4. Primers and Oligos .................................................................................................................. 43
Table 2.5. Accession numbers for relevant strains .................................................................................. 49
Table 3.1. Comparison of β-galactosidase expression levels between exponential and stationary phase of LT2_I*gttr:*lacZ reporter fusion strains ................................................................. 79
Table 4.1. Relative *gttrA* and STnc1870 expression of Path 891 +/- 1 mM H₂O₂ ................................. 103
Table 4.2. Relative *gttrA* and STnc1870 expression of Path 931 +/- H₂O₂ treatment ...................... 108
Table 4.3. mRNA targets of STnc1870 predicted by TargetRNA (Kery et al., 2014) ......................... 123
Table 5.1. Switch frequencies and *lacZ* expression levels of *S. Typhi* *gttr:*lacZ CRIM vector strains ........................................................................................................................................... 144
Table 5.2. Switch frequencies and *lacZ* expression levels of *S. Infantis_I* *gttr:*lacZ CRIM vector strains ............................................................................................................................................. 147
Table 5.3. Switch frequencies and *lacZ* expression levels of *S. Typhimurium* *gttr:*lacZ CRIM vector strains and *gttrABC::lacZ* fusion strains ........................................................................................................... 149
**Acknowledgements**

I would like to thank my supervisor, Marjan van der Woude, for all her support and guidance. Thanks go also to the members of my thesis advisory panel Maggie Smith and James Moir, for their valuable feedback and comments.

I also wish to thank all members of the van der Woude lab, past and present, for helpful discussions and experimental support. Further thanks go to Steven Watson who worked as a project student on the S. Infantis gtr project.

I am very grateful to the staff of the Technology Facility and in particular, Andrew Leech for help with the fluorescence measurements, and Sally James and Lesley Gilbert who provided vital support and training for the qRT-PCR experiments. Thanks to Phil Roberts from Biology photo-graphics for producing high-quality pictures of colonies.

Furthermore, I wish to thank Laurent Aussel and Frederic Barras of Aix-Marseille University (France), who provided the pLA40 plasmid. Thanks also to Lloyd Ruddock of the University of Oulu (Finland) for supplying the Erv1p/DsbC expression plasmid.

And finally, to my friends, family and especially Jill: thank you for everything and making this possible!
Author’s declaration

The work presented here is my own, except for the construction of the *S. Infantis* \( gtr: lacZ \) fusions CRIM vectors, which was carried out by Steven Watson under my supervision. Furthermore, the construction of the wildtype *S. Typhi* \( gtr: lacZ \) fusion CRIM vectors was performed by Edwin Kaptein. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.
CHAPTER 1
INTRODUCTION
1.1 **SALMONELLA BIOLOGY**

1.1.1 **General biology and burden of disease**

The Gram negative bacteria *Salmonella* causes intestinal or systemic illness in humans and other animals. It is classed among the family of Enterobacteriaceae, along with other clinically relevant organisms such as *Escherichia coli*, *Yersinia*, *Klebsiella* and *Shigella* (Williams et al., 2010). *Salmonella* is thought to have diverged from a common ancestor with *E. coli* between 120 and 160 million years ago (Ochman and Wilson, 1987).

The *Salmonella* genus consists of two species, *S. enterica* and *S. bongori*. *S. enterica* is subdivided into the following six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae* and *indica* (Tindall, 2005). 98% of clinically relevant infections are caused by *Salmonella enterica* subsp. *enterica*, which is further classified by the Kauffmann-White scheme for serotyping. *Salmonella* serotypes or serovars are distinguished by agglutination with antisera that bind components of the outer surface of the bacteria (= O-antigens, see 1.2.1). Over 2,500 different serovars have been identified (Lerouge and Vanderleyden, 2006).

Human *Salmonella* infections can be broadly distinguished between non-typhoidal salmonellosis which is restricted to the intestine of the host, and systemic, invasive typhoidal infections (reviewed in Crump et al., 2015). Non-typhoidal *Salmonella* infections were estimated to have caused up to 93.8 million infections and 155,000 deaths worldwide in 2006. The disease often goes unreported, especially in developing nations (Majowicz et al., 2010). Transmission of the gastro-intestinally restricted form of the disease (salmonellosis) usually occurs via the faecal-oral route (Madigan et al., 2005). Food contaminated either during production (e.g. infected animals) or preparation by an infected food handler is the most common source of infection. A survey of 7,120 flocks of broiler chickens conducted by the European Food Safety Agency (EFSA) found that 23.7% of flocks were infected with *Salmonella* (EFSA, 2007).

While typhoidal infections are less common, they caused an estimated 190,200 deaths worldwide in 2010 (Lozano et al., 2013). Typhoidal infections in humans are caused by the *S. enterica* subsp. *enterica* serovars Typhi and Paratyphi (referred to as *S. Typhi* and *S. Paratyphi*).
1.1.2 Gastrointestinal *Salmonella* infections

Non-typhoidal *Salmonella* serovars may establish infections that remain confined to the host intestinal environment. Ultimately, these infections result in replication of the bacteria and cause diarrhoea, which allow the infection to be disseminated to new hosts via the faecal-oral
route. The onset of symptoms such as abdominal cramps, fever and diarrhoea occurs 12 to 72 hours post infection (Santos et al., 2001).

*Salmonella* is faced with several hurdles that could prevent establishment of infection, such as competition for nutrients by the resident gut microbiota, iron limitation and anaerobic conditions in the gut lumen as well as the host immune system. The *Salmonella* genome encodes factors that allow these challenges to be overcome. In general, *Salmonella* hijacks and exploits host cell functions to create an environment that is conducive to its replication.

Following ingestion by a host, *Salmonella* bacteria that survived the acidic stomach environment enter the gastrointestinal tract. When the contents of the stomach enter the intestine, the stomach acid is neutralised by bile salts such as deoxycholate secreted by the gall bladder. This action has the added effect of causing osmotic shock to bacteria. *Salmonella* responds to this shock through the environmental sensor EnvZ and by preferentially expressing the outer membrane porin OmpC instead of OmpF (Chatfield et al., 1991; Methner et al., 2004). Porins allow the passage of small molecules between the periplasmic space and the exterior (Puente et al., 1995).

A further host defence concerns iron limitation in the intestinal lumen: the secreted peptide lipocalin-2 sequesters iron, depriving bacteria of this essential metal and suppressing growth. *Salmonella* secretes salmochelin, a high affinity iron (Fe$^{3+}$) chelator to avoid growth suppression (Bellet et al., 2013).

In order to manipulate the intestinal environment to allow *Salmonella* replication, some of the bacteria invade enterocyte cells of the intestinal lining as well as M cells covering the Peyer’s patches lymphoid tissue in the distal ileum (Carter and Collins, 1974). Flagellar expression allows the bacteria to traverse the mucus lining of the gut to access the enterocytes. Once the host cells are encountered, virulence genes encoded by a genomic island called the *Salmonella* Pathogenicity Island 1 (= SPI1) are transcribed to produce a type III secretion system needle. The *Salmonella* Pathogenicity Islands were likely acquired by horizontal gene transfer (reviewed in Agbor and McCormick, 2011).

The SPI1 needle secretes effectors into host cells such as SipA, SopB and SopE, which promote polymerisation of actin fibres causing membrane rearrangement and internalisation of the
bacteria by endocytosis (reviewed in Galán and Zhou, 2000). In human cells under normal conditions, membrane rearrangements are controlled by the Rho subfamily of actin-organising small GTP-binding proteins (GTPases). GTPases switch between an inactive, GDP bound form and an active GTP-bound form. The SPI1 effectors SopB and SopE activate two Rho GTPases, Cdc42 and Rac1 by promoting the switch to the GTP-bound form. Once activated, Cdc42 and Rac1 cause remodelling of the actin cytoskeleton and membrane ruffling that leads to internalisation of Salmonella bacteria. Injection of purified SopE into human cells also induces membrane ruffling (Hardt et al., 1998). The SipA effector is required to induce localised bundling of actin fibres. Without SipA, actin bundling is diffuse and Salmonella entry into the cell is restricted (Zhou et al., 1999). Therefore, SPI1 mediated cell invasion exemplifies how Salmonella manipulates the host environment for its own benefit.

Salmonella infections that remain confined to the intestine are self-limiting, as they actively induce the inflammation that eventually clears them from the host organ. The process of Salmonella internalisation into host cells causes production of the transcription factor NFκB and the release of pro-inflammatory IL-8 cytokines (Hobbie et al., 1997). In addition to its role in membrane rearrangement, the SipA protein also promotes recruitment of neutrophil cells of the host immune system. Neutrophils trigger inflammation of the gut lining and profuse diarrhoea which causes the bacteria to be shed to the environment (reviewed in Agbor and McCormick, 2011).

Inflammation of the gut lining leads to secretion of reactive oxygen species into the intestinal lumen by the immune system. The reactive oxygen species (such as hydrogen peroxide, H₂O₂) or the superoxide radical (O₂⁻) can damage DNA and are part of a wider attempt to kill the invading bacteria. To counter this oxidative burst, Salmonella produces catalases and superoxide dismutases which detoxify the compounds (Aussel et al., 2011; Hebrard et al., 2009).

Interestingly, Salmonella can exploit the secretion of reactive oxidative species to gain a growth advantage. The reactive oxygen species oxidise thiosulphate, which is abundant in the gut lumen, causing it to form tetrathionate. Salmonella is able to use tetrathionate as a terminal electron acceptor in anaerobic respiration, unlike the resident gut microflora which grows by fermentation. As respiration generates more energy than fermentation, Salmonella can outgrow competing organisms of the resident microbiota (Winter et al., 2010). Resistance to reactive oxygen species and the ability to grow by anaerobic respiration further highlight how
Salmonella not only survives host immune responses but also exploits them for its own advantage.

As previously explained, the following tasks must be completed by Salmonella to establish gastro-intestinal infection: host inflammation must be triggered and the bacteria need to multiply to maximise chances of spreading to a new host. A clonal Salmonella population achieves these tasks by establishing distinct subpopulations with different specialisations, which appear to allow a form of labour division (Stewart and Cookson, 2012).

For example, expression of flagella (a bacterial motility apparatus) is bi-stable, i.e. a population grown from the same ancestor cell can contain cells with or without flagella. This is achieved through the mutually repressing repressors FliZ and FliV. FliZ activates flagellar transcription, but represses ydiV, and in turn YdiV represses fliZ. An equilibrium is established between the two repressors – depending on which repressor gains the upper hand, flagella are either expressed or repressed (Stewart et al., 2011).

Furthermore, bi-stability of flagellar expression extends to the SPII virulence genes, as FliZ appears to be involved in activating SPII expression (Lucas et al., 2000). As a result, Salmonella can produce subpopulations with different motility and virulence capabilities. A subpopulation expressing both flagella and SPII could cross the mucus lining and invade enterocytes, promoting inflammation. The inflammation benefits the cells that do not express flagella or SPII, which remain in the intestinal lumen and are able to outcompete the resident microbiota (reviewed in Stewart and Cookson, 2012).

A non-motile, avirulent subpopulation has the added advantage of maintaining genomic homogeneity among the population. Without this population, cells with mutations disabling the motility and virulence genes could benefit from the improved conditions provided by the virulent cells, but without contributing to the establishment of infection. The avirulent, but genetically identical subpopulation ensures that any non-contributing mutants face growth competition and cannot become dominant (Diard et al., 2013).

A further advantage of specialised subpopulations concerns resistance to antibiotics. Using a fluorescent reporter system, it was found that non-growing or slow growing S. Typhimurium
were more resistant to antibiotic treatment than fast-growing cells in susceptible BALB/c mice after oral infection (Claudi et al., 2014).

In summary, *Salmonella* produces effectors to survive host defences (e.g. bile salts, iron limitation and reactive oxygen species) and cause inflammation to promote its replication (by anaerobic respiration using tetrathionate). The creation of subpopulations with different specialisations, such as high virulence or rapid growth could help *Salmonella* accomplish these tasks.

### 1.1.3 Typhoidal *Salmonella* infections

In *Salmonella* gastrointestinal infections, the bacteria invade cells of the intestinal lining, but do not disseminate to other sites of the body and do not breach the intestinal lamina propria. In typhoidal, invasive infections of humans, serovars *S*. Typhi and *S*. Paratyphi can spread to organs such as the liver, spleen and gall bladder. In these cases, the bacteria migrate via dendritic cells from the gut lumen to mesenteric lymph nodes via lymph drainage. The bacteria may also be transported from the gut to the liver or spleen via CD18+ phagocytic cells such as macrophage or dendritic cells. Bile produced in the liver can also be used to spread the bacteria to the gall bladder (reviewed in Watson and Holden, 2010). From the gall bladder, the bacteria can be shed into the intestine through bile secretion, allowing the bacteria to spread to new hosts via the faecal-oral route. A carrier state can be established in 1 – 4 % of typhoidal infections, which is characterised by asymptomatic long-term (more than a year) secretion of the bacteria (reviewed in Crump et al., 2015). In a human infection model, doses of $10^3$ or $10^4$ colony forming units of *S*. Typhi were found to be sufficient to induce typhoid fever in 55 or 65 % of patients, respectively (Waddington et al., 2014). Typhoidal infections result in sustained fever (>12 hours) and bacteremia which can become fatal without antimicrobial treatment in 10 – 30 % of cases. With antibiotic treatment, the fatality rate drops to less than 1 % (van den Bergh et al., 1999).

If the intestinal lining is crossed, *Salmonella* bacteria encounter macrophage cells of the immune system, in which they can persist inside a specialised compartment termed the *Salmonella* Containing Vacuole (SCV). Both broad-host range (e.g. *S*. Typhimurium) and host restricted (e.g. *S*. Typhi) serovars can produce SCV compartments in susceptible macrophage. However, *S*. Typhi cannot survive in murine macrophage under normal conditions (Spanò and
Galán, 2012). The SCV has characteristics of an endosome, although it does not fuse with actual lysosomes, which could kill the bacteria. To survive in the SCV, *Salmonella* uses a further pathogenicity island, SPI2.

SPI2 genes encode a second type III secretion system needle and effectors that manipulate the host cell cytoskeleton (reviewed in Abrahams and Hensel, 2006). For example, the SPI2 effector SpiC prevents fusion of the SCV with lysosomes and is required for virulence (Uchiya et al., 1999). Two other SPI2 effectors, SseG and SifA position the SCV next to the host cell nucleus and in proximity of the Golgi apparatus (Salcedo and Holden, 2003). Bacterial replication only occurs in SCV compartments associated with the Golgi, which may provide a source of nutrients. In addition, the host cell attempts to recruit NADPH oxidase to the SCV membrane, in order to secrete O$_2^-$ into the SCV. SPI2 mediated interference with this process prevents bactericidal concentrations of O$_2^-$ being produced (Vazquez-Torres et al., 2000). The SCV environment induces a subpopulation of *S. Typhimurium* to stop growing which may allow them to survive and persist in BALB/c murine macrophage better than faster growing cells (Helaine et al., 2014).

The ability to cross the intestinal barrier and persist inside host macrophage appears to be a required characteristic to allow systemic persistent infection. The serovar *S. Typhi* has the innate ability to systemically infect humans, but no other species. The cause of this host restriction of *S. Typhi* is difficult to determine, due to the lack of an animal model for the disease (reviewed in Spanò, 2014). Studies of systemic salmonellosis have relied on *S. Typhimurium* infections of susceptible mice (e.g. BALB/c or C57BL lines) which lead to systemic and persistent illness in these animals. Genetic susceptibility of these mice to *Salmonella* infection is due to the lack of a functional Nramp1 protein (Roy and Malo, 2002). Nramp1 is a metal transporter which localises to the SCV membrane and removes divalent cations such as Mn$^{2+}$, which are required for bacterial replication (Jabado et al., 2000). An alternative animal model uses calves to study *S. Typhimurium* induced gastro-enteritis (Tsolis et al., 1999).

*S. Typhi* evolved from *S. Typhimurium* through gene deletion, as well as acquisition. The genome of *S. Typhi* contains 204 pseudogenes (Parkhill et al., 2001). An important distinction to *S. Typhimurium* is the production of the capsular polysaccharide Vi antigen by *S. Typhi* (Looney and Steigbigel, 1986). The Vi antigen reduces binding of complement proteins of the immune system to *S. Typhi*, as well as increasing resistance to phagocytosis by
polymorphonuclear leukocytes (Hart et al., 2016; Looney and Steigbigel, 1986). Interestingly, while a vaccine based on purified Vi antigen confers protective immunity to S. Typhi, a further vaccine based on the live attenuated Ty21a strain, which does not express the Vi antigen, also provides immunity (see section 1.1.1). Studies of typhoid infection in a human model (using a Vi expressing S. Typhi strain) found that the antibody response to infection targeted the LPS (see section 1.2.1) and flagella components, but not the Vi antigen (Tacket et al., 1991; Waddington et al., 2014). Naturally acquired immunity to S. Typhi is therefore unlikely to be mediated by anti-Vi antibodies.

A further difference between the two strains is that S. Typhimurium encodes a phage-derived, SPI2 secreted effector called GtgE which is absent from S. Typhi (Ho et al., 2002). Expression of GtgE in S. Typhi allowed this strain to survive in C57BL/6 mouse macrophage when infected in vitro, which it is normally incapable of. Furthermore, GtgE was shown to degrade the host cell GTPase Rab32, which is involved in membrane trafficking. Knock-down of Rab32 expression by siRNA allowed S. Typhi to survive in C57BL/6 mouse macrophage. Rab32 may be involved in an antimicrobial process that leads to killing of bacteria (such as S. Typhi) if they do not express GtgE as a countermeasure (Spanò and Galán, 2012).

Another unique feature of S. Typhi is the production of typhoid toxin, an AB toxin which causes host cell cycle arrest, and is only produced after establishment of the SCV (Song et al., 2013). Once produced, typhoid toxin is transferred outside the host cell through a process requiring another GTPase, Rab29. Rab 29 is also cleaved by the GtgE effector of S. Typhimurium. Therefore, expression of GtgE could be disadvantageous for S. Typhi, as this would interfere with the secretion of typhoid toxin (Spano et al., 2011). This disadvantage could have incentivised the loss of the gtgE gene from the S. Typhi genome.

The loss of GtgE explains how S. Typhi could be restricted from infecting mouse macrophage, but it remains to be determined what the differences are between human and mouse macrophage that allow S. Typhi to survive in one but not the other. If S. Typhi requires GtgE to survive in mouse macrophage, why is it not required for survival in human macrophage?

Interestingly, the S. Typhimurium D23580 strain prevalent in sub-Saharan Africa has been found to be evolving into an invasive strain more similar to S. Typhi. The strain is classed as invasive non-typhoidal Salmonella (iNTS). It is thought that compromised immune systems
(through HIV infection and malnutrition) of the local population contribute to this process (Kingsley et al., 2009; Yang et al., 2015).

1.1.4 Prokaryotic gene regulation

A major focus of this work concerns the regulation of bacterial genes. The following section introduces relevant mechanisms of genetic regulation along with examples. Generally speaking, bacterial genes can be regulated at the level of transcription or translation, as well as post-translationally or post-transcriptionally (Madigan et al., 2005).

A well-studied example of transcriptional regulation is the lac operon of E. coli, which is required for the catabolism of lactose. If lactose is absent, the LacI repressor protein binds the lacO genetic region upstream of the lacZYA operon (which encodes the genes required for lactose catabolism) and prevents transcription. If lactose becomes available to the cell, the LacI repressor binds lactose, undergoes a conformational change and can no longer repress lacZYA transcription (Madigan et al., 2005).

In addition, the availability of glucose influences transcription of the lac operon via a process termed catabolite repression. A complex formed of CAP (catabolite activator protein) and cAMP (cyclic adenosine monophosphate) is required to initiate high levels of lacZYA transcription. The cAMP-CAP complex binds upstream of the promoter region of lacZYA and increases recruitment of RNA polymerase. Levels of the cAMP-CAP complex are low when glucose levels are high and vice versa. Therefore, reduced glucose levels lead to more cAMP-CAP complexes and increased lacZYA transcription (but only if lactose is also available) (Madigan et al., 2005).

A further important transcriptional regulator in E. coli and Salmonella is the OxyR protein which acts as an oxidative stress response regulator (Storz et al., 1990). OxyR regulates genes encoding enzymes such as catalase (katG) or alkyl hydroperoxide reductase (ahpC) that scavenge hydrogen peroxide (H₂O₂) and detoxify it to H₂O and O₂ (Aussel et al., 2011). In the absence of this defence mechanism, H₂O₂ can react with cellular iron in a Fenton reaction to produce the hydroxyl radical OH⁻ which in turn reacts with most biomolecules including DNA, leading to mutations (Imlay et al., 1988).
The OxyR protein senses the presence of reactive oxygen species through cysteine residues (C199 and C208) in its structure that may form disulphide bridges under oxidising conditions. Formation of such disulphide bridges results in a conformational change of the entire protein (termed ‘oxidised OxyR’) (Choi et al., 2001). Oxidised OxyR can bind promoters (such as *ahpC*) that the reduced form may not be able to and stimulate transcription by RNA polymerase (Zheng et al., 2001). This promoter selectivity is based on the spacing of half sites that OxyR contacts on the DNA. Two half sites must be contacted for binding. One half site consists of two adjacent major grooves of the DNA helix (with the appropriate OxyR binding sequence). If two half sites are not separated (thereby consisting of four adjacent major grooves), oxidised OxyR is more likely to bind. Alternatively, if the two half sites are separated by one helical turn of the DNA (roughly 10 bp), reduced OxyR is predicted to bind (Toledano et al., 1994).

In the above examples, transcription is controlled by regulatory proteins that repress or activate gene expression in response to specific signals such as lactose or oxidative stress levels. This process allows bacteria to adapt to changing environmental conditions. However in certain instances, gene expression can be activated or silenced as a result of a random switch. This random switch is termed phase variation and can be used to increase the phenotypic variety of a bacterial population without altering genetic homogeneity. Mechanistically, phase variation can be caused by site specific recombination, slipped strand mispairing or DNA methylation (van der Woude and Baumler, 2004). Examples of these mechanisms are given in section 1.3.2.

A final regulatory mechanism relevant to this work concerns short RNAs (sRNAs). sRNA molecules, typically 50 – 300 bp in size, commonly act to regulate expression of an mRNA target at the post-transcriptional level. sRNAs bind target mRNAs, often with the help of the RNA chaperone Hfq. Typically, sRNAs bind at or close to the ribosome binding site of an mRNA, thereby occluding it and blocking translation.

An example of sRNA regulation concerns the previously mentioned process by which *Salmonella* acquires iron in the host intestine, using the chelator salmochelin (see also section 1.1.2). Iron acquisition by salmochelin is regulated by two short RNA molecules: RhyB1 and RhyB2 bind and stimulate translation of the *iroN* mRNA, which encodes the outer membrane receptor for iron-bound salmochelin (Balbontín et al., 2015).
Furthermore, the short RNA GcvB regulates roughly 1 % of all *Salmonella* genes, including amino-acid metabolism (Sharma et al., 2011). In enterohemorrhagic *E. coli* (EHEC) a further sRNA, AgvB antagonizes the function of GcvB. An EHEC strain with a deletion of AgvB was less competitive compared to the wildtype ancestor during growth in bovine terminal rectal mucosa (Tree et al., 2014).

Alternatively, sRNAs may be required to allow expression of a particular mRNA, as in the case of the DsrA sRNA which is induced by low temperature in *E. coli* and binds the rpoS mRNA. The rpoS mRNA is normally folded in a way that occludes the ribosome binding site. Binding of DsrA and Hfq makes the ribosome binding site of rpoS available for translation (Battesti et al., 2011). sRNAs can also promote mRNA degradation via RNase III or RNase E (Nicholson, 2014). Finally, sRNAs can interact directly with proteins to regulate their activity (Storz et al., 2011).

1.2 THE LPS MOLECULE

1.2.1 Structure of LPS

As a Gram negative bacteria, *Salmonella* has a double lipid bilayer composed of phospholipids, separated by a layer of peptidoglycan (Madigan et al., 2005). The outer membrane contains the lipopolysaccharide (LPS) molecule which provides structural protection and covers up to 75 % of the outer surface area (reviewed in Lerouge and Vanderleyden, 2006). LPS is composed of the lipid A tail, the core oligosaccharide and the O-antigen (reviewed in Erridge et al., 2002). The lipid A is recognised by the human innate immune system through the Toll-like receptor 4 (TLR4). The other LPS components are not recognised by the innate immune system (Poltorak et al., 1998).

The lipid A molecule and the core oligosaccharide are synthesised in the bacterial cytoplasm and then translocated across the inner membrane. The O-antigen chain is then attached to the core oligosaccharide. The O-antigen is produced as separate subunits in the cytoplasm before being moved to the periplasm (at which point individual subunits may be linked together to form a repeating chain). The *S. Typhimurium* O-antigen consists of a galactose-rhamnose-mannose repeat, with the mannose sugar having an abequose side chain (Miller et al., 2005).
The same structure is found in *S.* Typhi, although instead of abequose, tyvelose is attached to the mannose sugar (Crawford et al., 2013). *S.* Typhimurium produces O-antigen chains with lengths of 16-35 subunit repeats as well as very long chains with more than 100 repeats. Chain length is under control of the Wzz proteins (Murray et al., 2003).

The periplasmic assembled LPS molecule is transported to the outer membrane via lipopolysaccharide transport proteins (LptA-G) (reviewed in Whitfield and Trent, 2014). The LptD-E complex inserts the LPS molecule into the outer membrane (Dong et al., 2014; Qiao et al., 2014). Approximately 200 LptD-E complexes are dispersed across the outer membrane of a bacteria and each one of these complexes can transport five LPS molecules per second (reviewed in Bishop, 2014).

The O-antigen is the most diverse LPS component among the *Salmonella* genus, as opposed to the highly conserved core oligosaccharide and lipid A moieties. Over 20 different carbohydrates, arranged in repeating subunits of three to five sugars are known to be incorporated in the O-antigen. The different combination of these sugars allows for the vast diversity of strain-specific O-antigen molecules, over 2,500 of which have been identified (Lerouge and Vanderleyden, 2006). The different O-antigens are part of the basis of *Salmonella* classification according to serovars in the Kauffmann-White scheme (Guibourdenche et al., 2010; Tindall, 2005).

### 1.2.2 Modifications of the LPS molecule

A multitude of microorganisms modify their outer surface structures as part of their survival strategy. By altering surface structures that are exposed to attack by predators or host immune systems, organisms can escape killing. For example, the parasite *Trypanosoma brucei* switches coat proteins through gene recombination to evade the antibody mediated immune response (Mehlert et al., 2002). The bacterium *Pseudomonas aeruginosa* is capable of modifying the lipid A subunit of the LPS to either induce or prevent activation of inflammation, depending on the type of modification performed (reviewed in Bryant et al., 2010). Similarly, *Neisseria gonorrhoeae* modifies the lipid A component of the lipooligosaccharide (LOS) by addition of phosphoethanolamine to increase resistance to antimicrobial peptides and complement mediated killing (Handing and Criss, 2014). *Bacteroides thetaiotaomicron* can remove a single phosphate
group from the lipid A, allowing the bacteria to persist in the gut lumen during inflammation, as this modification reduces susceptibility to antimicrobial peptides (Cullen et al., 2015).

Another pathogen in the Enterobacteriaceae, *Shigella flexneri* adds glucose to the O-antigen molecule, causing it to shorten and expose a type III secretion system needle. The glycosylation may shift the LPS structure from a linear to a helical conformation, leading to shortening. The type III secretion system needle can then inject virulence effectors into host cells. The shorter O-antigen is less effective at resisting killing by antimicrobial peptides expressed in the gut, which explains why the glycosylation is not performed constitutively (West et al., 2005; Cunliffe, 2003).

In *Salmonella*, the PhoP/Q and PmrA/B systems control modifications of the lipid A component of the LPS in response to conditions such as low magnesium, low pH and presence of antimicrobial peptides by adding phosphoethanolamine to the lipid A molecule (Kato et al., 2012; Navarre et al., 2005). *Salmonella* encounters these conditions in the SCV in macrophage and requires the PhoP/Q system to survive in this environment (Aranda et al., 1992). Furthermore, the oafA gene performs acetylation of the O-antigen subunits. Although oafA modification does not affect virulence or survival, it can inhibit binding of monoclonal antibodies that target unacetylated O-antigen (Slauch et al., 1995).

### 1.3 THE gtr OPERON

#### 1.3.1 Function of the gtr operon

Further LPS modifications in *Salmonella* are performed by glycosyltransferase genes (*gtr*) which add carbohydrates to the O-antigen subunit. In this way, the already vast diversity of *Salmonella* O-antigens is further enhanced. The *gtr* operon consists of three genes: *gtrA*, *gtrB* and *gtrC*. All three genes are thought to produce membrane proteins which modify the O-antigen subunits in the periplasm (reviewed in Allison and Verma, 2000). The *gtrC* protein performs the actual modification of the O-antigen, with both the site and type of modification differing between individual *gtrC* genes (Bogomolnaya et al., 2008; Davies et al., 2013).

Ten different families of *gtr* operon have been identified in *Salmonella*, in phage-derived or phage-associated sequences. *gtr* operons show evidence of recombination and not all
modifications performed by them are detected by standard serotyping assays. A *Salmonella* strain may carry between 1 and 4 different *gtr* operons (Davies et al., 2013).

What is the purpose of O-antigen modification by *gtr* operons? One function certainly seems to involve resistance to phage that use the O-antigen as a receptor for infection. The phage P22 *gtr* operon performs an $\alpha$ 1-6 glycosylation of the galactose sugar of the *S*. Typhimurium O-antigen, which confers resistance against superinfection by P22 (Kim and Ryu, 2012; Pedulla et al., 2003).

Similarly, the *S*. Typhimurium LT2_I *gtr* operon confers resistance to phage SPC35, by performing an $\alpha$ 1-4 glycosylation of the same galactose sugar (Bogomolnaya et al., 2008; Kim and Ryu, 2012). The LT2_I *gtr* operon is part of the novel *Salmonella* Pathogenicity Island 16 (Vernikos and Parkhill, 2006). Data published by Bogomolnaya et al., 2008 indicated that the LT2_I *gtr* operon could be required for long term intestinal persistence of *S*. Typhimurium in mice. Strains with deletions of LT2_I *gtrC* were less persistent compared to wildtype strains during competitive infections, produced by oral gavage. The use of a mouse strain resistant to *Salmonella* infection (CBA/J) raises questions about this data however (Plant and Glynn, 1976; Roy and Malo, 2002). In contrast, intravenous infections of *Salmonella* susceptible BALB/c mice with a library of transposon mutated *S*. Typhimurium showed no attenuation for strains with transposon insertions in the LT2_I *gtrABC* genes (Chaudhuri et al., 2013; Roy and Malo, 2002).

A further connection between *gtr* and phage resistance was found in research by Kintz et al., 2015. A role for the prophage BTP1 GtrC protein was identified, which modifies the O-antigen of *S*. Typhimurium D23580, the iNTS strain mentioned previously (section 1.2). The tailspike protein of the BTP1 prophage has endorhamnosidase activity and can therefore cleave and shorten the O-antigen. Modification of the O-antigen by BTP1 GtrC prevents cleavage.

Due to its exposed nature, the O-antigen is a convenient target for the antibody mediated response of the immune system and therefore *gtr* modifications could have important implications for the study of *Salmonella* infections. A final possible role for *gtr* modification concerns resistance to predatory amoebae. Certain amoebae present in vertebrate intestines display feeding preferences based on *Salmonella* O-antigen structures (Wildschutte and Lawrence, 2007). For example, amoebae of the genus *Encephalitozoon* are opportunistic
pathogens of humans (Wasson and Peper, 2000). gtr modification of the O-antigen could allow Salmonella to escape such predation.

1.3.2 Phase variation and gtr operons

As mentioned previously, Salmonella produces subpopulations with different specialisations to mount infections. Interestingly, gtr operons appear to follow a similar trend by enhancing phenotypic heterogeneity among a genetically identical population. 8 out of the 10 identified gtr families are likely to phase vary, which means their expression can be switched ON or OFF in a heritable but reversible manner (Broadbent et al., 2010; Davies et al., 2013). A single Salmonella bacteria with a switched OFF gtr operon can divide to produce a descendant cell with a switched ON gtr operon. The switched ON cell can in turn produce switched OFF descendants. As a result, a clonal Salmonella population can contain a mixture of cells with switched ON or OFF gtr operons. Cells with switched ON gtr operons produce the corresponding O-antigen modification, whereas cells with switched OFF gtr operons do not. The population therefore displays a heterogeneous mixture of different O-antigen molecules.

Previous work has identified two factors that regulate phase variation of the gtr operon by interacting with the upstream region: methylation of palindromic GATC sites by the enzyme Dam (Deoxyadenosine methylase), and binding of the oxidative stress response regulator OxyR to corresponding target sequences (Broadbent et al., 2010).

The gtr regulatory region contains three half sites for the OxyR regulator: OxyR A, OxyR B and OxyR C. OxyR must access two of these sites to bind. The OxyR C half site also overlaps with the -35 region of gtr. The GATC methylation target sites for Dam overlap with the OxyR A and OxyR C half sites. Methylation by Dam at these sites renders the sequence inaccessible to OxyR and thereby prevents it from binding. Should the GATC sites furthest from the -35 region of gtr (in the OxyR A half site) become methylated, OxyR binds the B and C half sites and gtr transcription is halted, possibly because the RNA polymerase can no longer bind. This state results in repression of the gtr operon. If the GATC sites in the OxyR C half site are methylated, OxyR binds the A and B half sites and gtr transcription occurs (Broadbent et al., 2010). A model of this system is shown in Fig.1.1.
The methylation state of the regulatory region remains unchanged until DNA replication is commenced, after which it is presumed that OxyR and Dam would be in competition to bind the newly synthesised, unmethylated strand of DNA. The methylation state of the template strand seems to exert influence on this action, as the previous methylation pattern is often inherited by the new strand. However, with a certain probability, alternate binding sites may become occupied by OxyR on the new strand, resulting in a change in expression state. In this way, phase variation occurs, as the expression shifts are heritable, but reversible. Bacterial populations derived from a single cell show heterogeneous expression for phase varying genes such as the *gtr* operon (Broadbent et al., 2010).

An additional phase-varying O-antigen modification system is used by *Salmonella*. The *S. Typhimurium* *opvAB* locus reduces the chain length of O-antigens, which usually contain 16-35, or more than 100 subunit repeats (Murray et al., 2003). *opvAB* expression reduces the number of O-antigen subunit repeats to 3-8 (Cota et al., 2012). *opvAB* expression phase varies from ON to OFF through Dam methylation of GATC sites and OxyR binding. Unlike *gtr* however, OxyR is required for *opvAB* expression (Cota et al., 2015a). Strains with constitutive expression of *opvAB* are resistant to infection by P22 phage. However, they have reduced survival in murine macrophage or in the presence of serum and are less virulent in oral and intraperitoneal infections of BALB/c mice compared to wildtype strains (Cota et al., 2015b, 2012). *opvAB* alteration of O-antigen chain length can therefore be beneficial or detrimental to the cell, depending on the situation.

The process of phase variation is not unique to *Salmonella* O-antigen modification. A variety of other bacterial surface structures such as pili, outer membrane proteins or flagella can also phase vary. Mechanistically, phase variation can be caused by site specific recombination, slipped strand mispairing or DNA methylation and examples of each are given below (van der Woude and Baumler, 2004).

The pyelonephritis associated pilus (*pap*) used by *E. coli* for attachment to host cells undergoes phase variation similar to *gtr* (Braaten et al., 1994; Van Der Woude et al., 1998). A region upstream of the *pap* promoter contains two GATC sites (GATC<sup>1</sup> and GATC<sup>2</sup>) which overlap with binding sites for the leucine-responsive regulatory protein Lrp. The Lrp sites surrounding GATC<sup>2</sup> also overlap with the *pap* promoter region. Methylation of either GATC<sup>1</sup> or GATC<sup>2</sup> by Dam prevents Lrp binding at the overlapping site. If GATC<sup>1</sup> is methylated, Lrp binds downstream at the sites surrounding GATC<sup>2</sup>, which stops transcription of *pap* (OFF state). If
GATC\(^2\) is methylated, Lrp binds upstream at the sites surrounding GATC\(^1\) and *pap* transcription occurs. A further regulator, PapB is required for the OFF to ON switch of *pap*. Sequence variation in the PapB binding site affects the sensitivity of *pap* operon activation (Totsika et al., 2008). Type 1 fimbriae of *E. coli* also phase vary by site specific recombination, causing inversion of the promoter of *fimA*, the main structural gene (Abraham et al., 1985). The frequency of switching is influenced by growth conditions such as temperature and media (Gally et al., 1993).

A further phase varying factor in *E. coli* is the outer membrane protein Ag43 which promotes biofilm formation. The upstream region of the *agn43* gene contains three GATC sites which overlap with OxyR binding sites. Methylation of all three GATC sites prevents OxyR binding and enables *agn43* transcription (ON state). If the GATC sites are unmethylated, OxyR can bind and *agn43* transcription is stopped (OFF state) (Wallecha et al., 2003, 2002).

The O-antigen molecule of *Helicobacter pylori* is modified by fucosyltransferases, which act to produce structures similar to the Lewis antigen expressed on the surface of human epithelial cells. This molecular mimicry could aid immune evasion of the bacteria and promote host colonisation (reviewed in Moran, 2008). The expression of fucosyltransferases is transient: repeat sequences in the corresponding genes are susceptible to slipped strand mispairing, whereby erroneous DNA replication either adds or deletes a base, causing frame-shifts which either prevent or enable correct translation (Wang et al., 2000).

Another example of slipped strand mispairing mediated phase variation concerns fimbriae of *Bordetella pertussis*, the causative agent of whooping cough. A ~15 bp stretch of repeating C bases in the promoter region of fimbrial subunit genes can be altered by replication errors inserting or removing bases. These changes can either allow or stop transcription, thereby switching fimbrial expression ON or OFF (Chen et al., 2010; Willems et al., 1990).

Other than simply switching expression ON or OFF, different variants of a surface structure can also be expressed. In *S. Typhimurium*, flagella show antigenic variation caused by switching between two flagellar subunit proteins: FliC and FljB, which produce the H1 and H2 antigens respectively. The flagellar antigen is usually determined along with the O-antigen as part of serotyping assays. Site specific recombination by the Hin recombinase at 26 bp inverted repeat sequences causes inversion of a 995 bp element containing the promoter for *fljBA*. If the
promoter is orientated toward the \textit{fljBA} operon, transcription occurs (Eom et al., 2012; Simon et al., 1980). The FljB protein forms the H2 flagellar antigen and FljA represses \textit{fliC} transcription and translation (Bonifield and Hughes, 2003). If the Hin recombinase inverts the promoter region in the opposite direction of the operon, FljBA are not expressed and \textit{fliC} translation is uninhibited, producing the H1 flagellar antigen.

In summary, phase variation is not only wide-spread among bacteria, but also occurs through a variety of vastly different mechanisms. The random nature of the process indicates that the advantage of phase variation is the creation of phenotypic heterogeneity among a population, while maintaining genetic homology.

1.3.3 Relevance of \textit{gtr} operons

If there are specific benefits to O-antigen modification such as phage resistance, why are 8 out of 10 \textit{gtr} families predicted to phase vary? If the action of \textit{gtr} was critical to the survival or virulence of all cells of a population at a certain stage in the \textit{Salmonella} life cycle, it is unlikely that such a mechanism would be subject to a random switch, but rather employed as a response to a given signal (similar to the \textit{Shigella flexneri} O-antigen glycosylation, or \textit{Salmonella} PhoP/Q mediated lipid A modification mentioned previously).

However, the phase variation aspect of the \textit{gtr} system does enable a genetically identical population of cells to display a large phenotypic diversity, as several different \textit{gtr} operons can be present in a single genome. This diversity in itself could be the desired effect. A particular \textit{Salmonella} strain will only have a single set of O-antigen biosynthesis genes, which, although possibly quite distinct from many other \textit{Salmonella} strains would still be susceptible to predatory phage or amoeba (Kim and Ryu, 2012, Wildschutte and Lawrence, 2007). In addition, antibodies of the immune system could arise that would target this specific O-antigen molecule and quickly neutralise the bacteria. The \textit{gtr} system could offer a means to avoid such dangers by altering the O-antigen chain’s immunogenicity.

Because the \textit{Salmonella} bacteria cannot anticipate which antibodies a host may express or which phage it might encounter, it is possible that it is vital for the \textit{gtr} system to be subject to random switching. This system allows for a diverse population to be maintained which can survive a wide variety of threats. Should a particular predator or antibody arise that targets the
Fig.1.1 *gtr* phase variation is controlled by GATC methylation and OxyR binding

GATC sequences are annotated and numbered 1, 2, 3 or 4. OxyR half-sites are coloured in red and annotated A, B or C in red letters. The *gtrABC* promoter region is in blue and the -35 and -10 sites are annotated. The OxyR regulator is in grey and annotated. Methylated GATC sites are in red and the A base is underlined. **A**, OxyR occupation of the OxyR A and B sites results in *gtrABC* transcription (ON state). **B**, OxyR occupation of the B and C sites halts *gtrABC* transcription (OFF state). Graph based on Broadbent et al., 2010. Not to scale.
O-antigen of only a subgroup of this population, the remaining members could escape. However, these remaining members may encounter a different threat subsequently, in which case the O-antigen of the previously targeted group may allow escape. In such a model, the ability to express a great variety of O-antigens is beneficial if not vital, but no particular O-antigen can ever become dominant, as there is no specific advantage associated with any O-antigen variant. To be brief, the advantage lies in variety, but no specific variety ever gains more than a temporary advantage.

Regarding the possible function of *gtr* in allowing *Salmonella* to escape an adversary, a further question concerns whether there may be advantages that a certain phenotype could benefit from during the infection of a human or animal. Given that members of the *Salmonella* genus can infect as either a persistent and systemic disease (typhoidal) or as self-limiting gastro-enteritis, the importance of *gtr* in this context should be considered. Specifically, could *gtr* enable seroconversion and immune evasion, thereby allowing for the persistence of a typhoidal infection? Or could a host that had previously experienced gastro-enteritis and established an antibody response to a certain O-antigen form of the causative strain become re-infected by the same strain expressing a different, *gtr*-modified O-antigen? If *gtr* modification allowed a strain to escape the antibody mediated immune response, this would have implications for vaccine development.

1.4 QUESTIONS

If *gtr* expression is influenced by factors other than Dam and OxyR, this could either enhance or limit the effects of phase variation. Specifically, if *gtr* is in the ON state, but *gtr* transcription is too low due to other influences, the cell may be in a phenotypic OFF state. Furthermore, if environmental signals impact *gtr* expression, the exact nature of such signals could help explain in which stage of the *Salmonella* life cycle *gtr* modifications are either important (or not). One example of environmental signals affecting *gtr* regulation was discovered in RNA sequencing data published by Kröger et al., 2013. Under conditions intended to mimic the *Salmonella* containing vacuole of the macrophage, a short RNA molecule was produced from the *gtr* regulatory region. The regulation and function of this short RNA molecule, as well as the relationship with the *gtr* operon are addressed in this work.
The role of \( gtr \) in protection against phage predation was previously established (Kim and Ryu, 2012; Kintz et al., 2015). However, the question whether \( gtr \) has an influence on \textit{Salmonella} host infection remains to be answered. By examining the genetic regulation of \( gtr \), new insights into the role of \( gtr \) during infection could be gained.

Finally, how is phase variation itself influenced? A number of naturally occurring \textit{Salmonella} isolates are predicted not to phase vary. How is this absence of phase variation achieved and could there be an advantage to losing \( gtr \) phase variation in certain environments? By extension, what is the advantage of retaining \( gtr \) phase variation? The differences in lifestyle between phase varying and non-phase varying \textit{Salmonella} strains could offer a further insight into the importance of \( gtr \). The work presented here focuses on the following questions:

- What influences \( gtr \) expression?
- What influences \( gtr \) phase variation?
CHAPTER 2
MATERIALS AND METHODS
### 2.1.1 Bacterial strains, plasmids, primers and genome sequences

*Salmonella* strains used in this work are listed in Table 2.1. *E. coli* strains are listed in Table 2.2. Plasmids are listed in Table 2.3 and all primers and oligos used in this work are listed in Table 2.4. The NCBI accession numbers of *Salmonella* genome sequences analysed in this work are listed in Table 2.5.

#### Table 2.1. *Salmonella* Typhimurium and *Salmonella* Typhi strains

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant genotype</th>
<th>Plasmid</th>
<th>Source</th>
<th>Strain</th>
<th>Primers used for lambdaRED</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 77</td>
<td>LT2 wildtype</td>
<td>ATCC number 19585</td>
<td>LT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path 79</td>
<td>Path 77</td>
<td>pINT-ts</td>
<td>LT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path 150</td>
<td>r(LT)- m(LT)+ r(SA)- m(SA)+ r(SB)- m(SB)+ <em>galE</em></td>
<td>Tsai et al., 1989</td>
<td>LT2 JR501</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path 83</td>
<td>Path 79, pMV 251 integrated at lambda <em>att</em> site</td>
<td>pMV 251</td>
<td>Broadbent et al., 2010</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 84</td>
<td>Path 79, pMV 252 integrated at lambda <em>att</em> site</td>
<td>pMV 252</td>
<td>Broadbent et al., 2010</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 175</td>
<td>Path 79, pMV 296 integrated at lambda <em>att</em> site</td>
<td>pMV 296</td>
<td>Broadbent et al., 2010</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 460</td>
<td>Path 79, pMV 341 integrated at lambda <em>att</em> site</td>
<td>pMV 341</td>
<td>Broadbent et al., 2010</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 805</td>
<td>Path 79, pMV 425 integrated at lambda <em>att</em> site</td>
<td>pMV 425</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-----------------------------------------------</td>
<td>---------</td>
<td>------------</td>
<td>-----</td>
<td></td>
</tr>
<tr>
<td>Path 943</td>
<td>Path 79, pMV 427 integrated at lambda <em>att</em> site</td>
<td>pMV 427</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 725</td>
<td>Path 83 Cm$^5$</td>
<td>pMV 251</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 726</td>
<td>Path 84 Cm$^5$</td>
<td>pMV 252</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 727</td>
<td>Path 175 Cm$^5$</td>
<td>pMV 296</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 728</td>
<td>Path 725</td>
<td>pMV 251, pFH255</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 730</td>
<td>Path 726</td>
<td>pMV 252, pFH255</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 732</td>
<td>Path 727</td>
<td>pMV 296, pFH255</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 760</td>
<td>Path 77</td>
<td>pLA40, pFH255</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 292</td>
<td>LT2_I <em>gtr</em> regulatory region replaced with Ptac promoter</td>
<td>Sarah Broadbent</td>
<td>LT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path 293</td>
<td>$\Delta$ oafA, $\Delta gtr$ LT2_I, $\Delta gtr$ LT2_II</td>
<td>Sarah Broadbent</td>
<td>LT2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path 86</td>
<td>Path 77</td>
<td>pKD46</td>
<td>Mark Davies</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 346</td>
<td>LT2 wildtype</td>
<td>This study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Path 823</td>
<td>Path 346</td>
<td>pKD46</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 827</td>
<td>Path 823, LT2_I <em>gtrC:tetRA</em></td>
<td>pKD46</td>
<td>This study</td>
<td>LT2</td>
<td>oMV 1057 + oMV 1058</td>
</tr>
<tr>
<td>Path 859</td>
<td>Path 827, LT2_I gtrC::lacZ</td>
<td>This study</td>
<td>LT2</td>
<td>oMV 1070 + oMV 1071</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------</td>
<td>------------</td>
<td>-----</td>
<td>---------------------</td>
<td></td>
</tr>
<tr>
<td>Path 869</td>
<td>Path 859, LT2_I gtrC::lacZ</td>
<td>pKD46</td>
<td>This study</td>
<td>LT2</td>
<td></td>
</tr>
<tr>
<td>Path 885</td>
<td>Path 859, LT2_I gtrC::lacZ, Δ rpoS::tetRA</td>
<td>This study</td>
<td>LT2</td>
<td>oMV 1033 + oMV 1034</td>
<td></td>
</tr>
</tbody>
</table>

**Chapter 4 strains**

<table>
<thead>
<tr>
<th>Path 380</th>
<th>ST4/74 wildtype</th>
<th>Rob Kingsley</th>
<th>ST4/74</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 382</td>
<td>Path 380</td>
<td>pKD46</td>
<td>This study</td>
</tr>
<tr>
<td>Path 887</td>
<td>Path 382, ST4/74_I gtrC::tetRA</td>
<td>pKD46</td>
<td>This study</td>
</tr>
<tr>
<td>Path 891</td>
<td>Path 887, ST4/74_I gtrC::lacZ</td>
<td>This study</td>
<td>ST4/74</td>
</tr>
<tr>
<td>Path 902</td>
<td>Path 891</td>
<td>pKD46</td>
<td>This study</td>
</tr>
<tr>
<td>Path 926</td>
<td>Path 902, ST4/74_I gtr::tetRA</td>
<td>pKD46</td>
<td>This study</td>
</tr>
<tr>
<td>Path 931</td>
<td>Path 926, ST4/74_I gtr OFF mutant</td>
<td>This study</td>
<td>ST4/74</td>
</tr>
<tr>
<td>Path 923</td>
<td>Path 891</td>
<td>pMV 449</td>
<td>This study</td>
</tr>
<tr>
<td>Path 953</td>
<td>Path 891</td>
<td>pLAC22</td>
<td>This study</td>
</tr>
<tr>
<td>Path 961</td>
<td>Path 891</td>
<td>pMV 458</td>
<td>This study</td>
</tr>
<tr>
<td>Path 941</td>
<td>Path 902, ST4/74_I gtrA::tetRA (short deletion)</td>
<td>pKD46</td>
<td>This study</td>
</tr>
</tbody>
</table>
| Path 942 | Path 902, ST4/74_I  
gtrA::tetRA (long deletion) | pKD46 | This study | ST4/74 | oMV 1236 +  
oMV 1235 |
|---------|----------------------------------|--------|------------|--------|----------------|
| Path 946 | Path 941, ST4/74_I gtr  
short deletion | This study | ST4/74 | oMV 1237 |
| Path 947 | Path 942, ST4/74_I Δ gtrA  
long deletion | This study | ST4/74 | oMV 1238 |
| Path 960 | Path 941, ST4/74_I gtr  
point mutations | This study | ST4/74 | oMV 1256 |
| Path 945 | Path 79, pMV 455  
integrated at lambda att site | pMV 455 | This study | ST4/74 |
| Path 948 | Path 79, pMV 456  
integrated at lambda att site | pMV 456 | This study | ST4/74 |
| Path 954 | Path 931 | pKD46 | This study | ST4/74 |
| Path 955 | Path 954, Δ oxyR::tetRA | pKD46 | This study | ST4/74 | oMV 808 +  
oMV 809 |
| Path 959 | Path 955, oxyR:C199S | This study | ST4/74 | oMV 810 |

Chapter 5 strains

<table>
<thead>
<tr>
<th>Path 636</th>
<th>Edwin Kaptein</th>
<th>LT2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 637</td>
<td>Edwin Kaptein</td>
<td>LT2</td>
</tr>
</tbody>
</table>
| Path 667 | S. Typhi 2_I gtr: lacZ fusion  
CRIM vector integrated at  
lambda att site | Edwin Kaptein | S. Typhi  
BRD948 |
| Path 806 | Path 79, pMV 426  
integrated at lambda att site | pMV 426 | This study | LT2 |
| Path 833 | Path 79, pMV 430 | pMV 430 | This study | LT2 |
Table 2.2. *E. coli* strains

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MV 1494</td>
<td>XL-1 Blue</td>
<td>pFH255</td>
<td>Edwin Kaptein</td>
</tr>
<tr>
<td>MV 475</td>
<td>BT340CGSC # 7629</td>
<td>pCP20</td>
<td>Barry Wanner</td>
</tr>
<tr>
<td>DL 433</td>
<td>DH5 α</td>
<td></td>
<td>Bethesda Research Labs</td>
</tr>
<tr>
<td>MV 1581</td>
<td>DL433</td>
<td>pLA40</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1085</td>
<td>Pir2</td>
<td></td>
<td>Goulian, MvdW</td>
</tr>
<tr>
<td>MV 1540</td>
<td>MV1085</td>
<td>pMV 389</td>
<td>Edwin Kaptein</td>
</tr>
<tr>
<td>MV 382</td>
<td><em>katF13</em>:Tn10 (<em>tetRA</em>)</td>
<td></td>
<td>Bonnie Manaski</td>
</tr>
<tr>
<td>MV 1103</td>
<td>DH5 α, pirsupE44 Δ lacU169</td>
<td>pMV 243</td>
<td>Ruth Verstraten</td>
</tr>
<tr>
<td>MV 1140</td>
<td>MV1085</td>
<td>pMV 252</td>
<td>Broadbent et al., 2010</td>
</tr>
<tr>
<td>MV 1596</td>
<td>MV1085</td>
<td>pMV 426</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1597</td>
<td>MV1085</td>
<td>pMV 425</td>
<td>This study</td>
</tr>
<tr>
<td>Name</td>
<td>Description</td>
<td>Antibiotic resistance</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>------------------------------------</td>
<td>------------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>MV 1378</td>
<td>MV1085</td>
<td>pMV 341</td>
<td>Broadbent et al., 2010</td>
</tr>
<tr>
<td>MV 1602</td>
<td>MV1085</td>
<td>pMV 427</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1615</td>
<td>MV1085</td>
<td>pMV 430</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1628</td>
<td>DL433</td>
<td>pLAC22</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1657</td>
<td>DL433</td>
<td>pMV 449</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1662</td>
<td>MV1085</td>
<td>pMV 455</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1664</td>
<td>MV1085</td>
<td>pMV 456</td>
<td>This study</td>
</tr>
<tr>
<td>MV 1665</td>
<td>DL433</td>
<td>pMV 458</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 2.3. Plasmids**

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Antibiotic resistance</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMV 243</td>
<td>Empty CRIM vector</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Haldimann and Wanner, 2001</td>
</tr>
<tr>
<td>pMV 252</td>
<td>LT2_I&lt;sub&gt;gtr&lt;/sub&gt; full length regulatory region CRIM vector</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Broadbent et al., 2010</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Description</td>
<td>Resistance</td>
<td>References</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>------------</td>
<td>------------</td>
</tr>
<tr>
<td>pMV 341</td>
<td>LT2_I gtr -10/-35 sites only CRIM vector</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Broadbent et al., 2010</td>
</tr>
<tr>
<td>pMV 251</td>
<td>P22 gtr full length regulatory region CRIM vector</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Broadbent et al., 2010</td>
</tr>
<tr>
<td>pMV 296</td>
<td>P22 gtr -10/-35 sites only CRIM vector</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Broadbent et al., 2010</td>
</tr>
<tr>
<td>pMV 425</td>
<td>PMV 252 point mutated at LT2_I gtr -10 site</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study, oMV 1043 + oMV 1044</td>
</tr>
<tr>
<td>pMV 427</td>
<td>PMV 341 point mutated at LT2_I gtr -10 site</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study, oMV 804 + oMV 716</td>
</tr>
<tr>
<td>pKD46</td>
<td>lambdaRed helper plasmid</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Datsenko and Wanner, 2000</td>
</tr>
<tr>
<td>pINT-ts</td>
<td>CRIM vector integration helper plasmid</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Haldimann and Wanner, 2001</td>
</tr>
<tr>
<td>pFH255</td>
<td>Erv1p + DsbC expression vector</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Van Dat Nguyen et al., 2011</td>
</tr>
<tr>
<td>pLA40</td>
<td>ahpC:gfp fusion</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Aussel et al., 2011</td>
</tr>
<tr>
<td>pCP20</td>
<td>Cm&lt;sup&gt;R&lt;/sup&gt; cassette excision from strains with CRIM vector insertion</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Cherepanov and Wackernagel, 1995</td>
</tr>
<tr>
<td>pLAC22</td>
<td>IPTG-inducible expression vector</td>
<td>Amp&lt;sup&gt;R&lt;/sup&gt;, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Warren et al., 2000</td>
</tr>
</tbody>
</table>

Chapter 4 plasmids
<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Selectant</th>
<th>Author(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMV 449</td>
<td>pLAC22 with STnc1870 insertion</td>
<td>AmpR</td>
<td>This study</td>
<td>oMV 1198 + oMV 1200</td>
</tr>
<tr>
<td>pMV 455</td>
<td>Path 931 (gtr) OFF mutant - 10/-35 sites only CRIM vector</td>
<td>CmR</td>
<td>This study</td>
<td>oMV 412 + oMV 1233</td>
</tr>
<tr>
<td>pMV 456</td>
<td>Path 931 (gtr) OFF mutant full length regulatory region CRIM vector</td>
<td>CmR</td>
<td>This study</td>
<td>oMV 412 + oMV 414</td>
</tr>
<tr>
<td>pMV 458</td>
<td>pMV 449 with point mutations in STnc1870 insert</td>
<td>AmpR</td>
<td>This study</td>
<td>oMV 1257 + oMV 1258</td>
</tr>
</tbody>
</table>

**Chapter 5 plasmids**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Selectant</th>
<th>Author(s)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMV 389</td>
<td>(S.\ Typhi\ 2_II gtr) full length regulatory region CRIM vector</td>
<td>CmR</td>
<td>Edwin Kaptein</td>
<td>oMV 1025 + oMV 1026</td>
</tr>
<tr>
<td>pMV 426</td>
<td>pMV 389 point mutated at (S.\ Typhi\ 2_II gtr) GATC(^4)</td>
<td>CmR</td>
<td>This study</td>
<td>oMV 1061 + oMV 1062</td>
</tr>
<tr>
<td>pMV 430</td>
<td>pMV 389 point mutated at (S.\ Typhi\ 2_II gtr) GATA(^4)</td>
<td>CmR</td>
<td>This study</td>
<td>oMV 1162 + oMV 1163</td>
</tr>
<tr>
<td>pMV 343</td>
<td>(S.\ Infantis_I gtr) full length regulatory region CRIM vector</td>
<td>CmR</td>
<td>This study</td>
<td>oMV 1164 + oMV 1165</td>
</tr>
<tr>
<td>pMV 450</td>
<td>pMV 343 point mutated at (S.\ Infantis_I gtr) TAAC(^1)</td>
<td>CmR</td>
<td>Steven Watson</td>
<td>oMV 1162 + oMV 1163</td>
</tr>
<tr>
<td>pMV 451</td>
<td>pMV 343 point mutated at (S.\ Infantis_I gtr) GATC(^1)</td>
<td>CmR</td>
<td>Steven Watson</td>
<td>oMV 1164 + oMV 1165</td>
</tr>
<tr>
<td>Name</td>
<td>Sequence 5’ → 3’</td>
<td>Orientation</td>
<td>Purpose</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>-------------</td>
<td>----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Chapter 3 primers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>oMV 1043</td>
<td>CAATTGTAGTGTTACACTCCAG</td>
<td>F</td>
<td>LT2_I gtr -10 site point mutation</td>
<td></td>
</tr>
<tr>
<td>oMV 1044</td>
<td>CTGGAGTGTAACACTACAAATTG</td>
<td>R</td>
<td>LT2_I gtr -10 site point mutation</td>
<td></td>
</tr>
<tr>
<td>oMV 804</td>
<td>GGTAGCTCTGCAGATCGTTTATAT CGATCAAAGCAATT</td>
<td>F</td>
<td>Cloning LT2_I gtr -10/-35 sites into CRIM vector</td>
<td></td>
</tr>
<tr>
<td>oMV 1033</td>
<td>GAAATCCGTAACCCCGCTCGTTA TTTACCGCAGCGATAATTAAGACC CACTTTACATT</td>
<td>F</td>
<td>rpoS deletion</td>
<td></td>
</tr>
<tr>
<td>oMV 1034</td>
<td>TTACTCGCGGAACACCGCTTCGAT ATTCAGCCCCTGCGCTCTAAGCAC TTGCTCTCCTG</td>
<td>R</td>
<td>rpoS deletion</td>
<td></td>
</tr>
<tr>
<td>oMV 1035</td>
<td>CCGGCACCAGCTCTACACGC</td>
<td>F</td>
<td>rpoS deletion screening</td>
<td></td>
</tr>
<tr>
<td>oMV 1036</td>
<td>GCTGCTGGCAGAGACAAAC</td>
<td>R</td>
<td>rpoS deletion screening</td>
<td></td>
</tr>
<tr>
<td>oMV 1063</td>
<td>CTGTTATCGCAGGGGCGCAACACA GCG</td>
<td>F</td>
<td>rpoS deletion screening</td>
<td></td>
</tr>
<tr>
<td>oMV 1064</td>
<td>CTGACGAACAGGTTCAGCGTGAA GACCG</td>
<td>R</td>
<td>rpoS deletion screening</td>
<td></td>
</tr>
<tr>
<td>oMV 1057</td>
<td>GGCAGGACAAATGAGAATATTA</td>
<td>F</td>
<td>LT2_I gtr C:tetRA</td>
<td></td>
</tr>
<tr>
<td>oMV</td>
<td>Sequence</td>
<td>Orientation</td>
<td>Insertion Type</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------</td>
<td>-------------</td>
<td>----------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>1058</td>
<td>CGGAAATAATTAATAATTAAGA CCCACTTCACATT</td>
<td>R</td>
<td>LT2-I <code>gtrC::tetRA</code> insertion</td>
<td></td>
</tr>
</tbody>
</table>
| 1070   | CCGCCGCCGCCGCCATTCCCATGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG
<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Amplification Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>oMV 1086</td>
<td>ATGCGCGCTTCACCTTTAAC</td>
<td>F</td>
<td>gtrA qPCR</td>
</tr>
<tr>
<td>oMV 1087</td>
<td>GCCAGGGAACATTTGTACGC</td>
<td>R</td>
<td>gtrA qPCR</td>
</tr>
<tr>
<td>oMV 1084</td>
<td>TGATCCGCTCGTTATCCGC</td>
<td>F</td>
<td>gtrB qPCR</td>
</tr>
<tr>
<td>oMV 1085</td>
<td>ATAGGAATGACCGCATCCCG</td>
<td>R</td>
<td>gtrB qPCR</td>
</tr>
<tr>
<td>oMV 1082</td>
<td>GGAAACCTATCCCGTGCTG</td>
<td>F</td>
<td>gtrC qPCR</td>
</tr>
<tr>
<td>oMV 1083</td>
<td>CCAATATGCCCTTACGCTG</td>
<td>R</td>
<td>gtrC qPCR</td>
</tr>
<tr>
<td>oMV 1099</td>
<td>AAGGATGCGGCTATCCCAA</td>
<td>F</td>
<td>lrp qPCR</td>
</tr>
<tr>
<td>oMV 1100</td>
<td>AGATAATGCGGGTTCAACAG</td>
<td>R</td>
<td>lrp qPCR</td>
</tr>
</tbody>
</table>

Chapter 4 primers

<table>
<thead>
<tr>
<th>Primer ID</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
<th>Amplification Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>oMV 1068</td>
<td>CTATTACGAGCCCTTTAATTGCC</td>
<td>F</td>
<td>lacZ gene amplification from Path 859</td>
</tr>
<tr>
<td>oMV 460</td>
<td>TCGGTGTTATGGTGCAATTGCC</td>
<td>R</td>
<td>lacZ gene amplification from Path 859</td>
</tr>
<tr>
<td>oMV 1194</td>
<td>TGGTACCGATCAATTGGGGC</td>
<td>F</td>
<td>STnc1870 qPCR</td>
</tr>
<tr>
<td>oMV 1195</td>
<td>CTTGGAAGCCTAACTTCAAAAC</td>
<td>R</td>
<td>STnc1870 qPCR</td>
</tr>
<tr>
<td>oMV 1196</td>
<td>CGGTGGCGTCTACTACCTCC</td>
<td>F</td>
<td>yceB qPCR</td>
</tr>
<tr>
<td>oMV 1197</td>
<td>TTGGCGCTTAAAGCGACG</td>
<td>R</td>
<td>yceB qPCR</td>
</tr>
<tr>
<td>oMV 1244</td>
<td>CGCGCTAACAATGCTTAC</td>
<td>F</td>
<td>ssaO qPCR</td>
</tr>
<tr>
<td>oMV 1245</td>
<td>ACCGGGCCATTGTTGTTC</td>
<td>R</td>
<td>ssaO qPCR</td>
</tr>
<tr>
<td>oMV 1198</td>
<td>ATCTGAAGATCTATCTCAATTGATCGTTG</td>
<td>F</td>
<td>Cloning STnc1870 into pLAC22</td>
</tr>
</tbody>
</table>
| oMV 1200 | CTGTATCGGCGCTGAAATGGAC  
|          | GACTATGAA          |
| oMV 1257 | ATTGCCTGCTGCTGTTTACAAA  
|          | ACGGGACACCAAAG       |
| oMV 1258 | TTTGTAAACCAGGGCGCAATC  
|          | AGTAGCCCCCAATTG       |
| oMV 789  | GTGAAAGAAAAAACCACCTGGC |
| oMV 1203 | ATAAACAATAAACTTTAAACTATTTGA  
|          | ATACCACATTATATTTAAGACC  
|          | CACTTTTCACATT        |
| oMV 1204 | TCGGAAAGTCTGAGTGTAAGCA  
|          | CTACAAATTTGCTTTTGATCTAAGCA  
|          | CTTGTCTCTCTG              |
| oMV 1208 | AATAACTTTAAACTATTTGAATACC  
|          | ACATTATGGATAGTTTATATCGAT  
|          | TAAAGCAATTGATGTGCTACACT  
|          | CCAGACCTTTCC               |
| oMV 1233 | GGTAGCTCTGCAGATAGTCTTATAT  
|          | CGATCAAAGCGCAAATT         |
| oMV 412  | CATGATGGAACCTTTCAACACATTAT  
|          | GAAAATCAGCGG             |
| oMV 414  | TTGATCTCCTGCAGCCACGGCTTA  
|          | GATGTTCCTGG              |

- **Cloning STnc1870 into pLAC22**
- **Point mutations of STnc1870 in pMV 449**
- **Point mutations of STnc1870 in pMV 449**
- **Point mutations of STnc1870 in pMV 449**
- **ST4/74_I gtr:tetRA insertion**
- **ST4/74_I gtr:tetRA insertion**
- **ST4/74_I gtr locked OFF mutation**
- **Locked OFF ST4/74_I gtr -10/-35 sites CRIM vector cloning**
- **Locked OFF ST4/74_I gtr CRIM vector cloning**
- **Locked OFF ST4/74_I gtr CRIM**
| oMV 1234 | TAGCGAATAACTTCAACATTATGAAATCAGCGGATTCGGTAGGTAAGACCCACTTTTCACATT | F | STnc1870 upstream deletion (short) |
| oMV 1235 | CTTTAAACTATTGAATACCACATTATGATCGTTTATATCCTAAGCACCATTGTCTCCTG | R | STnc1870 upstream deletion |
| oMV 1236 | GTGGCTTTTTCATTGAAGACCGGATACGACTAAAGATTTTAAGACCACCTTTTCACATT | F | STnc1870 upstream deletion (long) |
| oMV 1237 | CATTATGAAAATCAGCCCGGATTCGTTGAAGACCACCGCCGCGCGCGCAGCGACGGGGATACTGCTATAGTT | F | Short deletion of STnc1870 upstream region |
| oMV 1238 | TTCTTCAATTGAAGACCGGAAACGCATTAAATGTCTTTAAACGATCATAATGTGTTATTCAATAGTT | F | Long deletion of gtrA and STnc1870 upstream region |
| oMV 1256 | AATAACTTCAACATTATGAAAATCAGCGGATTCCACCTGCTGGAGGGACACCTACAACGTCGTTTGCTAGATATAACGATCATAATGTGTTATTCAATAGTT | F | Single bp changes upstream of STnc1870 |
| oMV 808 | GAGAAATTGTGCTGCTGAGAAGATTGGGCACTTTAAGACCACCTTTTACTT | F | OxyR C199S mutation |
| oMV 809 | TCCCGCTTTCAAAACAGAAACCCCATCGCCTGATCGCAGACATAGCAGATATGCTGGTCTTCTCCTG | R | OxyR C199S mutation |
| oMV 810 | GAGAAATTGTGCTGCTGAGAAGATTGGGCACTTTAAGACCACCTTTTACTT | F | OxyR C199S mutation |
| oMV 1025  | TTGATCGTTTATATCGATCAAAGC AATTTGTAGTAC | F | S. Typhi 2_II *gtr*  
GATC<sup>4</sup> point mutation |
| oMV 1026  | TAGTACTACAAATTTGCTTTGATCG ATATAAAACGATC | R | S. Typhi 2_II *gtr*  
GATC<sup>4</sup> point mutation |
| oMV 1061  | TTGATCGTTTATATCGATAAAAGC AATTTGTAGTAC | F | S. Typhi 2_II *gtr*  
GATA<sup>4</sup> point mutation |
| oMV 1062  | TAGTACTACAAATTTGCTTTTATCG ATATAAAACGATC | R | S. Typhi 2_II *gtr*  
GATA<sup>4</sup> point mutation |
| oMV 1162  | TCCAATATCAACCTAACGATGCA ACGATCG | F | S. Infantis_I *gtr*  
TAAC<sup>1</sup> point mutation |
| oMV 1163  | CGATCGTTGCAATCGTTAGGTTGA TATTTGA | R | S. Infantis_I *gtr*  
TAAC<sup>1</sup> point mutation |
| oMV 1164  | TTTCCAATATCAACCGATCGATTG CAACGAT | F | S. Infantis_I *gtr*  
GATC<sup>1</sup> point mutation |
| oMV 1165  | ATCGTTGCAATCGATCGTTTGATA TTGGAAA | R | S. Infantis_I *gtr*  
GATC<sup>1</sup> point mutation |
Table 2.5. Accession numbers for relevant strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>NCBI Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S.</em> Typhimurium strain LT2</td>
<td>NC_003197.1</td>
</tr>
<tr>
<td><em>S.</em> Typhimurium strain ST4/74</td>
<td>NC_016857.1</td>
</tr>
<tr>
<td><em>S.</em> Typhimurium strain SL1344</td>
<td>FQ312003.1</td>
</tr>
<tr>
<td><em>S.</em> Typhimurium strain DT104</td>
<td>HF937208.1</td>
</tr>
<tr>
<td><em>S.</em> Typhimurium strain D23580</td>
<td>FN424405.1</td>
</tr>
<tr>
<td><em>S.</em> Infantis</td>
<td>NZ_LN649235.1</td>
</tr>
<tr>
<td><em>S.</em> Gallinarum</td>
<td>NZ_CM001153.1</td>
</tr>
<tr>
<td><em>S.</em> Typhi strain CT18</td>
<td>NC_003198.1 and AL513382.1</td>
</tr>
<tr>
<td><em>S.</em> Typhi strain B/SF/13/03/195</td>
<td>CP012151.1</td>
</tr>
<tr>
<td><em>S.</em> Typhi strain PM016/13</td>
<td>CP012091.1</td>
</tr>
<tr>
<td><em>S.</em> Typhi strain Ty21a</td>
<td>CP002099.1</td>
</tr>
<tr>
<td><em>S.</em> Typhi strain P-stx-12</td>
<td>CP003278.1</td>
</tr>
<tr>
<td><em>S.</em> Typhi strain Ty2</td>
<td>AE014613.1</td>
</tr>
<tr>
<td><em>S.</em> Abaetetuba</td>
<td>CP007532.1</td>
</tr>
<tr>
<td><em>S.</em> Heidelberg</td>
<td>CP001120.1</td>
</tr>
<tr>
<td><em>S.</em> Javiana</td>
<td>CP004027.1</td>
</tr>
<tr>
<td><em>S.</em> Pullorum</td>
<td>CP012347.1</td>
</tr>
<tr>
<td><em>S.</em> Enteritidis</td>
<td>CP013097.1</td>
</tr>
<tr>
<td><em>S.</em> Bredeney</td>
<td>CP007533.1</td>
</tr>
<tr>
<td><em>S.</em> Montevideo</td>
<td>CP007530.1</td>
</tr>
</tbody>
</table>
2.2 MEDIA

2.2.1 LB media

LB broth was prepared by mixing 20 g of LB (Lennox) powder with one litre of RO water and autoclaving the solution. To make LB plates, 17.5 g of agar were added to the mixture. To grow *S. Typhi* (Path 667) supplements were added at the following final concentrations: 40 μM L-phenylalanine, 40 μM L-tryptophan, 40 μM L-tyrosine, 1 μM para-aminobenzoic acid and 1 μM 2,3-dihydroxybenzoic acid.

2.2.2 SOC medium

SOC media was prepared by mixing 2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM NaCl and 2.5 mM KCl into 1 litre of RO water. The solution was autoclaved and allowed to cool before adding 10 mM MgCl$_2$, 10 mM MgSO$_4$ and 20 mM D-Glucose. The media was then filter sterilised.

2.2.3 tet$^8$ medium

To make 500 ml of tet$^8$ media used in lambda Red counterselection experiments, two flasks (A and B) were prepared according to the method of Bochner et al., 1980. Flask A contained 7.5 g of agar, 2.5 g of tryptone, 2.5 g of yeast extract and 25 mg of chlortetracycline in 450 ml of RO water. Flask B contained 5 g of NaCl (1.71 M) and 5 g of NaH$_2$PO$_4$·H$_2$O (0.72 M) in 50 ml of RO water. Both flasks were autoclaved and cooled to 55°C. 110 μl of a 55 mg/ml stock of fusaric acid (dissolved in dimethylformamide) and 2.5 ml of a 20 mM ZnCl$_2$ stock were added to flask B. Flask A and B were mixed together before pouring into sterile Petri plates. Throughout the process, care was taken to minimise exposure of the plates to light. Plates were always prepared fresh on the day of a counterselection experiment.
2.2.4 M9 medium

For 1 litre of liquid media, 11.3 g of M9 salts (Sigma) were mixed with 1 litre of RO water and autoclaved. The solution was cooled to 55°C, before adding 200 µl of Iron-citrate (0.3 % w/v, 12.20 mM), 1 ml CaCl₂ (0.1 M), 1 ml MgSO₄ (1.0 M), 1 ml Vitamin B1 (1 % w/v) and 10 ml D-Glucose (20 % w/v, 1.11 M). To make 1 litre of solid M9 media, 500 ml of RO water were mixed with 11.3 g of M9 salts and autoclaved. In a separate flask, 17.5 g of agar were mixed with 500 ml of RO water and autoclaved simultaneously. Both flasks were cooled to 55°C, before adding the supplements listed above to the M9 salts flask. If required, 2 ml of X-Gal (5-bromo-4-chloro-3-indyl β-D-galactoside, 2 % w/v in dimethylformamide) and appropriate antibiotics were also added. The agar and M9 flasks were then combined, mixed and poured into plates.

2.2.5 InSPI2 medium

InSPI2 media was prepared according to the recipe of Löber et al., 2006. For 1 litre of liquid media, the following ingredients were combined in 500 ml of MilliQ water: 80 mM MES (morpholino-ethane-sulphonic acid), 4 mM Tricine, 100 µM FeCl₃, 376 µM K₂SO₄, 50 mM NaCl, 22.2 mM D-Glucose, 15 mM NH₄Cl, 1 mM MgSO₄, 0.01 mM CaCl₂, 10 nM Na₂MoO₄, 10 nM Na₂SeO₃, 4 nM H₃BO₃, 300 nM CoCl₂, 100 nM CuSO₄, 800 nM MnCl₂, 1 nM ZnSO₄. 8.5 ml of K₂HPO₄ (0.4 mM) and 91.5 ml of KH₂PO₄ (0.4 mM) were mixed to create a stock solution with a pH of 5.8, of which 10 ml were added to the InSPI2 media. The InSPI2 media was titrated (using NaOH) to pH = 5.8 and the volume made up to 1 litre before filter sterilising. The media was stored at 4°C.

2.2.6 NonSPI2 medium

This media was prepared in the same manner as InSPI2 media, with the following exceptions (Löber et al., 2006): 80 mM MOPS (morpholino-propane-sulphonic acid) was used instead of MES. Additionally, 80.2 ml of K₂HPO₄ (25 mM) and 19.8 ml of KH₂PO₄ (25 mM) were mixed to create a stock solution with a pH of 7.4, of which 10 ml were added to the NonSPI2 media. The solution was titrated to a pH of 7.4. The media was stored at 4°C.
2.2.7 Antibiotics

To select for multicopy plasmids, antibiotics were added to growth media at the following final concentrations: ampicillin (100 µg/ml), tetracycline (15 µg/ml) or chloramphenicol at 30 µg/ml.

To select for single copy resistance markers inserted in the bacterial chromosome, 12.5 µg/ml tetracycline or 8 µg/ml chloramphenicol were added.

2.2.8 Catalase solution

A stock of catalase solution was prepared by mixing 50 ml of MilliQ water with 0.5 g of catalase (2000 – 5000 Units/mg, Sigma). Following filter sterilisation, aliquots were stored at -20°C. 100 µl of thawed catalase solution were spread on plates used for growing strains with mutations in the oxyR gene.

2.3 PCR TECHNIQUES

2.3.1 Colony PCR

Individual colonies were picked from plates and transferred to 25 µl of sterile MilliQ water in Eppendorf tubes. The tubes were then heated at 95°C for 7 minutes. The resulting solution was used directly as template in either Q5 or GoTaq PCR reactions.

2.3.2 Q5 PCR

For PCRs of long products (>1000 bp) or amplifications requiring high fidelity, the proof reading Q5 Hotstart polymerase (NEB) was used. A single reaction with a total volume of 25 µl was set up as follows: a mix of 5 µl 5x Q5 buffer, 0.5 µl dNTPs (10 mM each), 1.25 µl primer 1 (10 µM, Sigma), 1.25 µl primer 2 (10 µM), 5 µl 5x High G/C enhancer and 11.5 µl MilliQ water was prepared. 0.25 µl of template DNA (10-100 ng/µl concentration) was added, followed by 0.25 µl Q5 Hotstart polymerase. The following PCR cycles were used: an initial denaturation was carried out at 98°C for 3 minutes, then 34 cycles of denaturation (98°C, 10
seconds), annealing (55 – 72°C dependent on primers used, 30 seconds), extension (72°C, 30 seconds per kilobase of product). The final extension step was 72°C for 2 minutes followed by an unlimited hold at 4°C. Appropriate annealing temperatures were determined either by gradient PCR, or by using the New England Biolabs Tm calculator (found online at http://tmcalculator.neb.com/#/).

### 2.3.3 GoTaq PCR

For PCRs of short products (<1000 bp) that did not require high fidelity, GoTaq G2 flexi polymerase (Promega Corporation) was used. A single 25 µl reaction contained: 5 µl 5x GoTaq buffer, 2 µl MgCl2, 0.5 µl dNTPs (10mM each), 0.75 µl primer 1 (10 µM), 0.75 µl primer 2 (10µM) and 13.375 µl MilliQ water. 2.5 µl DNA template (10 – 100 ng/µl concentration) were added followed by 0.125 µl of GoTaq G2 flexi polymerase. PCR cycles were as follows: initial denaturation at 95°C for 3 mins, preceding 34 cycles of denaturation (95°C, 30 seconds), annealing (55°C, 30 seconds) and extension (72°C, 1 minute per kilobase of product). The final extension was 95°C for 5 minutes before a 4°C unlimited hold.

### 2.3.4 qRT-PCR

qPCR experiments made use of the Applied Biosystems Fast qPCR machine and mastermix. A single reaction contained 10 µl of mastermix, 7 µl nuclease free water, 0.5 µl of each primer (0.25 µM final concentration) and 2 µl of cDNA template or water for negative controls. The following amplification cycles were used: initial denaturation (95°C, 20 seconds) followed by 40 cycles of 95°C for 3 seconds and a combined annealing and extension step of 60°C for 30 seconds. A melt curve was also produced, consisting of a 15 second 95°C denaturation, a 60°C step for 1 minute and a gradual increase by 0.3°C until reaching 95°C and holding for 15 seconds. To perform absolute quantitations of transcripts, a standard curve was generated using previously amplified PCR products of target genes with known copy number. Copy number of PCR products was calculated using the following formula: ((amount of PCR product in ng) x 6.022 x 10^{23}) / ((length of PCR product in bp) x (1 x 10^9) x 660). An appropriate dilution range of PCR product was then produced for use as a standard curve (typically in the range of 10^2 – 10^6 copies/sample).
2.4 NUCLEIC ACID TECHNIQUES

2.4.1 Genomic DNA extraction

Genomic DNA was isolated following the protocol of Aljanabi and Martinez (1997), from bacterial cultures grown to stationary phase. 1.5 ml of culture was centrifuged for 2 minutes at 13000 rpm in a benchtop microcentrifuge. The supernatant was discarded and the pellet resuspended in 400 µl of sterile salt homogenising buffer (0.4 M NaCl, 10 mM Tris-HCl, 2 mM EDTA, pH = 8.0). 40 µl of a 20 % w/v SDS (sodium dodecyl sulphate) solution was added along with 4 µl of Proteinase K (Roche, 200 µg/ml final concentration). The solution was mixed and incubated overnight at 60°C. 300 µl of 5 M NaCl was added before vortexing at maximum speed for 30 seconds. The sample was centrifuged for 30 minutes at 10,000 g and the supernatant transferred to a fresh tube. An equal volume of isopropanol was added. The mixture was vortexed and incubated at -20°C for 1 hour. Centrifugation was performed at 10,000 g and 4°C. The supernatant was discarded, and the pellet was washed in 100 µl 70 % v/v ethanol. Ethanol was then removed by pipetting and the pellet was left to air dry. Final resuspension of the pellet was performed using 100 µl of sterile MilliQ water. The tube was incubated at 65°C for 1 hour to achieve complete resuspension and then stored at -20°C.

2.4.2 DNA gel separation

Agarose gels used to visualise DNA fragments were prepared containing 1 % w/v agarose mixed with TAE buffer (40 mM Tris, 20 mM Acetic acid, 1 mM EDTA). The solution was boiled in a microwave oven. After cooling, ethidium bromide was added at a final concentration of 0.5 µg/ml. DNA samples were mixed with loading dye before applying them to the gel. Electrophoresis was performed at 100 V for 40 minutes.

2.4.3 DNA/RNA quantification

The concentrations of nucleic acid samples were determined using a Nanodrop machine. After blanking the machine with the same media used in the extraction of the nucleic acid, samples were loaded and measured. Concentrations given in ng/µl as well as ratios of 260/280 nm and 260/230 nm wavelength were recorded to check for presence of contaminants in the sample. For
DNA, a 260/280 ratio of 1.8 was considered an indication of sufficient purity, whereas for RNA a ratio of 2.0 was desired. For the 260/230 ratio, a value of 2.0 – 2.2 was aimed for.

2.4.4 DNA gel extraction

To purify DNA fragments, samples were loaded on to a 50 ml TAE 0.8 % w/v agarose gel containing 10 µl of Nancy-520 dye (Sigma). Electrophoresis was performed at 44 V for 2 hours, or until sufficient separation was achieved. DNA bands were visualised using a source of blue light and excised with a clean scalpel. The excised DNA bands were purified using the QIAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions. DNA was eluted in 30 µl of sterile MilliQ water.

2.4.5 DNA restriction digests

All enzymes and reagents used in restriction digests were supplied by New England Biolabs. Reactions were set up containing up to 1 µg DNA, 5 µl reaction buffer, 1 µl enzyme and nuclease free water made up to a 50 µl total volume. Reactions were incubated at 37°C for one hour, then analysed on agarose gels.

2.4.6 DNA precipitation

To concentrate DNA samples, precipitation by sodium acetate and ethanol was used. 3 M sodium acetate was added at 1/10 of the volume of the DNA sample, along with 2 volumes of 100 v/v % ethanol. The sample was placed at -20°C for 20 minutes, and then centrifuged at 13000 rpm in a cooled (4°C) benchtop microfuge. The supernatant was removed and 1 ml of 70 % v/v ethanol was added. The tube was centrifuged once more and the supernatant removed. The pellet was air-dried on the bench, and then resuspended in the required volume of MilliQ water.
2.4.7 RNA extraction

RNA was extracted from bacterial cultures using the Qiagen RNeasy minikit according to the manufacturer’s instructions (available at www.qiagen.com). Briefly, the protocol was as follows: cultures were grown in sterile, RNAzap treated baffle flasks at 200 rpm in a shaking incubator at 37°C. All cultures were started by diluting an overnight culture 100-fold into 50 ml of fresh media. At OD_{600} = 0.3, 2.5 ml of culture were removed and added to 5 ml of Bacteriaprotect Reagent (Qiagen). After 5 minutes of incubation at room temperature, samples were centrifuged for 10 mins at 5000 g. Supernatants were removed and pellets were resuspended in 200 μl TE buffer containing 15 mg/ml lysozyme (Sigma) and 3 μl proteinase K (Roche, 330 μg/ml final concentration). The solution was transferred to a nuclease-free Eppendorf and vortexed heavily every 2 minutes for a total time of 10 minutes. 700 μl of buffer RLT from the Rneasy kit was added, followed by vortexing. 500 μl of 100 % ethanol were added next. Half the total volume (700 μl) were added to a spin column and centrifuged for 15 seconds at 8000 g. All following centrifugation steps were carried out under the same conditions, unless otherwise indicated. The flow through was discarded and the process was repeated with the remaining 700 μl. Next, 350 μl of buffer RW1 were added, followed by a further centrifugation step and discarding of the flow through. DNase treatment was performed on column using the RNase free DNase kit supplied by Qiagen. 10 μl of DNase I was added to 70 μl of buffer RDD and gently mixed by pipetting before application to the column. DNase treated columns were incubated at room temperature for 15 minutes. 350 μl of buffer RW1 were added next and the columns were left to stand for a further 5 minutes before centrifugation. The spin column was placed in a clean collection tube and 500 μl of RPE buffer (consisting of 100 μl RPE stock mixed with 400 μl 100 % ethanol) were added. After centrifugation and discarding of the flow through, the process was repeated using a 2 minute centrifugation step. Finally, the spin column was placed in a clean Eppendorf tube and 40 μl of RNase-free water were added to the column. After a 1 minute centrifugation step, the eluted RNA was stored at -80°C.

2.4.8 RNA testing for genomic DNA presence

RNA samples were tested for the presence of contaminating genomic DNA using the following method: a small aliquot of RNA was resuspended in 125 μl of sterile buffer solution (60mM Tris-HCl, 1mM EDTA, pH = 6.8) and 2 μl of DNase-free RNase (Roche) were added. This
procedure was repeated for a positive control consisting of 2 μl of a genomic DNA extract. Samples were incubated at 37°C for 4-5 hours and then used as templates in a GoTaq PCR reaction using the same primers to be used in subsequent qPCR experiments. Amplification in the genomic DNA positive control, but not the RNA samples, was considered evidence that the RNA samples were free of genomic DNA.

2.4.9 cDNA synthesis

All ingredients were supplied by Thermo Fisher Scientific (2016), and the manufacturer’s protocol was followed. (available at: https://tools.thermofisher.com/content/sfs/manuals/superscriptIII_man.pdf).

To synthesise cDNA from RNA samples, a mixture of 9 μl RNase free water, 1 μl dNTPs (10mM of each nucleotide) and 1 μl of random primers (250 ng/μl concentration) was added to 2 μl of RNA in a PCR tube (0.2 ml volume). The mixture was heated to 65°C for 5 minutes in a PCR thermocycler and then cooled to 4°C for 1 minute. A second mix of 4 μl 5x First Strand synthesis buffer, 1 μl 0.1 M dithiothreitol and 1 μl RNaseOUT Inhibitor was added. In the final step, 1 μl Superscript III Reverse Transcriptase was added before incubation at 25°C for 5 minutes, then 50°C for 60 minutes, followed by inactivation at 70°C for 15 minutes. cDNA samples were stored at -20°C.

2.4.10 Lambda RED recombination

For lambda Red mediated genetic recombination, the method of Datsenko and Wanner (2000) was used. Cells were grown as described under the protocol for generating electrocompetent cells (section 2.7.2). The only exception was the addition of 0.2 % w/v L-arabinose (13.32 mM, final concentration) to the culture at an OD₆₀₀ of 0.4 - 0.6, followed by one hour further growth. The lambda Red helper plasmid pKD46 used in this procedure is temperature sensitive and therefore requires growth at 30°C in media supplemented with ampicillin (100 μg/ml final concentration). All genetic recombination work involved the insertion of the tetRA resistance marker as a primary step. Furthermore, it was necessary to retain the pKD46 plasmid in these steps for future recombination work. Cells were therefore plated on LB media with tetracycline (12.5 μg/ml final concentration) as well as ampicillin (100 μg/ml final concentration) and grown at 30°C to select for pKD46.
2.4.11 Lambda RED counterselection

In order to replace a chromosomally inserted \textit{tetRA} cassette with alternate DNA sequences, lambda Red mediated counterselection was used according to the method of Datsenko and Wanner (2000). In this process either DNA-oligos or PCR products were used. For both methods, the DNA had 35-40 bp overhangs that matched the chromosomal sequences flanking the \textit{tetRA} cassette. Strains carrying both the \textit{tetRA} cassette in the chromosomal region to be altered and the lambda Red helper plasmid were grown under the following conditions: An overnight culture of the required strain containing 4 ml LB media supplemented with ampicillin (100 mg/ml final concentration) and tetracycline (12.5 mg/ml final concentration) was used to inoculate 50 ml LB in a sterile baffled flask, also with ampicillin (concentration as above), but without tetracycline. Lambda RED genes were induced by L-arabinose addition as described in section 2.4.10. Growth conditions and preparation of electrocompetent cells were as described in section 2.7.2. Cells were transformed by electroporation with 2 μl (~200 ng) DNA or water as a control. Following resuspension in 1 ml prewarmed SOC (37°C) cells were incubated for 1 hour at 37°C and 220 rpm in a shaking incubator. 100 μl aliquots of both the undiluted outgrowth and a 10^-2 dilution were plated on 9 tet^5 plates each. The water transformed control was diluted 10^-5 fold and 100 μl plated in duplicate on both tet^5 and LB media with tetracycline (12.5 μg/ml final concentration). All plates were incubated at 42°C for 18-20 hours. Any colonies appearing on plates of the DNA transformed sample were transferred to a single LB reference plate without antibiotics and numbered. After overnight growth at 37°C, the numbered colonies were streaked for single colonies on to one LB plate each and incubated at 37°C overnight. Single colonies derived from these plates were then picked to a second LB reference plate, numbered and simultaneously tested for tetracycline resistance by streaking onto LB plates supplemented with 12.5 μg/ml tetracycline and growth overnight at 37°C. In this manner up to 40 colonies were screened. Absence of growth on tetracycline plates indicated a possible loss of the \textit{tetRA} cassette. Any colonies shown to be tetracycline sensitive were tested further by PCR and sequencing. Colonies with successful recombination were picked from the second reference plate, streaked to a fresh plate and frozen as described in section 2.7.4.

2.4.12 CRIM vector integration

Integration of CRIM vector into the \textit{Salmonella} chromosome followed the method of Haldimann and Wanner (2001). \textit{S. Typhimurium} strains carrying the temperature sensitive pINT-ts integration helper plasmid were grown at 30°C in 50 ml LB media supplemented with
ampicillin (100 mg/ml final concentration). Other growth conditions and the procedure for generating electrocompetent cells were as described in section 2.7.2. 2μl (~200 ng) of the CRIM vector were transformed into electrocompetent cells, followed by resuspension in 1 ml pre-warmed SOC media (37°C) and transfer to a Universal tube. Outgrowth at 37°C for one hour, and then 42°C for 30 minutes was performed in a shaking incubator at 220 rpm. Selection of cells with successful CRIM vector integration was performed by plating on LB media with chloramphenicol (8 μg/ml final concentration). Integration of CRIM vectors was confirmed by GoTaq PCR using the following primer pairs: oMV472/473, oMV472/430, oMV431/473 and oMV430/431.

### 2.4.13 Plasmid DNA extraction

4 ml overnight cultures in LB media with appropriate antibiotics were set up and grown at the required temperature in a shaking incubator set to 220 rpm. The entire volume was collected by centrifugation at 13000 rpm for 3 minutes in a benchtop microcentrifuge. All further steps were performed using the QIAPrep spin miniprep kit (QIAGEN) according to the manufacturer’s instructions (available at https://www.qiagen.com/gb/shop/sample-technologies/dna/dna-preparation/qiaprep-spin-miniprep-kit/). The purified plasmid DNA was eluted in 30 μl of sterile MilliQ water.

### 2.4.14 PCR purification

PCR products used for sequencing or restriction digests were purified using the QIAquick PCR purification kit (QIAGEN) according to the manufacturer’s instructions (available at https://www.qiagen.com/gb/shop/sample-technologies/dna/dna-clean-up/qiaquick-pcr-purification-kit/). The purified DNA was eluted in 30 μl of sterile MilliQ water.

### 2.4.15 DNA Ligation

T4 Ligase and buffer were acquired from NEB. Ligations of insert and vector DNA were performed according to the manufacturer’s instructions (available at https://www.neb.com/protocols/1/01/01/dna-ligation-with-t4-dna-ligase-m0202) using a 3:1 molar ratio.
2.4.16 Sequencing

All newly constructed plasmids and mutated chromosomal regions were checked by DNA sequencing. Sequencing was performed by Eurofins. Reactions were submitted using the SmartSeq kit (Eurofins) as instructed by the manufacturer. Purified PCR products were used as templates and diluted in nuclease free water.

2.4.17 PCR site directed mutagenesis (SOE-PCR)

The introduction of point mutations in gtr regulatory regions contained in CRIM vectors, followed the method of Ho et al., 1989. Complementary primers were designed containing the desired point mutation with overhangs of 15-18 bp on either side. Two separate Q5 PCR reactions were performed using one of the mutagenesis primers as well as ‘end’ primers which bound the CRIM vector DNA at a location several hundred bp either up- or downstream of the point mutation site. The resulting PCR products were purified by gel excision and extraction as described in section 2.4.4. The two products were then mixed, diluted and added as template in a further Q5 PCR, using only the ‘end’ primers. The template PCR products contained a region of overlap introduced by the mutagenesis primers, which allowed annealing of the strands and subsequent amplification of a single product by the end primers. The single, point mutated product was digested by restriction enzymes and ligated into a CRIM vector.

2.5 REPORTER ASSAY TECHNIQUES

2.5.1 β-galactosidase assays

β-galactosidase assays were performed as described by Miller, 1972. Cultures derived from two independent colonies were grown in M9 minimal media supplemented with glucose. 50 ml of fresh M9 media supplemented 8 µg/ml chloramphenicol were inoculated with 500 µl of overnight S. Typhimurium culture and incubated under shaking conditions (200 rpm) in baffle flasks at 37°C. 50 µl samples were collected in quadruplicate between OD_{600} = 0.3-1.9 and mixed with 950 µl Z-buffer (0.06 M Na_{2}HPO_{4}, 0.04 M NaH_{2}PO_{4}, 0.01 M KCl, 0.001 M MgSO_{4}, 0.05 M β-mercaptoethanol, pH = 7.0) in clean glass tubes. Samples were chilled to 4°C before addition of 50 µl 0.1 % w/v SDS and 100 µl chloroform and vortexing for 20 seconds at
maximum speed. A 5 minute incubation step at 28°C was followed by addition of 200 µl ONPG solution (0.061 M Na₂HPO₄, 0.039 M NaH₂PO₄, 0.0133 M o-nitrophenyl-β-galactoside, pH = 7.0) and brief vortexing. Samples were then incubated at 28°C until a pale yellow colour developed. At this point, 500 µl 1 M Na₂CO₃ was added and mixed by vortexing. Samples were transferred to 1.5 ml Eppendorf tubes and centrifuged for 15 minutes at 13 000 rpm in a tabletop microcentrifuge to remove cell pellets. Supernatants were transferred to cuvettes and their optical absorbance at 420 nm wavelength was measured in a spectrophotometer. The β-galactosidase activity (given in Miller Units) was calculated using the formula (1000 x OD₄₂₀) / (T x V x OD₆₀₀). OD₄₂₀ is the absorbance of the culture supernatant after the β-galactosidase assay, T is the time in minutes between ONPG solution addition and Na₂CO₃ addition, V is the volume of culture used in the assay (given in ml) and OD₆₀₀ is the optical absorbance at 600 nm of the culture when the sample was taken. To account for phase variation, 10⁻⁶ and 10⁻⁷ dilutions of the measured culture were plated in triplicate on M9 agar supplemented with 8 µg/ml chloramphenicol (if required) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Blue and white colonies were counted after 48 hours of incubation at 37°C to determine the percentage of gtr phase ON cells in the population.

2.5.2 Fluorescence measurement

Strains expressing GFP were grown in M9 media with appropriate antibiotics to OD₆₀₀ = 0.3 before collecting 50 µl samples. The samples were pipetted into a 96-well plate and chilled to 4°C before commencing measurements. OD₆₀₀ values of the culture were recorded after each sample extraction. Fluorescence measurement was performed using a FluoSTAR OPTIMA (BMG Labtechnologies) plate reader set with an excitation filter at 485 nm and an emission filter at 520 nm wavelength.

2.6 LPS ANALYSIS TECHNIQUES

2.6.1 LPS sample preparation

LPS samples were prepared in the following manner: 1 colony of the relevant strain was picked to 4 ml of M9 minimal media (supplemented with appropriate antibiotics) and incubated on a shaker (200 rpm) at 37°C overnight. 1ml of the overnight culture was pelleted by centrifugation
for 5 minutes at 13,000 rpm. The supernatant was discarded and the pellet was resuspended in 1ml of a sterile 0.9 % w/v NaCl solution. This wash step was repeated 2 more times. After the final wash, the pellet was resuspended in 125 μl of a sterile 60mM Tris-HCl, 1mM EDTA, 2 % w/v SDS (pH=6.8) solution, vortexed and boiled at 95°C for 5 minutes. 875 μl of a sterile 60mM Tris-HCl, 1mM EDTA (pH=6.8) solution were added followed by 2 μl each of RNase and DNase. The sample was then incubated at 37°C for 4-5 hours. 5 μl of Proteinase K (110 μg /ml final concentration, Roche) were added and the sample was incubated overnight at 50°C. Prior to loading on TSDS-PAGE gels, 60 μl of a sample prepared in the above way was mixed with 40 μl of loading buffer (6 % w/v SDS, 6 % v/v β-mercaptoethanol, 10 mM dithiothreitol, 46 % v/v glycerol, 60 mM Tris (pH=8.0) and 0.1 % w/v bromophenol blue).

2.6.2 TSDS-PAGE

Gels for separation of LPS samples were prepared in the following manner (modified from Lesse et al., 1990). Two glass plates were cleaned successively with detergent, acetone and 70 % v/v ethanol. The plates were bound into a cassette using binder clips and separated by spacers of ~2 mm thickness. The edges of the cassette were sealed using 2 % w/v agarose. A 10.5 % separating gel was prepared containing 8.3 ml of gel buffer (3.0 M Tris, pH = 8.45, 0.3 % w/v SDS), 6.25 ml 40 % acrylamide (BioRad, 29:1 ratio), 2.6 ml glycerol and 7.85 ml MilliQ water in a total volume of 25 ml. 150 μl of an aqueous 10 % w/v ammonium persulphate solution (0.44 M) and 15 μl of TEMED (tetramethylethylenediamine) were added to start polymerisation. The separating gel was poured into the gel cassette. An aqueous solution of 50 % v/v butanol was poured on top to exclude air during polymerisation. A 4 % stacking gel was prepared containing 3.1 ml of gel buffer, 1.25 ml of acrylamide and 8.15 ml of MilliQ water. 100 μl of 10 % w/v ammonium persulphate and 10 μl of TEMED were added to start polymerisation. The stacking gel was poured onto the separating gel (after removing the butanol by decanting) and a comb was placed on top to form wells. The top of the gel cassette was wrapped in plastic foil to exclude air during polymerisation. To run the gel, the lower end was placed in a tank (after removing the spacer at this end) containing the anode buffer (0.2 M Tris, pH = 8.9) and the upper end was placed in a tank containing cathode buffer (0.1 M Tris, 0.1 M Tricine, 0.1 % w/v SDS, pH = 8.25). The comb was removed and the wells were washed by injecting cathode buffer. After sample loading (40 μl per well), the gel was electrophoresed at 50 V until the sample had passed through the stacking gel (~4 hours). Following this, the voltage was increased to 100 V until the samples reached the end of the separating gel (~18 hours).
2.6.3 Silver staining

TSDS-PAGE gels of separated LPS samples were silver stained similar to the method described in Kittelberger and Hilbink, 1993. Gels were placed in thoroughly cleaned trays with fixative solution (30 % v/v ethanol, 10 % v/v glacial acetic acid, 60 % v/v ultrapure MilliQ water, 200 ml total) and left for a minimum of two days with gentle agitation on an orbital shaker. The fixative solution was discarded and replaced by fresh fixative after one day. All the following steps were carried out using the same tray. Ultrapure MilliQ water was used to make all the solutions. For each step, the previous solution was removed from the tray and the gel resuspended in the appropriate new solution. Gels were oxidised in oxidiser solution [30 % v/v ethanol, 10 % v/v acetic acid, 60 % v/v water, 1.4 g periodic acid (H₅IO₆ 30.71 mM), 200 ml total] for 10 minutes. Three 15 minute washes were performed using 400 ml of water before silver staining for 30 minutes. The silver stain solution consisted of 200 ml water with 0.2 g silver nitrate (AgNO₃, 5.89 mM). During silver staining, the tray was wrapped in aluminium foil. After the silver stain and a brief rinse of the gel in water, the developer solution was added (200 ml MilliQ water, 6 g Na₂CO₃, 0.02 % v/v formaldehyde). The gel was kept in the developer solution until the LPS bands were sufficiently stained (normally for ~40 minutes). The staining reaction was stopped by washing the gel several times in MilliQ water. Gels were then sealed in plastic sheets and scanned.

2.7 MISCELLANEOUS TECHNIQUES

2.7.1 Switch frequency determination

Switch frequency analysis was performed according to the method of Blyn et al., 1989. Single blue or white colonies were picked from M9 plates with added X-Gal and resuspended with thorough vortexing in 1 ml sterile liquid M9 media. From this, 10⁻⁴ and 10⁻⁵ serial dilutions were produced and plated on M9 plates with X-Gal and antibiotics if required. For both the 10⁻⁴ and 10⁻⁵ dilutions, 5 replicate plates were prepared by spreading 150 µl onto each plate. After incubation at 37°C for 24 hours, blue and white colonies were counted. The switch frequency was calculated using the formula (M/N)/g. M is the number of colonies that switched colour compared to the ancestor colony, N is the total number of colonies counted and g is the number of generations. g is calculated as the log of the total number of cells in a dilution sample divided by log2.
2.7.2 Preparing electrocompetent cells

Electrocompetent cells of *E. coli* and *S. Typhimurium* were prepared by inoculating overnight cultures at 100-fold dilution into 50 ml of appropriate growth media with antibiotics as required in a baffle flask (250 ml volume). Growth at the required temperature (37°C for most applications, 30°C for strains containing temperature sensitive plasmids pKD46 or PINT-ts) at 200 rpm in a shaking incubator continued until the OD\(_{600}\) of the culture reached 0.4 – 0.6. At this point, cultures were placed on ice for 20 minutes with occasional swirling, and then transferred to chilled Falcon tubes. Centrifugation at 4000 rpm in a tabletop centrifuge was performed for 15 minutes at 4°C. Supernatants were discarded and cell pellets gently resuspended in equal volumes of ice-cold sterile MilliQ water before centrifugation at 4000 rpm for 8 minutes at 4°C. This washing process was repeated 3 – 5 times. After the final wash step, pellets were resuspended in 75 – 100 µl sterile ice-cold MilliQ water.

2.7.3 Electroporation

50 µl of electrocompetent cells were mixed by pipetting with 2 µl of DNA or MilliQ water (as a control) in chilled Eppendorf tubes, left to stand for 1 minute and then transferred to chilled Biorad cuvettes. Transformation was performed by a Biorad Electroporator. The machine was set to the Ec1 setting for transformation of *E. coli* and Ec2 for *Salmonella*. Following electroporation, cells were quickly resuspended in 1 ml of pre-warmed SOC media (37°C or 30°C depending on the application). Resuspended cultures were transferred to Universal tubes and incubated for 1 hour at the appropriate temperature at 220 rpm in a shaking incubator. Cultures were plated on appropriate media with antibiotics as required.

2.7.4 Freezer stock preparation

Any new strains generated in this work were stored for future use by freezing. A single colony was streaked across the entire surface of an LB plate with antibiotics as required and incubated at the appropriate temperature overnight. A sterile cotton swab was used to collect the bacterial growth and transfer it to a 2 ml screw cap Corning tube containing 1.5 ml filter sterilised LB media with 15 % v/v glycerol. All tubes were labelled and stored at -80°C.
2.8 BIOINFORMATIC TECHNIQUES

2.8.1 Primer design

Primers used in qPCR experiments were designed using the NCBI primer design tool found online at http://www.ncbi.nlm.nih.gov/tools/primer-blast/. The chosen parameters were an annealing temperature of 58 - 60°C and a length of 20 +/- 3 bp, with a product size between 80 and 150 bp.

2.8.2 Sequence alignments

Alignments of two sequences were generated using the BLAST tool, found online at http://blast.ncbi.nlm.nih.gov/Blast.cgi. Alignments of more than two sequences were generated using the ClustalW2 Multiple Sequence Alignment tool found online at http://www.ebi.ac.uk/Tools/msa/clustalw2/.

2.8.3 Phylogenetic trees

Phylogenetic trees were generated using the MUSCLE tool found online at http://www.ebi.ac.uk/Tools/msa/muscle/.

2.8.4 sRNA target prediction

To predict potential interaction targets of sRNA molecules, the TargetRNA2 program was used (found online at http://tempest.wellesley.edu/~btjaden/TargetRNA2/index.html) (Kery et al., 2014). A further program, RNAfold was used to predict RNA structures (Gruber et al., 2008; Lorenz et al., 2011).
CHAPTER 3
INFLUENCE OF GROWTH CONDITIONS ON $gtr$ REGULATION
3.1 INFLUENCE OF OXIDATIVE STRESS ON gtr EXPRESSION

3.1.1 The OxyR regulator

Phase variation of gtr operons requires binding of the OxyR protein to the gtr regulatory region (Broadbent et al., 2010). As mentioned, the OxyR protein performs an important role in the cell as an oxidative stress response regulator (Storz et al., 1990). OxyR shifts conformation via formation of a disulphide bridge between cysteine residues in response to H$_2$O$_2$ exposure (Choi et al., 2001).

Salmonella is typically exposed to H$_2$O$_2$ in two environments it occupies during its life cycle. Firstly, when entering the host intestine, Salmonella virulence genes trigger an inflammatory response by the immune system which involves secretion of reactive oxygen species (ROS) into the lumen. ROS include the superoxide radical O$_2^-$ and H$_2$O$_2$, which are produced by the enzyme NADPH oxidase of neutrophil cells (Babior, 2004). As mentioned previously, the presence of ROS benefits Salmonella as it utilises tetrathionate formed in the lumen under these conditions to grow by anaerobic respiration and outcompete the resident microbiota (see chapter 1, section 1.1.2) (Winter et al., 2010).

The second environment with H$_2$O$_2$ exposure is the Salmonella containing vacuole (SCV) that the bacteria establish when infecting macrophage cells. Macrophage produce around 10 μM of H$_2$O$_2$ as one of several mechanisms intended to kill bacteria (Aussel et al., 2011). The oxidative stress response of OxyR helps Salmonella survive in both the intestine and the SCV.

Unless reactive oxygen species are applied to Salmonella from external sources, the bacterial cytoplasm is thought to be a reducing environment. OxyR would therefore predominately be present in the reduced form. Cellular disulphide bond reducing pathways (gor andtrxB) are likely to contribute to maintaining the reduced form of OxyR (Van Dat Nguyen et al., 2011). Reduced OxyR is known to be sufficient for phase variation (Broadbent et al., 2010). However, an area worth further study concerns the interaction of oxidised OxyR with the gtr regulatory region.
The spacing of the OxyR binding half sites in the gtr regulatory region is consistent with the binding pattern of reduced OxyR (two half sites separated by one turn of the DNA helix, Broadbent et al., 2010; Toledano et al., 1994). This raised the following questions: can oxidised OxyR (which binds four adjacent half sites) bind the gtr regulatory region? Or is this prevented by the spacing of the OxyR half sites? If oxidised OxyR can bind, or is prevented from doing so, this could affect gtr phase variation or transcription.

To address these questions a method was required to induce the oxidised form of OxyR while measuring transcriptional activity as well as phase variation of the gtr regulatory region. Although a variety of oxidising chemicals are readily available (such as H$_2$O$_2$) these may be rapidly detoxified by the cell, making their effects short-lived. The ON or OFF state of gtr is thought to be maintained until genome replication commences (Broadbent et al., 2010). Several bacterial divisions would need to occur before any difference in switch frequency becomes noticeable. The ideal method would therefore induce persistently oxidised OxyR over a long time period but without being detrimental to cell growth.

### 3.1.2 Inducing persistently oxidised OxyR

As previously mentioned, oxidised OxyR is characterised by the formation of disulphide bonds between cysteine residues. Disulphide bonds within proteins can also be formed by disulphide bond isomerases and sulfhydryl oxidases. These enzymes were used by Van Dat Nguyen et al. (2011) to induce disulphide bond formation in a variety of eukaryotic proteins expressed within *E. coli*. It was hypothesised, that disulphide bond isomerase and sulfhydryl oxidase be used to induce the oxidised form of OxyR.

In order to test this idea, the plasmid pFH255 (provided by L. Ruddock), expressing the Erv1p sulfhydryl oxidase and the DsbC disulphide bond isomerase under transcriptional control of an L-arabinose inducible promoter was transformed into *S. Typhimurium* LT2. To determine whether Erv1p and DsbC could induce the oxidised form of OxyR, a further plasmid, pLA40 (provided by F. Barras) was transformed into the same strain (designated as Path 760). Plasmid pLA40 contains the regulatory region of the *Salmonella ahpC* gene in a transcriptional fusion to the *gfp* reporter gene. Oxidised OxyR stimulates RNA polymerase transcription of the *ahpC* gene. Therefore, in strains carrying the pLA40 plasmid, oxidative stress causes oxidised OxyR to increase transcription of the *gfp* gene, which can be measured as elevated fluorescence.
(Aussel et al., 2011). It was hypothesised that Erv1p and DsbC expression could have a similar effect.

Four separate Path 760 cultures were grown in M9 minimal media. After reaching an optical density of \( \text{OD}_{600} = 0.3 \), fluorescence and optical density were measured at intervals of 20-40 minutes (Fig. 3.1). Following the second measurement, two of the cultures were treated with L-arabinose (leaving the other two cultures as untreated controls) to induce production of Erv1p and DsbC, and measurements continued.

The results showed increased fluorescence (measured as fluorescence/optical density) in the induced cultures 100 minutes after L-arabinose addition, compared to the untreated controls. This trend continued for subsequent measurements, with fluorescence increasing up to an average of 51,944 units for the L-arabinose induced cultures, compared to 28,090 units for the uninduced control cultures. L-arabinose addition therefore clearly resulted in increased fluorescence produced by Path 760. This data supported the hypothesis that Erv1p and DsbC could oxidise OxyR, which in turn stimulated transcription of \( \text{ahpC} \), as evidenced by elevated GFP fluorescence.

GFP contains two cysteine residues (C49 and C71) that Erv1p and DsbC could potentially act upon. However, in correctly folded GFP, these residues are too far displaced (2.4 nm) to form a disulphide bond (Ormo et al., 1996). Should a disulphide bond still form, it would compromise the tight \( \beta \)-barrel structure required for GFP fluorescence. It is therefore unlikely that the observed increase in fluorescence was caused by Erv1p and DsbC assisting GFP folding. As mentioned, Erv1p and DsbC were previously used to assist in the folding of eukaryotic proteins with disulphide bridges expressed in the \( \text{E. coli} \) cytoplasm (Van Dat Nguyen et al., 2011).

Importantly, the increased fluorescence of the induced cultures was maintained over a period of ~100 minutes, which indicated that OxyR was kept in a persistently oxidised state during this time. The cultures all continued growth during the experiment as well. The two main criteria of this method to induce oxidised OxyR (long term oxidation while allowing cell growth) were therefore satisfied. This method was applied to study \( \text{gtr} \) expression in the presence of oxidised OxyR.
Fig. 3.1. Fluorescence levels of Path 760 containing pFH255 (Erv1p + DsbC) and pLA40 (ahpC:gfp) with and without arabinose addition

Four biological duplicate cultures were grown to OD$_{600}$ = 0.3 in M9 minimal media, before extracting samples for fluorescence measurement. After the second timepoint 0.5 % w/v L-arabinose was added to two of the cultures (indicated by red arrow). Growth was continued with periodic sample extraction for fluorescence measurement until stationary phase was reached.
3.1.3 Influence of oxidised OxyR on \textit{gtr} phase variation

To study \textit{gtr} phase variation and expression in the presence of oxidised OxyR, the pFH255 plasmid was transformed into \textit{S. Typhimurium} LT2 strains with fusions of a \textit{gtr} regulatory region and the \textit{lacZ} reporter gene. The \textit{gtr:lacZ} fusions were contained in plasmids that integrated as single copies into the chromosome (Haldimann and Wanner, 2001). The \textit{gtr} regulatory regions used consisted of either the 275 bp ‘full length’ P22 \textit{gtr} regulatory region (Path 728), or a truncated version (Path 732) (Broadbent et al., 2010). The full length version contained the entire sequence required for phase variation, i.e. OxyR and GATC sites, along with the -10 and -35 sequences necessary for RNA polymerase recruitment. The truncated version contained only the OxyR C site and the -35 and -10 sites (Fig. 3.3).

Initially, the potential effect of persistently oxidised OxyR on \textit{gtr} phase variation was explored. A Path 728 colony, containing the full length P22 \textit{gtr: lacZ} fusion construct and the pFH255 plasmid was resuspended, diluted and spread plated on M9 minimal media agar plates supplemented with X-Gal with or without L-arabinose. If expressed, the \textit{lacZ} gene produces \(\beta\)-galactosidase which cleaves X-Gal and releases a blue dye, causing colonies to grow blue. A blue colony phenotype indicates that the \textit{gtr} regulatory region controlling expression of \textit{lacZ} is in the ON state. Should the \textit{gtr} region be OFF, \textit{lacZ} is not expressed in high enough levels and the colony grows white.

Following incubation, the blue and white Path 728 colonies were counted. If induction of Erv1p and DsbC from pFH255 by L-arabinose caused a difference in switch frequency, it would result in a difference in the ratio of ON/OFF colonies, compared to colonies on plates without L-arabinose. On plates with arabinose, 89% of colonies had a switched ON phenotype (total counted = 646). On plates without arabinose, 93% of colonies were switched ON (total counted = 511). A similar result was obtained for Path 730, containing the full length LT2_I \textit{gtr: lacZ} fusion and pFH255. A single colony that was resuspended and plated on M9 X-Gal plates with or without L-arabinose gave the following counts: 55% ON (total counted = 244) on L-arabinose supplemented plates and 50% ON for non-arabinose plates (total counted = 437). These differences were not considered substantial enough to warrant further investigation. It was concluded that expression of Erv1p and DsbC did not affect \textit{gtr} switch frequency.
3.1.4 Influence of oxidised OxyR on gtr transcription

The strains used in the previous experiments (Path 728, 730, 732) were next used to determine the effect of Erv1p and DsbC expression on the level of gtr transcription. Blue colonies of each strain (indicating switched ON gtr regulatory regions upstream of lacZ) were picked to set up separate cultures, which were grown in M9 minimal media to an optical density of OD$_{600} = 0.3$ (Fig.3.2a/b/c). Samples were taken at 30-60 minute intervals to determine the amount of β-galactosidase enzyme produced from the lacZ gene, which provided a measure of the transcriptional activity of the upstream gtr region. After two measurements, each culture was split equally to separate flasks. To induce expression of pFH255 oxidising factors, L-arabinose was added to one half each, keeping the other half as an untreated control. This procedure was necessary due to phase variation of the gtr: lacZ fusions; comparison of β-galactosidase expression levels between induced and uninduced cultures would only be meaningful if both cultures had the same percentage of ON cells. Splitting a single population ensured this was the case.

The β-galactosidase levels (given in Miller Units) of the induced and uninduced cultures of Path 728 showed significant differences at 150 minutes after arabinose addition ($P < 0.05$, t-Test). The preceding and subsequent timepoints did not show significant differences ($P > 0.05$, t-Test) (Fig.3.2a). For Path 730, significant differences between induced and uninduced cultures were measured at 180 and 255 minutes after arabinose addition ($P < 0.05$, t-Test). The preceding timepoints did not show significant differences ($P > 0.05$, t-Test) (Fig.3.2b).

Although some significant differences were measured, the data did not appear to show a clear trend, especially when compared to the experiments with the ahpC:gfp reporter system (see section 3.2.1). Furthermore, the truncated P22 gtr: lacZ fusion strain (Path 732) which did not contain OxyR binding sites upstream of lacZ showed no significant differences between induced and uninduced cultures (t-Test, $P > 0.05$) (Fig.3.2c). On a final note, the optical densities (OD$_{600}$) of arabinose induced and uninduced cultures of Path 728, 730 and 732 did not show substantial differences. It was concluded that expression of Erv1p and DsbC probably did not affect gtr: lacZ expression levels under these conditions.
Fig. 3.2a. β-galactosidase assay of ‘full length’ P22 gtr: lacZ fusion (Path 728) with expression of Erv1p and DsbC.

After growth in M9 minimal media to OD$_{600}$ = 0.3 samples were extracted periodically for β-galactosidase measurement (given in Miller Units). The culture was split equally after the 30 minute timepoint and 0.5 % w/v L-arabinose was added to one half to induce expression of Erv1p and DsbC. Sample collection (from both cultures) for the β-galactosidase assay was continued. Statistical analysis was by t-Test. * $P < 0.05$. Error bars show +/- 2 standard deviation for 3 technical repeats.
Fig.3.2b. β-galactosidase assay of ‘full length’ LT2_I gtr: lacZ fusion (Path 730) with expression of Erv1p and DsbC.

After growth in M9 minimal media to OD₆₀₀ = 0.3 samples were extracted periodically for β-galactosidase measurement (given in Miller Units). The culture was split equally after the 30 minute timepoint and 0.5 % w/v L-arabinose was added to one half to induce expression of Erv1p and DsbC. Sample collection (from both cultures) for the β-galactosidase assay was continued. Statistical analysis was by t-Test. * P < 0.05. Error bars show +/- 2 standard deviation for 3 technical repeats.
Fig.3.2c. β-galactosidase assay of ‘truncated’ P22 gtr: lacZ fusion (Path 732) with expression of Erv1p and DsbC.

After growth in M9 minimal media to OD$_{600}$ = 0.3 samples were extracted periodically for β-galactosidase measurement (given in Miller Units). The culture was split equally after the 30 minute timepoint and 0.5 % w/v L-arabinose was added to one half to induce expression of Erv1p and DsbC. Sample collection (from both cultures) for the β-galactosidase assay was continued. Error bars show +/- 2 standard deviation for 3 technical repeats.
3.2 INFLUENCE OF STATIONARY PHASE GROWTH ON gtr EXPRESSION

3.2.1 Expression of LT2_I gtr:lacZ increases between exponential and stationary phase

In the preceding experiments it was noticed that β-galactosidase expression of all strains increased during growth from exponential to stationary phase, regardless of arabinose treatment. This information was considered worth investigating further.

*S. Typhimurium* LT2 carrying the full length LT2_I gtr:lacZ reporter was utilised (Path 84, Broadbent et al., 2010) (Fig.3.3). Duplicate cultures of Path 84 were grown in M9 minimal media to an optical density of OD\(_{600}\) = 0.3. This point was termed early exponential phase (‘EEP’), and a sample was taken for β-galactosidase measurement. Further growth continued to late exponential phase (‘LEP’, OD\(_{600}\) = 1.0) and up to early stationary phase (‘ESP’, OD\(_{600}\) = 1.8). Additional β-galactosidase measurements were taken at LEP and ESP. The results are shown in Fig.3.4a. Relative to early exponential phase, expression of β-galactosidase increased by 105% in early stationary phase (Table 3.1). This result indicated that transcription from the LT2_I gtr regulatory region increased between exponential and stationary phase.

3.2.2 The promoter region is sufficient for LT2_I gtr:lacZ expression increases

A further experiment sought to determine whether the observed relative increase in LT2_I gtr:lacZ expression between exponential and stationary growth phase was dependant on the presence of the phase variation sequences (OxyR and GATC sites), or required only the promoter region, i.e. the -10 and -35 sequences. The Path 460 *S. Typhimurium* LT2 strain contains a single copy inserted fusion of the LT2_I gtr promoter region (only the -10/-35 sequences) to the lacZ gene and does not phase vary (Broadbent et al., 2010) (Fig.3.3). Duplicate cultures of Path 460 were grown under identical conditions as described previously in section 3.3.1. A β-galactosidase assay was performed on samples collected during early and late exponential phase as well as early stationary phase. The results are shown in Fig.3.4b. In this case, a 118% increase in β-galactosidase expression was measured between early exponential and early stationary phase (Table 3.1). The absence of the LT2_I gtr phase variation sequence probably did not affect the relative expression increases of the gtr:lacZ reporter fusion. It was
Fig. 3.3. Graph of gtr regulatory regions used in this chapter as part of reporter fusions with the lacZ gene contained in the CRIM vector

The red box delineates gtr regulatory sequences inserted into the CRIM vector for transcriptional studies. OxyR half sites are in red. GATC sites, gtr -35, -10 and +1 sites are annotated. The lacZ gene is represented by a white arrow. A, full length gtr regulatory region containing all three OxyR half sites and the -10/-35/+1 sites present in Path 84, Path 805, Path 728 and Path 730. B, truncated gtr regulatory region containing only the -35/-10/+1 sites present in Path 460, Path 943 and Path 732. Graph not to scale.
Fig. 3.4. β-galactosidase levels of gtr: lacZ fusion strains grown from exponential to stationary phase

All strains were grown in M9 minimal media. EEP, Early Exponential Phase (OD$_{600}$ = 0.3), LEP, Late Exponential Phase (OD$_{600}$ = 1.0), ESP, Early Stationary Phase (OD$_{600}$ = 1.9). Miller Units are calculated for 100% ON cultures where applicable. Results are representative of 2 biological repeats. Error bars show +/- 2 standard deviation for 4 technical repeats.

A, Path 84 (full length LT2_I gtr regulatory region lacZ fusion). B, Path 460 (only -10/-35 sites LT2_I gtr regulatory region lacZ fusion). C, Path 805 (full length, point mutated -10 site LT2_I gtr regulatory region lacZ fusion). D, Path 943 (only -10/-35 sites, point mutated -10 site LT2_I gtr regulatory region lacZ fusion).
Table 3.1. Comparison of β-galactosidase expression levels between exponential and stationary phase of LT2_I gtr: lacZ reporter fusion strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Miller Units – Early Exponential Phase$^a$</th>
<th>Miller Units – Early Stationary Phase$^a$</th>
<th>$P$ value</th>
<th>Relative increase (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 84 (full length LT2_I gtr: lacZ)</td>
<td>890 (80)</td>
<td>1827 (117)</td>
<td>&lt; 0.01</td>
<td>105 %</td>
</tr>
<tr>
<td>Path 460 (only -10/-35 sites LT2_I gtr: lacZ)</td>
<td>224 (6)</td>
<td>488 (20)</td>
<td>&lt; 0.001</td>
<td>118 %</td>
</tr>
<tr>
<td>Path 805 (full length LT2_I gtr: lacZ,-13 C→T point mutation)</td>
<td>1245 (95)</td>
<td>1565 (103)</td>
<td>&lt; 0.05</td>
<td>26 %</td>
</tr>
<tr>
<td>Path 943 (only -10/-35 sites LT2_I gtr: lacZ,-13 C→T point mutation)</td>
<td>421 (13)</td>
<td>666 (32)</td>
<td>&lt; 0.01</td>
<td>58 %</td>
</tr>
</tbody>
</table>

$^a$ Miller Units are given as averages of 4 technical repeats, with the standard deviation in brackets. Path 84 and Path 805 results were calculated for 100% ON cultures (originally 75% and 72% ON, respectively). Statistical analysis was by t-Test.
concluded that the LT2_I gtr promoter region was sufficient to yield growth phase dependent expression increases.

3.2.3 *gtr* operons have a conserved -10 site that is characteristic of $\sigma^{38}$ dependent promoters

The LT2_I gtr promoter region was found to have a -10 site with a C base immediately upstream at the -13 position. This configuration is a common feature of promoters that are bound by an alternate sigma factor known as $\sigma^{38}$. Sigma factors bind the -10 and -35 sites of genes before recruiting RNA polymerase, allowing transcription to occur (Madigan et al., 2005). A C base at the -13 position is conserved among >70% of all experimentally confirmed $\sigma^{38}$ promoters (Weber et al., 2005).

$\sigma^{38}$ (or RpoS) is commonly referred to as the ‘stress response sigma factor’. It enables cells to respond to adverse conditions such as starvation, temperature shock, pH shock or DNA damage by increasing transcription of genes that help survival of the stress situation (reviewed in Battesti et al., 2011). The sigma factor used by the cell predominately under non-stress conditions is the ‘housekeeping’ sigma factor $\sigma^{70}$. $\sigma^{38}$ and $\sigma^{70}$ can often bind the same promoter regions, but certain features (such as a C base at position -13 or a T at -14) can make a promoter more specific for $\sigma^{38}$. In the absence of stress, $\sigma^{38}$ is present in low amounts. Stress conditions results in $\sigma^{38}$ amounts increasing through both increased transcription and translation as well as reduced degradation. $\sigma^{38}$ then binds promoters of the genes required for the stress response and competes with $\sigma^{70}$ for recruitment of RNA polymerase to initiate transcription (reviewed in Typas et al., 2007). Should *gtr* be a $\sigma^{38}$ dependant promoter, it would mean that *gtr* expression is highest under stress conditions.

The conservation of the C base at the -13 position was explored among a dataset of 60 predicted *gtr* operons (derived from Davies et al., 2013). The C base was present in 45 *gtr* operons (Fig.3.5). One interesting exception was the *S. Infantis*_II gtr, which had a T base at the -13 position, but otherwise showed high similarity to the other *gtr* sequences, as shown in an alignment of the regulatory regions. The T base could alter expression of *S. Infantis*_II gtr, specifically by reducing the ability of the promoter to recruit $\sigma^{38}$ under stress conditions. By extension, replacing the C base at -13 with a T base in LT2_I gtr could reduce the potential increases of the $\beta$-galactosidase levels measured between exponential and stationary phase.
Fig. 3.5. Alignment of the regulatory regions of 46 gtr operons (dataset derived from Davies et al., 2013)

The predicted -35 and -10 sites are bold and underlined. The conserved C base preceding the -10 site is highlighted green. The S. Infantis_II gtr operon contains a T base at this position (highlighted in red).
3.2.4 Altering the -13 position of LT2_I gtr reduces proportional expression increases

A point mutation was introduced in the full length LT2_I gtr regulatory region upstream of the lacZ gene by changing the C base at the -13 site to a T. This substitution produced a -10 site that matched the -10 site present in S. Infantis_II gtr. The point mutated vector was integrated as a single copy into the S. Typhimurium LT2 chromosome (= Path 805). A β-galactosidase assay performed using this strain showed a relative increase of 26 % between early exponential and early stationary phase (Fig.3.4c, Table 3.1). By comparison, the same construct with C at the -13 position showed a 105 % increase (Path 84). This was considered an indication that altering the promoter in this way may have reduced affinity of the σ38 factor to the gtr regulatory region.

In a further experiment, the -10/-35 sequence only LT2_I gtr: lacZ fusion was altered by introducing a point mutation in the same manner as above (-13 C → T) and integrated as a single copy into the S. Typhimurium LT2 chromosome (= Path 943). A β-galactosidase assay of this strain showed a 58 % increase between early exponential and early stationary phase (Fig.3.4d, Table 3.1) which was lower than the same LT2_I gtrC: lacZ fusion with C at the -13 position (= Path 460, 118 % increase). These experiments indicated that the C base at -13 was important to achieve higher relative β-galactosidase expression in stationary phase compared to exponential phase. This pointed to the possibility that σ38 was involved in LT2_I gtr transcription.

3.2.5 Analysis of LT2_I gtr expression using qRT-PCR

It was decided that an ideal method to confirm the β-galactosidase findings on gtr expression would make use of quantitative RT-PCR to determine the actual transcript levels of the chromosomal gtrABC operon. qRT-PCR was likely to produce more accurate data than β-galactosidase assays, which measured the amount of reporter enzyme produced by an artificial fusion of the gtr regulatory region to the lacZ gene. A fusion of lacZ to the gtrABC operon could also be used to select colonies containing either modified or unmodified O-antigen molecules. This would provide a useful tool to study e.g. whether gtr modification can provide a survival benefit in certain environments.
To study transcription of the gtr operon using qRT-PCR, the phase varying LT2_I gtr operon was chosen as a model system. This presented the following challenge: measuring gtr expression would require a population of cells that were actually expressing the gtr operon at the time of measurement. Phase variation of gtr could complicate this, as e.g. absence of expression under a particular condition could be caused either by low transcription, or simply switched OFF gtr regulatory regions. A method was therefore needed to identify and select for gtr ON cells.

The lacZ gene was inserted as a transcriptional fusion downstream of the LT2_I gtrABC operon in S. Typhimurium LT2 using lambdaRed recombination (= Path 859, Datsenko and Wanner, 2000). The gtrC stop codon was separated from the lacZ start codon by 20 bp, which contained an additional ribosome binding site. This way, if gtr switched ON, both the gtr operon as well as the lacZ gene would be transcribed. The strain could therefore be grown on X-Gal containing media, with ON colonies growing blue and OFF colonies growing white (Fig. 3.6).

To confirm that the blue or white phenotype indicated the ON/OFF state of gtr operons of cells in a colony, culture samples derived from blue and white colonies were lysed and the LPS molecules were extracted. The LPS samples were separated on TSDS-PAGE gels and visualised by silver staining (Fig. 3.7). Blue colonies were predicted to contain more cells expressing gtrABC::lacZ than white colonies. Addition of glucose to the O-antigen subunits by gtrABC produces an LPS molecule with higher overall molecular weight. As a result, blue colonies were likely to produce more of the modified, larger LPS molecules than white colonies. The larger LPS molecules are retained more by the TSDS-PAGE gel, which causes shifts when compared to unmodified LPS molecules.

LPS molecules on TSDS-PAGE gels form a ladder, as they separate according to size. The lowest ‘rungs’ of the ladder contain LPS molecules with the lowest amount of O-antigen subunits attached. The rung above will contain LPS molecules with one extra O-antigen subunit, and so on. The ladder patterns formed by LPS molecules of blue colonies matched with the pattern formed by a positive control strain (constitutive LT2_I gtr expression, compare lanes 4 and 5), but not with the pattern formed by a negative control strain (knockout of all gtr operons, compare lanes 3 and 4). The patterns formed by white colonies did not match with either the positive or negative control strains, but appeared to be an intermediate of the two (compare lanes 1, 2 and 3). This could be explained by the fact that phase variation still occurred in this strain, therefore a small fraction of the white (OFF) population would likely still
Diluted culture samples were spread plated on M9 media supplemented with X-Gal to count the percentage of blue (gtr ON) and white (gtr OFF) colonies.
Fig. 3.7. TSDS-PAGE gel separation of Path 859 (gtrC: lacZ fusion) LPS samples visualised by silver stain

Lanes 1 and 5, Path 292 (positive control, constitutive LT2_I gtr modification). Lane 2, Path 859 white colony. Lane 3, Path 293 (negative control, knockout of all known gtr operons). Lane 4, Path 859 blue colony. Regions in which gel shifts are apparent are highlighted by red boxes. Results are representative of 3 biological repeats.
Fig.3.8. qRT-PCR results of gtrC:lacZ fusion strains grown in M9 minimal media from exponential to stationary phase

A, Path 859 (LT2_1 gtrC:lacZ fusion). B, Path 885 (LT2_1 gtrC:lacZ fusion, ΔrpoS). EEP, Early Exponential Phase (OD\textsubscript{600} = 0.3), ESP, Early Stationary Phase (OD\textsubscript{600} = 1.9). Expression levels of gtrABC were normalised to the lrp gene. Results are representative of two biological repeats.
be expressing the LT2_I gtr modification. This result supported the interpretation that the gtr operon was transcribed more in blue colonies than in white colonies.

ON colonies of Path 859 were selected and grown in minimal media from exponential to stationary phase. RNA samples were extracted for qRT-PCR during early exponential (OD$_{600} = 0.3$) and early stationary phase (OD$_{600} = 1.8$). Primers used in qPCR amplified from within the open reading frames of gtrA (see Table 2.4, oMV1086/1087) gtrB (oMV1084/1085) and gtrC (oMV1082/1083). Surprisingly, the qRT-PCR results showed that expression of gtrA, gtrB and gtrC was actually reduced in stationary phase, relative to exponential phase (0.4, 0.4 and 0.1 relative quantity, respectively; $P < 0.01$, t-Test) (Fig.3.8a).

This result by itself did not rule out the possibility of gtr transcription in stationary phase involving $\sigma^{38}$. If the gtr promoter was transcribed by both $\sigma^{70}$ and $\sigma^{38}$, it may show lower amounts of expression in the stationary phase. Furthermore, $\beta$-galactosidase results showed that gtr was expressed during exponential phase. Therefore, gtr was not considered likely to be exclusively dependent on $\sigma^{38}$ for transcription.

To fully determine whether or not $\sigma^{38}$ was required for gtr transcription in stationary phase, the rpoS gene coding for $\sigma^{38}$ was deleted by lambdaRed recombination (Path 885) (Datsenko and Wanner, 2000). The resulting strain was grown from exponential to early stationary phase as described above and RNA was extracted. A growth defect of Path 885 was not observed, which could mean that $\sigma^{38}$ may not be required for survival under these conditions. The qRT-PCR results showed very little difference to the wildtype strain, with relative quantities of gtrA, gtrB and gtrC of 0.4, 0.3 and 0.2 respectively, in stationary phase compared to exponential phase ($P < 0.01$, t-Test) (Fig.3.8b). Should $\sigma^{38}$ be required for gtr expression in stationary phase, there would likely have been a larger reduction in relative expression compared to the wildtype strain. This led to the conclusion that gtr expression is probably $\sigma^{38}$ independent.

### 3.3 INFLUENCE OF LOW pH ON LT2_I gtr

A particularly interesting aspect of Salmonella’s life cycle concerns the infection of macrophage. Following entry into the macrophage, a specialised compartment is formed (the Salmonella containing vacuole, SCV) that the bacteria survive in. Conditions in the SCV are
quite adversarial, involving nutrient limitation and injection of factors intended to kill the bacteria. Among these killing mechanisms are reactive oxygen species as previously mentioned (see section 3.2), as well as antimicrobial peptides and acidification.

Acidification (pH ~ 5.8) of the SCV, micromolar concentrations of divalent cations (Mg$^{2+}$, Ca$^{2+}$, Mn$^{2+}$) and antimicrobial peptides induce the regulatory system PhoPQ. Once activated, PhoPQ acts to induce surface remodelling of the bacteria, including LPS modification, which provides resistance against antimicrobial peptides (Navarre et al., 2005). PhoPQ mediated forms of LPS modifications include palmitoylation or addition of acyl groups to the lipid A molecule (reviewed in Ernst et al., 2001).

PhoQ is a histidine kinase located in the inner membrane, with a sensor domain extending into the periplasm as well as a cytoplasmic region performing kinase and phosphatase functions. PhoQ autophosphorylates when given the correct signals and subsequently, phosphate is transferred to PhoP. Phosphorylated PhoP in turn binds DNA to either repress or induce transcription (Monsieurs et al., 2005).

PhoP also influences a further transcriptional regulator, SlyA. Many of the genes required for antimicrobial peptide resistance that are regulated by PhoPQ, are co-regulated by SlyA. The consensus SlyA binding site has been denoted as TTAGCAAGCTAA, which forms an imperfect inverted repeat (Stapleton et al., 2002). The LT2_I gtr regulatory region was found to contain a sequence between the 2nd GATC site and the OxyR B site, with 9 of the 12 bp matching the SlyA consensus (TTAatAAGaTAA, mismatching bases are in lower case). Should SlyA be able to bind LT2_I gtr, it would potentially interfere with transcription or phase variation.

An experiment was designed that made use of Path 84 (containing the full length LT2_I gtr: lacZ fusion) and Path 460 ( -10/-35 sites only LT2_I gtr: lacZ). The gtr: lacZ fusion in Path 84 contains the predicted SlyA site, while the gtr: lacZ fusion in Path 460 does not. Should SlyA have a noticeable effect on LT2_I gtr: lacZ expression, it would only be visible in Path 84.

To induce the PhoPQ and SlyA systems, a specialised defined media named InSPI2 was used (Löber et al., 2006). InSPI2 media has a pH of 5.8 and contains 1 mM MgSO_4_, 10 μM CaCl_2_ and 0.8 μM MnCl_2_. A further media, NonSPI2 with a pH of 7.4 and different buffer, but
otherwise identical composition was used to grow non-induced control cultures (Kröger et al., 2013). Transcription of \textit{phoP}, \textit{phoQ} and \textit{slyA} is increased in InSPI2 media compared to NonSPI2 media (1.57, 1.55 and 2.09 –fold upregulation, respectively; Kröger et al., 2013).

Path 84 and 460 were grown in NonSPI2 media to an optical density of OD600 = 0.3. At this point, samples were taken to measure β-galactosidase activity. The cultures were then split equally and one half was centrifuged to collect the cells, while the other half was allowed to continue growth. The centrifuged cells were resuspended in InSPI2 media, to induce higher expression of PhoPQ and SlyA. β-galactosidase measurements were continued at 30 and 60 minutes after culture splitting. The results of this assay showed no significant difference in β-galactosidase levels between samples from InSPI2 and NonSPI2 cultures ($P > 0.05$, t-Test) (Fig. 3.9). This result was obtained for both Path 84 and Path 460. Furthermore, plate counts of Path 84 showed very little difference in the percentage of ON cells in cultures grown in NonSPI2 media (64 % ON, total counted 166) and InSPI2 media (69 % ON, total counted 220). These results led to the conclusion that low pH does not noticeably affect \textit{LT2\_I gtr} expression or phase variation.

### 3.4 DISCUSSION

The expression of Erv1p and DsbC did not alter \textit{gtr: lacZ} expression or phase variation. However it did appear that Erv1p and DsbC were capable of inducing the oxidised form of OxyR, as evidenced by data obtained using the \textit{ahpC:gfp} reporter construct. Three possible conclusions can be drawn from this. Perhaps oxidised OxyR does not affect \textit{gtr} expression in any way that is noticeably different from the reduced form. On the other hand, reduced OxyR may have remained bound to the \textit{gtr} regulatory region and not become oxidised by Erv1p and DsbC. Alternatively, the method used to induce oxidised OxyR affects \textit{ahpC} differently than \textit{gtr}.

The spacing of OxyR half sites in the \textit{ahpC} regulatory region is consistent with binding of oxidised OxyR (Zheng et al., 2001a). In the absence of oxidative stress (or Erv1p/DsbC expression) OxyR is likely to be in the reduced form, in which it may not be able to bind the \textit{ahpC} regulatory region. However, once oxidised, OxyR could bind \textit{ahpC} without competition and stimulate transcription.
Fig. 3.9. β-galactosidase assay of gtr: lacZ fusion strains grown in NonSPI2 (pH = 7.4) and InSPI2 media (pH = 5.8)

Path 84 (full length LT2_I gtr: lacZ fusion) and Path 460 (only -10/-35 sites LT2_I gtr: lacZ fusion) were grown in NonSPI2 media (pH = 7.4) before splitting cultures at OD$_{600}$ = 0.3 and resuspending half in InSPI2 media (pH = 5.8). β-galactosidase measurements were taken immediately before and 30 and 60 minutes after the cultures were split. Results are representative of two biological repeats. Error bars show +/- 2 standard deviation for 4 technical repeats.
On the other hand, OxyR half sites in the gtr regulatory region are spaced in a way that is more consistent with binding of reduced OxyR. It is also known that persistently reduced OxyR is sufficient to allow gtr phase variation and expression (Broadbent et al., 2010). OxyR binding at the gtr regulatory region is maintained until genome replication commences. This is exemplified by the OFF state of gtr. The absence of gtr expression in the OFF phase indicates that OxyR persistently binds gtr under non-oxidising conditions.

Expression of Erv1p and DsbC may cause cytoplasmic (non DNA bound) OxyR to become oxidised. However, it is not known whether Erv1p and DsbC can oxidise OxyR already bound to DNA. Should this not be possible, the gtr bound OxyR would remain in the reduced state. OxyR bound to gtr is only predicted to leave during genome replication. At this point, oxidised OxyR may have the opportunity to bind gtr. However, the spacing of the half sites suggests that this may not be possible.

If every OxyR molecule in the cell were oxidised, it could impact on gtr expression or phase variation, but neither showed differences in the experiments presented here. It is possible that Erv1p and DsbC oxidise a fraction of the cell’s OxyR molecules, but the reducing environment of the cytoplasm ensures that a number of reduced OxyR molecules are still available for gtr binding. This could explain why no differences were observed under these conditions.

The experiments using NonSPI2 and InSPI2 media showed that there was little difference in gtr expression between these two conditions. As other experiments showed that growth in InSPI2 media coupled with a H₂O₂ shock caused gtr expression to decrease (see chapter 4), it was important to note that InSPI2 media did not inherently cause reduced gtr expression. Ideally this should be confirmed by qRT-PCR.

qRT-PCR revealed that LT2_1 gtr expression is reduced under stationary phase growth. This result contradicted those obtained using the gtr: lacZ fusion system. However the qRT-PCR experiment is likely to have produced the more reliable data, as it measured transcription of the gtr operon, as opposed to expression levels of the β-galactosidase protein. The lack of difference between the qRT-PCR data of the wildtype and Δ rpoS deletion mutant showed that σ₃₈ is probably not required for gtr transcription during stationary phase. The increases seen in the lacZ experiments may be explained by accumulation of the β-galactosidase enzyme in the growth media and the cells. Regarding the point mutations, these may have affected how the σ₇₀

91
housekeeping sigma factor transcribes *gtr* and therefore given misleading results as to the importance of the C base at position -13. In a similar example, G at the -14 position was found to enhance overall promoter activity (Barne et al., 1997).

Overall, the chromosomal *gtr*:lacZ fusion strain proved to be a useful tool for any future studies on *gtr* expression, as it allowed more accurate qRT-PCR experiments to be performed as well as determining the switch frequency of the chromosomal *gtr* operon. Finally, no indication was found of low pH influencing *gtr* transcription or phase variation. This would indicate that PhoPQ and SlyA are not involved in *gtr* regulation. Furthermore, as the InSPI2 media used is intended to mimic the SCV compartment of the macrophage, this would indicate that *gtr* modification of the O-antigen is possible in this environment.
CHAPTER 4

INVESTIGATION OF A SHORT RNA MOLECULE PRODUCED BY THE $gtr$
REGULATORY REGION
4.1 INTRODUCTION

The original incentive for the work in this chapter came from the publication of RNA sequencing data by Kröger et al., (2013). The data showed that a short (110 bp) RNA designated ‘STnc1870’ was transcribed from the S. Typhimurium ST4/74 gtr regulatory region under conditions that mimic the Salmonella Containing Vacuole (SCV) of macrophage. The conditions used involved growth in InSPI2 media, which induces expression of the Salmonella Pathogenicity Island 2 (SPI2) genes, as well as addition of 1 mM hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}).

The RNA sequencing data also showed that high expression of STnc1870 (7,830 transcripts per million) coincided with very low expression of gtrABC (~3 transcripts per million). This information could be interpreted in two ways: either high expression of STnc1870 coincided with downregulation of gtrABC, or the sample that was used for RNA sequencing only contained cells in the gtr OFF phase. It was not possible to exclude either option with only the RNA sequencing data or protocol details available.

This chapter focuses on the regulation of STnc1870 as well as gtr during growth in InSPI2 media with H\textsubscript{2}O\textsubscript{2} treatment. Furthermore, the possible function of STnc1870 in the cell is explored.
4.2 THE \textit{gtr} REGULATORY REGION PRODUCES A SHORT RNA MOLECULE UNDER MACROPHAGE-LIKE CONDITIONS

A BLAST search found that the STnc1870 sequence was conserved (98-100\% identical) in the following \textit{Salmonella} serovars: Typhimurium (including strains LT2, ST4/74, SL1344, DT104, D23580), Abaetetuba, Heidelberg, Javiana, Gallinarum, Pullorum, Enteritidis, Bredeney and Montevideo. The predicted 5' → 3' translation of the STnc1870 sRNA sequence did not contain start codons or open reading frames. The transcriptional start site of STnc1870 was located between the OxyR A and B half sites (Fig.4.1 and Fig.4.2). The predicted -10 and -35 sites were placed within the OxyR B and C sites, respectively. Previous work showed OxyR binding to the \textit{gtr} regulatory region was persistent. It was inferred that OxyR binding was released during genome replication (Broadbent et al., 2010). OxyR binding in the OFF position involves occupation of the \textit{gtr} -35 site and as a result \textit{gtr} transcription is halted. It was thought that STnc1870 transcription could also be repressed by OxyR binding in either the ON or OFF position, as either state requires occupation of the OxyR B half site and therefore the STnc1870 -10 site.

Initially, the conditions of the RNA sequencing experiment were replicated to confirm that upregulation of STnc1870 transcription occurred following growth in InSPI2 media with \textit{H}_2\textit{O}_2 treatment. A further aim was to precisely quantify any potential effects of these conditions on \textit{gtr} phase variation and expression, as this data could not be acquired from the RNA sequencing data. \textit{S. Typhimurium} ST4/74 was chosen as model organism as this was the same strain used in the RNA sequencing experiments. ST4/74_I \textit{gtrABC} is identical to LT2_I \textit{gtrABC}.

To measure potential effects on \textit{gtr} phase variation, the \textit{lacZ} gene was inserted downstream of the chromosomal \textit{gtrC} gene using lambdaRed recombination, generating the Path 891 strain (Datsenko and Wanner, 2000). This approach created a transcriptional fusion of ST4/74_I \textit{gtrABC::lacZ}, similar to the LT2_I \textit{gtrABC::lacZ} fusion strain produced in section 3.3.5 (the only difference between the two was the strain background). A 20 bp gap between the \textit{gtrC} stop and \textit{lacZ} start codon contained an additional ribosome binding site to improve \(\beta\)-galactosidase production. Phase variation of the \textit{gtrABC::lacZ} fusion produced either blue or white colonies on X-Gal media (Fig.4.3a). This enabled selection of colonies containing cells with predominately ON (blue phenotype) or OFF (white phenotype) \textit{gtr} operons.
**Fig. 4.1. Overview of the gtr regulatory region**

Graph shows annotated GATC sites, OxyR half sites (in red) and -10/-35 sites of gtrABC and STnc1870. Arrowheads indicate position of transcriptional start sites. Graph not to scale.

**Fig. 4.2. Detailed sequences of the gtr regulatory region.**

The -10, -35 and +1 sites of gtr and STnc1870 are marked in red and annotated. GATC sites are marked by grey boxes. OxyR sites are underlined and annotated A, B or C. The STnc1870 and gtrABC mRNA sequences are marked by red and white arrows, respectively. The Path 931 mutation performed in this chapter is marked by a black box around red letters.
To confirm that the blue or white phenotype indicated the ON/OFF state of \textit{gtr} operons of cells in a colony, culture samples derived from blue and white colonies were lysed to extract LPS molecules. The LPS samples were separated on TSDS-PAGE gels and visualised by silver staining.

Blue colonies were predicted to contain more cells expressing \textit{gtrABC::lacZ} than white colonies. As a result, blue colonies were likely to produce more of the \textit{gtr} modified, larger LPS molecules than white colonies. The larger LPS molecules are retained more by the TSDS-PAGE gel, which causes shifts when compared to unmodified LPS molecules. The ladder patterns formed by LPS molecules of blue colonies matched with the pattern formed by a positive control strain (constitutive LT2\_I \textit{gtr} expression, compare lanes 2 and 3), but not with the pattern formed by a negative control strain (knockout of all \textit{gtr} operons, lane 1) (Fig.4.4a). The patterns formed by white colonies did not match with either the positive or negative control strains, but appeared to be an intermediate of the two (compare lanes 3, 4 and 5). This could be explained by the fact that phase variation still occurred in this strain, therefore a small fraction of the white (OFF) population would likely still be expressing the ST4/74\_1 \textit{gtr} modification. This result supported the interpretation that the \textit{gtr} operon was transcribed more in blue colonies than in white colonies.

Two blue and two white colonies of Path 891 were grown in InSPI2 media before splitting each culture equally. 1 mM H\textsubscript{2}O\textsubscript{2} was added to one half of each culture. This method accounted for phase variation of \textit{gtr}, by ensuring that both H\textsubscript{2}O\textsubscript{2} treated cultures and untreated controls contained (at least initially) the same proportion of ON cells (Fig.4.5). Growth was continued for 10 minutes followed by RNA extraction. The cultures were allowed to continue growth for ~2 hours and were diluted and spread plated on X-Gal media to determine the percentage of ON cells by counting (ON cells produce blue colonies). The optical density of H\textsubscript{2}O\textsubscript{2} treated cultures did not increase during this period and plate counts showed that approximately 80 % of cells were killed by the treatment. Subsequent experiments with lower amounts (0.1 and 0.01 mM) of H\textsubscript{2}O\textsubscript{2} showed that this absence of growth after treatment was dose-dependant – for example, addition of 0.01 mM H\textsubscript{2}O\textsubscript{2} did not noticeably affect growth (Fig.4.5). Plate counts showed only marginal differences in the percentage of ON cells between 1 mM H\textsubscript{2}O\textsubscript{2} treated and untreated cultures.

qRT-PCR was performed on the extracted RNA samples using absolute quantitation (via a standard curve of PCR products with known copy number) and relative quantitation (with the
Fig. 4.3. Colony phenotypes of S. Typhimurium ST4/74_1 gtrC: lacZ strains

Fig. 4.4. TSDS-PAGE gel separation of gtrABC::lacZ fusion strains Path 891 and Path 931 LPS samples visualised by silver stain.

Regions in which gel shifts are apparent are highlighted by red boxes. Representative of three biological repeats.

A, Lanes 1 and 5, Path 293 (negative control, knockout of all known gtr operons). Lane 2, Path 891 blue colony. Lane 3, Path 292 (positive control, constitutive LT2_I gtr modification). Lane 4, Path 891 white colony.

B, Lanes 1 and 5, Path 293 (negative control, knockout of all known gtr operons). Lanes 2 and 4, Path 931 white colonies (biological replicates). Lane 3, Path 292 (positive control, constitutive LT2_I gtr modification).
Fig. 4.5. Growth curve of Path 891 in InSPI2 media +/- H₂O₂ treatment

Red arrow indicates splitting of culture and addition of 1 mM, 0.1 mM or 0.01 mM hydrogen peroxide to separate samples. An untreated control was also used.
The yceB gene as reference – expression of this gene was not affected under the tested conditions, Kröger et al., 2013). The results showed that STnc1870 transcription increased significantly ($P < 0.05$, t-Test) (12.82 – 25.33 relative quantity) after peroxide addition, regardless of the percentage of ON cells in the tested culture. Concurrently, gtrA expression was reduced after peroxide addition (0.05 – 0.18 relative quantity, $P < 0.05$, t-Test) in all H$_2$O$_2$ treated samples (Fig.4.6 and Table 4.1).

The above experiment confirmed the RNA sequencing data and also established that STnc1870 transcription could be induced from cultures with predominately ON or OFF cells. In addition, STnc1870 induction by H$_2$O$_2$ treatment did not heritably alter the percentage of ON cells in a culture.

4.3 USING A LOCKED OFF MUTANT gtr TO TEST STnc1870 TRANSCRIPTION

The next question concerned transcriptional control of STnc1870. It appeared that the mechanism allowing STnc1870 transcription was not influenced by gtr phase variation. Furthermore, the optical density of H$_2$O$_2$ treated cultures did not increase during this period (Fig.4.5). This suggested that ongoing genome replication was not required for STnc1870 transcription.

The promoter region of STnc1870 overlaps with the OxyR B and C half sites in the gtr regulatory region (Fig.4.2). OxyR binding in either the ON or OFF state would require occupation of the B site which is likely to cause repression of STnc1870. This argument is supported by the gtr OFF state – OxyR binding of the B and C half sites coincides with gtr repression (Broadbent et al., 2010).

If OxyR binding of the gtr regulatory region caused STnc1870 repression, it would probably do so persistently: a gtr OFF state could not be established without persistent OxyR mediated repression. Genome replication was thought to be the only condition under which OxyR ceases gtr binding, although this was not required for STnc1870 transcription. So, if genome replication was the only way for OxyR to cease gtr binding, STnc1870 should have stayed repressed under the tested conditions. This was clearly not the case for H$_2$O$_2$ treated cultures.
Fig. 4.6. Absolute *gtrA* and STnc1870 expression of Path 891 (*gtrABC::lacZ*) +/- 1 mM H$_2$O$_2$ treatment

qRT-PCR results showing absolute quantities of transcript copy numbers. ON or OFF indicates the phenotype of the colony the culture was derived from, i.e. blue or white, respectively. Samples taken from hydrogen peroxide treated cultures are annotated ‘+ H2O2’. Results are representative of two biological repeats. Error bars show +/- 2 standard deviation for 4 technical repeats.
Table 4.1. Relative gtrA and STnc1870 expression of Path 891 +/- 1 mM H₂O₂

<table>
<thead>
<tr>
<th>Sample</th>
<th>% ON</th>
<th>+/- H₂O₂</th>
<th>Target</th>
<th>Relative Quantitya (RQ)</th>
<th>RQ Minimum</th>
<th>RQ Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>85 % ON cultureb</td>
<td></td>
<td></td>
<td>gtrA</td>
<td>1</td>
<td>0.63</td>
<td>1.59</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>STnc1870</td>
<td>1</td>
<td>0.72</td>
<td>1.39</td>
</tr>
<tr>
<td>86 %</td>
<td></td>
<td></td>
<td>gtrA</td>
<td>0.05</td>
<td>0.04</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>STnc1870 12.82</td>
<td>9.92</td>
<td>16.58</td>
<td></td>
</tr>
<tr>
<td>10 % OFF cultureb</td>
<td></td>
<td></td>
<td>gtrA</td>
<td>1</td>
<td>0.60</td>
<td>1.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>STnc1870</td>
<td>1</td>
<td>0.63</td>
<td>1.59</td>
</tr>
<tr>
<td>13 %</td>
<td></td>
<td></td>
<td>gtrA</td>
<td>0.18</td>
<td>0.12</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>STnc1870 25.33</td>
<td>20.49</td>
<td>31.32</td>
<td></td>
</tr>
</tbody>
</table>

a. For each culture, the non - H₂O₂ treated sample was used with the yceB gene as a reference to calculate relative quantities of gtrA and STnc1870 in H₂O₂ treated samples. Results are representative of two biological repeats.

b. ON or OFF indicates the phenotype of the colony the culture was derived from, i.e. blue or white, respectively, with plate counts used to give % ON.
Could an alternative mechanism remove OxyR from *gtr* in H$_2$O$_2$ treated cultures, thereby allowing STnc1870 transcription?

The next experiments aimed to test the hypothesis that H$_2$O$_2$ treatment caused OxyR removal from the *gtr* regulatory region. Specifically, a method was needed to indicate that OxyR was actually bound to the *gtr* regulatory region before attempting to remove it by H$_2$O$_2$ treatment. It was decided to lock OxyR in the *gtr* repressing OFF state, as the resulting lack of *gtr* transcription would produce a distinctive phenotype. This in turn could be used to infer persistent OxyR binding of the *gtr* regulatory region. If H$_2$O$_2$ treatment could still induce STnc1870 transcription, this would confirm that OxyR removal from *gtr* was possible.

The locked OFF mutation was chosen in favour of a locked ON mutant, because *gtr* transcription is possible without OxyR (as evidenced by fusions of just the -10/-35 *gtr* sites to the *lacZ* gene (Broadbent et al., 2010)). *gtr* repression however is thought to be completely dependent on OxyR binding.

The Path 891 *gtrC*:lacZ fusion strain was mutated using lambdaRed recombination to alter the 3rd and 4th GATC sites, which overlap with the OxyR C site (Datsenko and Wanner, 2000). Previous results had shown that point mutations in both GATC sites in a single OxyR half site (without altering the OxyR binding site consensus sequence) abrogated phase variation (Broadbent et al., 2010). The 3rd and 4th GATC sites were changed to GATA and GATT, respectively, which generated the Path 931 strain (Fig.4.2). The altered sites were predicted to be unavailable for Dam methylation, while still allowing OxyR binding. Theoretically, OxyR would be able to access all three OxyR binding sites in this mutated *gtr* regulatory region, allowing phase variation to occur. In fact, phase variation was not detectable when plating this strain on X-Gal media, as all colonies (over 4000 screened) had a white (Lac-) phenotype (Fig.4.3b). In addition, TSDS-PAGE gel separation of LPS samples revealed that locked OFF mutant colonies did not produce modified LPS molecules (Fig.4.4b). This supported the interpretation that OxyR persistently bound the mutated *gtr* regulatory region in the OFF state.

The point mutations used to create the locked OFF *gtr* mutant also altered a single base in the *gtr* -35 site (Fig.4.2). If this mutation, although unlikely, prevented RNA polymerase recruitment to *gtr*, it could result in an OFF phenotype without OxyR repression. To exclude
this scenario, the ‘full length’ gtr OFF mutant regulatory region (containing all OxyR sites) was fused to the lacZ gene and inserted into a CRIM vector. This vector was integrated as a single copy into the chromosome of S. Typhimurium LT2, generating Path 948 (Haldimann and Wanner, 2001). The same procedure was performed for a lacZ fusion of the ‘promoter only’ gtr OFF mutant regulatory region, which contained only the -10 and -35 sequences (= Path 945).

The promoter only fusion, which did not contain a complete OxyR binding site, was predicted to show constitutive lacZ expression, unless the altered -35 site interfered with transcription. The full length fusion however, should remain repressed by OxyR binding. A β-galactosidase assay showed that the promoter only region was expressed at an average level of 343 Miller Units (which was very similar to the Path 460 wildtype LT2_I gtr (identical to ST4/74_I gtr) promoter only fusion), whereas the full length version showed an average of only 3 Miller Units (Fig.4.7). This demonstrated that the promoter region was functional and allowed RNA polymerase recruitment and transcription. The full length version was therefore more likely to be repressed due to OxyR binding rather than a non-functional promoter. These conclusions were expanded to the chromosomal gtr OFF mutant – the absence of gtrABC expression in the locked OFF gtr Path 931 strain was more likely to be caused by OxyR repression of gtrABC (and STnc1870) rather than a non-functional -35 site.

To test whether STnc1870 could still be induced in the locked OFF gtr mutant Path 931, the previous qRT-PCR experiment was replicated (i.e. growth in InSPI2 media to OD600 = 0.3, followed culture splitting, 1 mM H2O2 treatment of half and RNA extraction after 10 minutes). Cultures did not increase in optical density after H2O2 treatment. qRT-PCR performed on the extracted RNA samples showed that STnc1870 was still highly induced after H2O2 treatment, compared to untreated samples (53.39 and 93.80 relative quantity, P < 0.001, t-Test). Interestingly, gtrA expression was not reduced and in one sample increased slightly after H2O2 treatment, compared to untreated samples (1.45 and 2.39 relative quantity – P < 0.05 and not significant, respectively, t-Test.) (Fig.4.8 and Table 4.2).

The locked OFF phenotype of Path 931 gave a good indication of persistent OxyR binding to the B and C half sites, which was predicted to repress STnc1870. The finding that STnc1870 was still inducible by H2O2 treatment supported the hypothesis that an unknown mechanism removed OxyR from the gtr regulatory region to allow STnc1870 transcription.
Fig. 4.7. β-galactosidase results of *gtr: lacZ* fusion strains to test functionality of Path 931 *gtr* promoter region

Path 460 (-10/-35 sites only wildtype LT2_I *gtr: lacZ*), Path 945 (-10/-35 sites only *gtr* OFF mutant *lacZ* fusion) and Path 948 (full length *gtr* OFF mutant *lacZ* fusion) were grown in minimal M9 media from exponential to stationary phase. EEP, Early Exponential Phase (OD$_{600} = 0.3$), LEP, Late Exponential Phase (OD$_{600} = 1.0$), ESP, Early Stationary Phase (OD$_{600} = 1.9$). Results are representative of 2 biological repeats. Error bars show +/- 2 standard deviation for 4 technical repeats.
Fig. 4.8. Absolute *gtrA* and STnc1870 expression of Path 931 +/- 1 mM H$_2$O$_2$ treatment

qRT-PCR results showing absolute quantities of transcript copy numbers. Error bars show +/- 2 standard deviation for 4 technical repeats.
Table 4.2. Relative gtrA and STnc1870 expression of Path 931 +/- H$_2$O$_2$ treatment

<table>
<thead>
<tr>
<th>Sample</th>
<th>+/- H$_2$O$_2$</th>
<th>Target</th>
<th>Relative Quantity$^a$ (RQ)</th>
<th>RQ Minimum</th>
<th>RQ Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture 1</td>
<td>-</td>
<td>gtrA</td>
<td>1</td>
<td>0.91</td>
<td>1.10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>STnc1870</td>
<td>1</td>
<td>0.63</td>
<td>1.60</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>gtrA</td>
<td>1.45</td>
<td>1.27</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>STnc1870</td>
<td>53.39</td>
<td>49.94</td>
<td>57.07</td>
</tr>
<tr>
<td>Culture 2</td>
<td>-</td>
<td>gtrA</td>
<td>1</td>
<td>0.82</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>STnc1870</td>
<td>1</td>
<td>0.70</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>gtrA</td>
<td>2.39</td>
<td>2.04</td>
<td>2.81</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>STnc1870</td>
<td>93.80</td>
<td>88.83</td>
<td>99.05</td>
</tr>
</tbody>
</table>

$^a$. For each culture, the untreated sample was used with the yceB gene as a reference to calculate relative quantities of gtrA and STnc1870 in H$_2$O$_2$ treated samples.
4.4 THE OXYR C199S MUTANT REPRESSES STnc1870 TRANSCRIPTION AFTER H₂O₂ TREATMENT

A possible mechanism by which OxyR could be removed from the gtr regulatory region involves cysteine residues (at positions 199 and 208) in the protein structure which form disulphide bridges in response to oxidative stress (see also chapter 3). The formation of disulphide bridges causes a conformational change of the protein, which results in different DNA binding patterns (Choi et al., 2001).

Could H₂O₂ treatment cause an OxyR protein bound to the gtr regulatory region to change conformation? As the spacing of the OxyR half sites in the gtr regulatory region is consistent with the reduced form binding, would oxidised OxyR be forced to leave? If so, this could explain the regulation of STnc1870: in the absence of oxidative stress, reduced OxyR could bind the gtr regulatory region, enabling phase variation while blocking STnc1870 transcription. Oxidative stress (e.g. H₂O₂ treatment) could induce disulphide bridge formation, causing OxyR to change conformation to the oxidised form, which may not be able to bind gtr. If this caused OxyR to leave gtr, the STnc1870 promoter could become accessible for transcription.

Should the above hypothesis be correct, preventing the formation of disulphide bridges in the OxyR protein would result in a permanently reduced OxyR protein, unable to leave the gtr regulatory region in response to oxidative stress. In this case, STnc1870 transcription would not increase in response to H₂O₂ treatment.

A common method to produce permanently reduced OxyR consists of making a point mutation in the corresponding gene to replace the cysteine residue with the structurally similar, but sulphur-deficient serine (‘C199S mutation’, Sun and Hattman, 1996). This mutant form is no longer capable of shifting to the oxidised state, as disulphide bridges are impossible. Could the C199S mutation cause OxyR to maintain repression of STnc1870 after H₂O₂ treatment?

C199S OxyR is capable of binding the gtr regulatory region, resulting in phase variation (Broadbent et al., 2010). For the following experiments, it was more advantageous to perform the OxyR C199S mutation on the non-phase varying, locked OFF gtr Path 931 strain, as this could provide a phenotype indicating OxyR binding to the gtr regulatory region. The C199S
mutation was therefore performed on Path 931 by lambdaRed recombination (Datsenko and Wanner, 2000).

Generating a strain with a chromosomally encoded $\text{oxyR}(C199S)$ mutation required the insertion of the tetracycline resistance cassette ($\text{tetRA}$) into the $\text{oxyR}$ gene during a preliminary step (= Path 955). Effectively, this produced an insertional knockout of OxyR, as it would be unlikely that this strain could produce a functional OxyR protein. This was supported by the fact that the strain required catalase (which detoxifies $\text{H}_2\text{O}_2$) addition to the growth media. Path 955 plated on X-Gal media showed uniform blue colonies, in stark contrast to the ancestor strain (Path 931) which grew entirely white (Fig.4.3c). The likely cause was that unlike Path 931, Path 955 did not have functional OxyR and could not repress $\text{gtrABC}::\text{lacZ}$ transcription (although Path 955 had the same $\text{gtr}$ OFF mutation as Path 931).

In a subsequent step, the tetracycline resistance cassette inserted in $\text{oxyR}$ in Path 955 was replaced with a point mutation that changed the coding sequence of the gene to produce OxyR C199S, generating Path 959. This mutation restored the OFF phenotype (white colonies) on X-Gal media, but was still dependant on catalase addition for growth on plates (Fig.4.3d). The likely reason was that restoring (persistently reduced) OxyR to the strain re-enabled repression of $\text{gtrABC}::\text{lacZ}$.

Path 931 (wildtype OxyR) and Path 959 (C199S OxyR) were grown in InSPI2 media to $\text{OD}_{600} = 0.3$, followed by splitting of the cultures and $\text{H}_2\text{O}_2$ treatment of one half each. RNA was extracted after 10 minutes. qRT-PCR results showed high increases for STnc1870 in the Path 931 wildtype OxyR ancestor, compared to an untreated control sample (254.66 relative quantity, $P < 0.001$, t-Test). On the other hand, the C199S mutant OxyR strain showed a much smaller increase in STnc1870 expression after $\text{H}_2\text{O}_2$ treatment, compared to an untreated control sample (3.02 relative quantity, $P < 0.05$, t-Test) (Fig.4.9). Compared to untreated controls, $\text{H}_2\text{O}_2$ treated samples of both strains showed either minor or non-significant changes in $\text{gtrA}$ expression: 1.54 relative quantity for Path 959 ($P < 0.05$, t-Test) and 1.25 relative quantity for Path 931 (not significant, t-Test).

Given that the relative increase in STnc1870 transcription after $\text{H}_2\text{O}_2$ treatment was 84 times higher in the wildtype OxyR strain compared to the C199S OxyR strain, it is likely that cysteine 199 in OxyR is required for high levels of transcription of STnc1870 after $\text{H}_2\text{O}_2$ treatment.
Fig. 4.9. Relative \textit{gtr}A and STnc1870 expression of Path 931 and Path 959 +/- 1 mM H$_2$O$_2$ treatment

qRT-PCR results showing relative quantities of \textit{gtr}A and STnc1870 transcripts in samples of Path 931 (wt OxyR) and Path 959 (C199S OxyR) grown with or without H$_2$O$_2$ treatment. For each culture, the non-H$_2$O$_2$ treated sample was used with the \textit{yce}B gene as a reference to calculate relative quantities of \textit{gtr}A and STnc1870 in H$_2$O$_2$ treated samples. Results are representative of two biological repeats. Statistical analysis was by t-Test. * \( P < 0.05 \). Error bars show +/- 2 standard deviation for 4 technical repeats. Note log scale on y-axis.
It appeared, that without the C199 cysteine residue OxyR could not change conformation and may have remained bound to the gtr regulatory region, continuing repression of STnc1870.

4.5 A POTENTIAL ALTERNATIVE OXYR SITE COULD BE REQUIRED TO ACTIVATE STnc1870 TRANSCRIPTION

H$_2$O$_2$ treatment appeared to abrogate wildtype OxyR repression of the STnc1870 promoter. Could an additional mechanism stimulate STnc1870 transcription after H$_2$O$_2$ treatment? The typical role of OxyR in the cell is to regulate transcription of oxidative stress response genes. The high transcription of STnc1870 in response to H$_2$O$_2$ treatment raised the question whether OxyR could activate STnc1870 transcription.

Closer analysis of the gtr regulatory region showed that a possible alternative site for OxyR was present surrounding the gtrA -10 site (Fig.4.10). If OxyR became oxidised after H$_2$O$_2$ treatment and moved to this alternative position it could potentially activate STnc1870 transcription. If true, deleting or mutating this alternative OxyR site would cause less STnc1870 to be transcribed in response to H$_2$O$_2$ treatment.

Three different mutant strains were produced by lambdaRed recombination using Path 891 (wildtype gtr regulatory region, gtrC: lacZ) as an ancestor (Datsenko and Wanner, 2000). For the first strain (Path 947) the entire alternate OxyR site and the gtrA gene were deleted. For the second strain (Path 946) the alternate OxyR site was replaced with 37 bp of random DNA sequence while maintaining the spacing of the region. In the final strain (Path 960), 12 bp predicted to be part of the alternate OxyR binding site consensus sequence were altered by replacing each purine with a non-complementary pyrimidine base and vice versa (A → C, T → G, etc.) (Fig.4.10 and Fig.4.11). These strains showed an OFF phenotype on X-Gal media, despite having the gtrC: lacZ fusion (Fig.4.12). This may have been caused by mutations affecting the gtr -10 and -35 sites, which could have left the promoter region non-functional.

Path 946, 947, 960 and the wildtype strain Path 891 (as a positive control) were grown in InSPI2 media to OD$_{600}$ = 0.3 followed by culture splitting and H$_2$O$_2$ addition to one half each. RNA was extracted after 10 minutes. qRT-PCR measurements of STnc1870 transcription showed an increase of 20.20 relative quantity for the H$_2$O$_2$ treated Path 891 sample, compared
Fig. 4.10. Sequence of the predicted alternate OxyR site in the \textit{gtr} regulatory region

The STnc1870 -35 and \textit{gtr} -10, -35 and +1 sites are marked in red and annotated. GATC sites are marked by grey boxes. The OxyR C half site is underlined and annotated. The key bases that form the potential alternate OxyR site are marked by grey boxes. The 12 key bp mutated to produce Path 960 are marked by a black box around red letters. For Path 946, the entire 37 bp sequence of the predicted alternate site was replaced by random DNA. See also Fig. 4.2.
Fig. 4.11. Overview of strains used to investigate alternate OxyR site hypothesis

The confirmed and predicted alternate OxyR sites are in red. The gtr and STnc1870 -10 and -35 sites are annotated as well as the gtrA, gtrB and gtrC genes. Based on Broadbent et al., 2010. Graph not to scale. See also Fig. 4.1 and Fig. 4.10. A, Path 891, wildtype strain with OxyR A, B and C half sites as well as the predicted alternate OxyR site and the full gtrABC operon. B, Path 947, containing a deletion of gtrA and the predicted alternate OxyR site. C, Path 946 containing a deletion of the predicted alternate OxyR site, but with the full gtrABC operon. D, Path 960 containing 12 bp point mutations in the predicted alternate OxyR site, but with the full gtrABC operon.
Fig. 4.12. Colony phenotypes of strains with mutations of the predicted alternate OxyR site

to an untreated control ($P < 0.01$, t-Test). In samples of the H$_2$O$_2$ treated mutant strains Path 946, 947 and 960, STnc1870 transcription increased by 6.70, 7.95 and 4.03 relative quantity respectively, compared to untreated control samples (all $P < 0.01$, t-Test) (Fig.4.13). This result showed that the mutated sequences were important to achieve high levels of STnc1870 transcription following H$_2$O$_2$ treatment. The data from Path 960 (the 12 bp mutant) was particularly intriguing, as it suggested that OxyR could indeed bind this region to initiate higher levels of STnc1870 transcription.

### 4.6 THE EFFECT OF STnc1870 OVEREXPRESSION ON gtrA

Further work focused on what the function of STnc1870 could be. It was observed that gtrA expression was reduced simultaneously with STnc1870 upregulation after H$_2$O$_2$ treatment. It was hypothesised that the STnc1870 sRNA could reduce gtrA expression, possibly by binding the mRNA and targeting it for degradation by RNase III (Nicholson, 2014). An example of such an interaction is the OxyS sRNA, which is transcribed divergently from the OxyR regulatory region and acts to repress rpoS expression (Altuvia et al., 1997).

To analyse the potential function of STnc1870 and its possible influence on gtrA, it was decided to overexpress STnc1870 from an inducible promoter on a plasmid. Although H$_2$O$_2$ treatment was obviously effective at inducing high amounts of STnc1870 transcription, this method also killed a large number of cells (~80 %) and was therefore likely to induce a variety of other transcriptional responses that would not be exclusive to STnc1870.

The STnc1870 gene was therefore inserted into the pLAC22 vector under control of the IPTG - inducible pLAC promoter (generating plasmid pMV449, Fig.4.14a). IPTG addition would result in overexpression of STnc1870. Possible effects this may have on gtrA could then be measured by qRT-PCR. Furthermore, by taking LPS samples before and after IPTG addition, potential effects on the LPS structure could be determined.

Plasmid pMV449 was transformed into Path 891 (gtrC::lacZ), generating Path 923. This strain was grown on X-Gal media and blue colonies were selected and grown in InSPI2 media to OD$_{600}$ = 0.3 before splitting cultures equally and adding 1 mM IPTG to one half each. Growth continued and RNA was extracted (from both cultures) at 5, 10, 15 and 106 minutes after IPTG
Fig. 4.13. Relative STnc1870 expression of Path 891, Path 946 and Path 947 +/- 1 mM H$_2$O$_2$ treatment

qRT-PCR results showing relative quantities of STnc1870 transcripts in samples of Path 891, Path 946, Path 947 and Path 960 with or without H$_2$O$_2$ treatment. For each culture, the non-H$_2$O$_2$ treated sample was used with the yceB gene as a reference to calculate relative quantities of STnc1870 in H$_2$O$_2$ treated samples. Results are representative of two biological repeats. Statistical analysis was by t-Test. ** $P < 0.01$. Error bars show +/- 2 standard deviation for 4 technical repeats.
Fig. 4.14. Diagram of plasmid pMV449 and description of the predicted ssaO binding site in STnc1870

A, pMV449 (STnc1870 overexpression plasmid). Annotations include STnc1870 coding region in black, pLAC promoter (‘lacI’) in green and ampicillin resistance marker in yellow (‘amp marker’), origin of replication (‘pBR322 origin’, black) and BglII/EagI restriction sites used to insert STnc1870. Graph to scale. Image created using Plasmapper (Dong et al., 2004).

B, STnc1870 sequence predicted to interact with ssaO mRNA (Kery et al., 2014). Numbers indicate coordinates relevant to either start codon of the ssaO mRNA or +1 site of STnc1870.

C, Point mutations in STnc1870 sequence of pMV458 compared to ssaO mRNA.
addition. LPS samples were also taken directly before (0’) and 106 minutes after IPTG addition (3.5 generation times). Culture samples from IPTG-induced and non-induced cultures were spread plated on X-Gal media (without IPTG) at the same timepoints as the LPS sample collection to estimate the percentage of gtr ON cells and determine whether heritable changes had occurred to gtr phase variation.

qRT-PCR performed on the RNA samples showed that STnc1870 transcription increased in IPTG treated samples across all timepoints (11.17-15.27 relative quantity) compared to non-IPTG treated samples. However, gtrA expression showed only minor differences in expression in IPTG treated samples compared to untreated samples: at the 5 minute timepoint a small increase was measured (1.15 relative quantity, $P < 0.01$, t-Test) and the 10 minute sample showed no significant differences, compared to non-IPTG treated samples. The 15 and 106 minute samples showed minor, but significant reductions: 0.68 ($P < 0.001$, t-Test) and 0.63 ($P < 0.05$, t-Test) relative quantity compared to non-IPTG treated samples, respectively (Fig.4.15). IPTG-induced overexpression of STnc1870 therefore appeared unlikely to have affected gtrA expression.

Furthermore, separation of LPS molecules extracted from IPTG-induced and uninduced cultures of Path 923 by TSDS-PAGE gels did not show differences in band patterns (Fig.4.16). All Path 923 samples (lanes 2, 4 and 6) showed band patterns similar to a control strain which constitutively produced the gtr O-antigen glycosylation modification (lanes 1 and 5). This data indicated that IPTG-induced overexpression of STnc1870 did not affect O-antigen modification by gtrABC in Path 923 in the tested interval. Ideally, a period of more than 3.5 generation times should be assessed for changes in LPS modification.

Finally, Path 923 samples spread plated on X-Gal media before (0 minutes) and after (106 minutes) IPTG addition did not show differences in the percentage of ON colonies. The 0 minute sample contained 84 % ON colonies (total counted 362), identical to the IPTG-induced 106 minute sample, which also contained 84 % ON colonies (total counted 417). The non-IPTG treated 106 minute sample contained 83 % ON colonies (total counted 156). In summary, IPTG-induced overexpression of STnc1870 had only a minor effect on gtrA expression and did not affect O-antigen modification or gtr phase variation.
Fig. 4.15. Relative gtrA and STnc1870 expression of Path 923 +/- 1 mM IPTG treatment

qRT-PCR results showing relative quantities of gtrA and STnc1870 transcripts in samples of Path 923 over a timecourse with and without IPTG treatment. For each timepoint, the non-IPTG treated sample was used with the yceB gene as a reference to calculate relative quantities of gtrA and STnc1870 in IPTG treated samples. Results are representative of two biological repeats. Statistical analysis was by t-Test. *** P < 0.001; ** P < 0.01; * P < 0.5; n.s., not significant. Error bars show +/- 2 standard deviation for 4 technical repeats.
Fig. 4.16. TSDS-PAGE gel separation of Path 923 (\textit{gtrC:}lacZ, pMV449) LPS samples visualised by silver stain

Regions in which gel shifts are apparent are highlighted by red box. Representative of 2 biological repeats. Lanes 1 and 5, Path 292 (positive control, constitutive LT2_I \textit{gtr} modification). Lane 2, Path 923, 0 minute sample (non-IPTG treated). Lanes 3 and 7, Path 293 (negative control, knockout of all known \textit{gtr} operons). Lane 4, Path 923 106 minute sample, IPTG treated. Lane 6, Path 923 106 minute sample, non-IPTG treated.
In search of other potential targets of STnc1870, the web-based programme TargetRNA which predicts possible interaction targets of short RNAs was used (Kery et al., 2014). RNA sequence, structure and conservation among related species are used by TargetRNA to generate a list of mRNAs that could be bound by a short RNA. One of the predicted target mRNAs was \textit{ssaO}, which encodes a structural component of the SPI2 type III secretion system needle, used by \textit{Salmonella} to inject virulence factors into the macrophage (reviewed in Figueira and Holden, 2012) (Table 4.3).

The potential target \textit{ssaO} was considered to be worth further investigation as the RNA sequencing data showed that \textit{ssaO} expression decreased to 0.03 relative quantity (33 – fold reduction) after H$_2$O$_2$ treatment, compared to an untreated sample (Kröger et al., 2013). Expression of the other mRNA targets predicted by TargetRNA did not decrease as much as \textit{ssaO} under these conditions (Table 4.3). This was interesting, although H$_2$O$_2$ treatment can have very wide-ranging effects on the cell, so a variety of factors could have caused the \textit{ssaO} reduction. However, if the TargetRNA prediction was correct, STnc1870 could bind \textit{ssaO} and cause it to be degraded, for example via RNaseIII (reviewed in Nicholson, 2014).

TargetRNA predicted that a 13 bp sequence of STnc1870 could bind the \textit{ssaO} mRNA at the coordinates of -35 to -47 bp upstream of the \textit{ssaO} start codon (Fig.4.1). This actually placed the binding site of STnc1870 within \textit{ssaN}, the gene preceding \textit{ssaO}. Both \textit{ssaO} and \textit{ssaN} are transcribed as part of an operon of SPI2 genes (Kröger et al., 2013).

To test whether high expression of STnc1870 affected \textit{ssaO} expression, strain Path 923 (containing the STnc1870 overexpression vector pMV449) was used again. In addition, a new negative control strain was needed for the following reason: the pLAC promoter is considered ‘leaky’ and allows a certain amount of expression in the absence of the inducer. Therefore STnc1870 was likely to be constitutively expressed at a low level in Path 923. During previous experiments on \textit{gtrA}, the constitutive low-level expression of STnc1870 in Path 923 had to be accepted – the phase variation of \textit{gtr} meant that the control and experiment samples had to be grown from the same colony.
Table 4.3. mRNA targets of STnc1870 predicted by TargetRNA (Kery et al., 2014)

<table>
<thead>
<tr>
<th>Predicted mRNA target</th>
<th>$P$ value</th>
<th>Predicted interaction coordinates</th>
<th>Relative expression levels&lt;br&gt;IndoSPI2 media&lt;br&gt;InSPI2 + 1 mM H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>htpG</em> chaperone Hsp90, heat shock protein</td>
<td>0.001</td>
<td>STnc1870 72 52</td>
<td>1 0.2</td>
</tr>
<tr>
<td><em>entB</em> 2,3-dihydro-2,3-dihydroxybenzoate synthetase</td>
<td>0.001</td>
<td>STnc1870 63 51</td>
<td>1 1</td>
</tr>
<tr>
<td><em>yieH</em> 6-phosphogluconate phosphatase</td>
<td>0.003</td>
<td>STnc1870 65 54</td>
<td>1 0.33</td>
</tr>
<tr>
<td>STM474_2889 putative hexulose 6 phosphate synthase</td>
<td>0.006</td>
<td>STnc1870 64 54</td>
<td>1 1</td>
</tr>
<tr>
<td>ssaO secretion system apparatus protein</td>
<td>0.006</td>
<td>STnc1870 62 48</td>
<td>1 0.03</td>
</tr>
<tr>
<td><em>ybhC</em> putative pectinesterase</td>
<td>0.007</td>
<td>STnc1870 74 59</td>
<td>1 0.21</td>
</tr>
<tr>
<td><em>stjB</em> putative fimbrial usher protein</td>
<td>0.007</td>
<td>STnc1870 71 58</td>
<td>1 1</td>
</tr>
<tr>
<td><em>rhaA</em> L-rhamnose isomerase</td>
<td>0.011</td>
<td>STnc1870 62 51</td>
<td>1 1</td>
</tr>
<tr>
<td><em>pgtC</em> phosphoglycerate transporter</td>
<td>0.013</td>
<td>STnc1870 68 56</td>
<td>1 1</td>
</tr>
<tr>
<td><em>creC</em> sensor protein</td>
<td>0.014</td>
<td>STnc1870 61 45</td>
<td>1 1</td>
</tr>
<tr>
<td><em>copA</em> copper exporting ATPase</td>
<td>0.018</td>
<td>STnc1870 65 51</td>
<td>1 0.8</td>
</tr>
</tbody>
</table>

a. Interaction coordinates indicate distance (in bp) to the +1 site of STnc1870 or the start codon of the predicted mRNA target.

b. Relative expression levels for the predicted target mRNAs are compared between cultures grown in IndoSPI2 media and cultures grown in IndoSPI2 media with a 1 mM H$_2$O$_2$ shock. The *ssaO* predicted target is in red. Data derived from Kröger et al., 2013.
However, for the experiment on *ssaO* it was considered better to use a separate control strain with the same genetic background as Path 923 but containing ‘empty’ pLAC22 (without the STnc1870 insert) instead of pMV449. pLAC22 was therefore transformed into Path 891, generating Path 953. The strain was grown in InSPI2 media to OD\textsubscript{600} = 0.3 and treated with 1 mM IPTG. RNA was extracted at 5, 10, 15 and 106 minutes after IPTG treatment. The samples were compared using qRT-PCR to the previously collected samples of Path 923 at the same timepoints after IPTG treatment (see section 4.6).

The results showed a 2 – fold reduction (0.5 relative quantity) of *ssaO* at 5, 10 and 15 minutes after IPTG treatment in Path 923 compared to Path 953 (Fig.4.17). This was a very modest reduction compared to the 33 – fold reduction of *ssaO* expression after H\textsubscript{2}O\textsubscript{2} treatment measured in the RNA sequencing data (Kröger et al., 2013). However, as mentioned, H\textsubscript{2}O\textsubscript{2} treatment is likely to have wide-ranging effects on the cell that could cause this reduction. Overexpressing STnc1870 in isolation appeared to reduce *ssaO* expression in two different cultures. This could mean that STnc1870 contributed to *ssaO* downregulation during H\textsubscript{2}O\textsubscript{2} shock.

### 4.8 A 13 bp SEQUENCE IN STnc1870 IS REQUIRED FOR *ssaO* DEGRADATION

13 bp of the STnc1870 sRNA were predicted by TargetRNA to bind the *ssaO* mRNA. If the predicted interaction was responsible for *ssaO* downregulation after STnc1870 overexpression, changing the 13 bp should remove the effect. The 13 bp of STnc1870 in the pMV449 vector were therefore altered by changing each purine to a non-complementary pyrimidine and vice versa (A \rightarrow C, T \rightarrow G, etc.) (Fig.4.14c). This altered vector (pMV458) was transformed into Path 891, generating Path 961. Duplicate cultures of Path 923 (*gtrC::lacZ*, pMV449), Path 953 (*gtrC::lacZ*, pLAC22) and Path 961 (*gtrC::lacZ*, pMV458) were grown in InSPI2 media. At OD\textsubscript{600} = 0.3, RNA samples were taken (0’ sample), before adding 1 mM IPTG to each culture. Growth was continued and further RNA samples were taken at 10 and 15 minutes after IPTG addition. qRT-PCR was then performed using the 0 minute samples as references. The results showed that in Path 923, *ssaO* had 0.09 relative quantity compared to the 0 minute sample at 15 minutes. Path 953 and Path 961 on the other hand had 1.27 and 0.75 relative quantity, respectively (Fig.4.18). This result indicated that the 13 bp of STnc1870 identified by TargetRNA may be required to reduce *ssaO* expression after STnc1870 overexpression and supported the conclusion that *ssaO* is a target of STnc1870.
Fig.4.17. Relative \textit{ssaO} and STnc1870 expression of Path 923 and Path 953 + 1 mM IPTG treatment

qRT-PCR results showing relative quantities of \textit{ssaO} and STnc1870 transcripts in samples of Path 923 (+ pMV449, STnc1870 overexpression plasmid) and Path 953 (+ pLAC22) over a timecourse with IPTG treatment. For each timepoint, the Path 953 sample was used with the \textit{yceB} gene as a reference to calculate relative quantities of \textit{ssaO} and STnc1870 in IPTG treated samples. The dotted horizontal line indicates 0.5 relative quantity. Results are representative of two biological repeats. Statistical analysis was by t-Test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.5$. Error bars show +/- 2 standard deviation for 4 technical repeats. Note log scale on y-axis.
Fig. 4.18. Relative *ssaO* and STnc1870 expression of Path 923, Path 953 and Path 961 +/- 1 mM IPTG treatment

qRT-PCR results showing relative quantities of *ssaO* and STnc1870 transcripts over a timecourse with IPTG treatment. For each timepoint, the 0’ minute sample (non-IPTG treated) was used with the *yceB* gene as a reference to calculate relative quantities of *ssaO* and STnc1870 in IPTG treated samples at 10’ and 15’ minutes. **A**, Path 923 (pMV449, STnc1870 overexpression plasmid). **B**, Path 961 (pMV458, mutated STnc1870 overexpression plasmid). **C**, Path 953 (‘empty’ pLAC22 plasmid without STnc1870 insert). Results are representative of two biological repeats. Statistical analysis was by t-Test. **P** < 0.01; n.s., not significant. Error bars show +/- 2 standard deviation for 4 technical repeats.
4.9 DISCUSSION

The results presented in this chapter demonstrate the transcriptional versatility of the gtr operon. The RNA sequencing data gave the first indication of the short RNA STnc1870 being produced from the gtr regulatory region in response to H$_2$O$_2$ shock (Kröger et al., 2013). qRT-PCR using the gtrC: lacZ fusion strain confirmed this result while also showing that STnc1870 transcription occurred regardless of the percentage of ON cells in the tested culture.

The predicted -10 and -35 sites of STnc1870 overlapped with the OxyR B and C sites. As OxyR was predicted to bind the B site persistently, how was STnc1870 transcription possible? This discrepancy was addressed by the hypothesis that H$_2$O$_2$ treatment forced OxyR to cease binding of the gtr regulatory region. The gtr OFF mutant (Path 931) was produced as a tool to test this hypothesis. The Lac-, unmodified phenotype of Path 931 indicated persistent OxyR binding in the OFF position, in all cells of the population. H$_2$O$_2$ induction of STnc1870 transcription was clearly still possible in this strain, which supported the idea that OxyR ceased gtr binding in response to H$_2$O$_2$ treatment.

A possible alternative explanation was that OxyR for some unknown reason did not bind to a subset of gtr regulatory regions in a population. This subset could then be responsible for the increases of STnc1870 observed after H$_2$O$_2$ treatment. The results of the OFF mutant experiments did not exclude this scenario, although they did allow for a simpler explanation, which was more in line with previous data about gtr regulation.

Another interesting aspect of the OFF mutant experiments was that, after H$_2$O$_2$ treatment, relative increases of STnc1870 were much higher compared to the wildtype. This may have simply been caused by variation between qRT-PCR experiments. However, the point mutations used to create this strain allowed OxyR to bind the C half site without competition from Dam. Furthermore the match between the C half site and the OxyR binding site consensus was increased by one bp. It is possible this caused OxyR to bind this position with higher affinity, resulting in stronger repression of STnc1870 in the absence of H$_2$O$_2$ treatment. STnc1870 transcription after H$_2$O$_2$ treatment could be as high in absolute amounts as in the wildtype, but the relative increase compared to the untreated control could be higher in the OFF mutant. Finally, it is possible that absolute amounts of STnc1870 transcription after the H$_2$O$_2$ shock are
actually higher in the OFF mutant than in the wildtype, although there is no obvious explanation for this.

Further evidence showed that the C199 cysteine residue in the OxyR protein was required to allow STnc1870 transcription to increase in response to H$_2$O$_2$ treatment. It is well established that this residue is required for the OxyR protein to switch conformation between the reduced and oxidised form (Sun and Hattman, 1996). Therefore, it appeared that OxyR must alter conformation for STnc1870 transcription to occur.

As mentioned, the spacing of the OxyR half sites in the gtr regulatory region (= 17 bp) is consistent with binding of the reduced form of OxyR. The reduced form of OxyR is also sufficient to allow phase variation of gtr (Broadbent et al., 2010). Experiments with the C199S mutant indicated that OxyR must switch to the oxidised form for STnc1870 repression to be lifted. Is it possible that after changing conformation, oxidised OxyR cannot bind the gtr regulatory region because the spacing of the half sites does not permit this? This would represent the first evidence of oxidised OxyR influencing gtr expression differently than the reduced form.

Oxidised OxyR eventually returns to the reduced state if the oxidative stress is removed (Choi et al., 2001). At this point, OxyR could resume binding of the gtr regulatory region in the ON or OFF state. Culture samples plated after H$_2$O$_2$ shock showed phase variation. If the cell was able to replicate during the oxidative stress, could this influence phase variation, as OxyR may be bound outside the usual position? Unfortunately, it may be difficult to design an experiment in which H$_2$O$_2$ addition is high enough to oxidise OxyR and induce STnc1870 transcription measurably, but also low enough to allow cell replication.

It was also found that a region upstream of the predicted STnc1870 -35 site was important for achieving a high relative increase of STnc1870 transcription after H$_2$O$_2$ treatment. Intriguingly, changing just 12 bp, which were predicted to form an alternative OxyR site, was sufficient to reduce the relative increases in STnc1870 transcription. The OxyR half sites of this predicted alternative site were separated by 10 bp, unlike the established OxyR sites in the gtr regulatory region which were separated by 17 bp. Although this suggested that oxidised OxyR could stimulate RNA polymerase transcription of STnc1870, better evidence would be needed. The challenge in finding such evidence is that OxyR could act as both repressor and activator of
STnc1870 transcription. For example, a good experiment would be to show whether C199S OxyR is capable of activating STnc1870 transcription in response to H₂O₂ treatment. However, based on previous results C199S OxyR is likely to remain bound to the gtr region and repress STnc1870. It would be ideal to analyse OxyR repression or activation of STnc1870 in isolation of each other, to determine their individual contributions. A possible approach would be to generate a strain with a gtr regulatory region that cannot be bound by OxyR. This could be achieved through point mutations that alter key bp of the OxyR B site. The predicted alternate OxyR site should not be mutated (except for control purposes). If OxyR cannot bind the A, B, or C half sites, STnc1870 may not be repressed.

STnc1870 transcription from this mutated gtr operon could be measured in strains with wildtype OxyR, C199S OxyR or a ΔoxyR deletion without H₂O₂ treatment. A strain with both wildtype gtr and OxyR should be used as control. If strains with the mutated gtr regulatory region (and either wt OxyR, C199S OxyR or ΔoxyR) show higher STnc1870 expression without H₂O₂ treatment than the wildtype control strain, this would indicate that OxyR binding of gtr is required for STnc1870 repression. Similarly, a ΔoxyR strain with a wildtype gtr regulatory region should also show higher STnc1870 transcription in the absence of H₂O₂ treatment.

The mutated gtr strains could then be tested for STnc1870 transcription in response to H₂O₂ treatment. If OxyR does indeed become oxidised and activates STnc1870 transcription from the alternate binding site, STnc1870 expression should increase in the strain with wt OxyR. Furthermore, in the C199S OxyR strain, STnc1870 transcription should not increase in response to H₂O₂ treatment (this may also be the case with a ΔoxyR strain – however this strain may not survive the H₂O₂ treatment). In short, this method could be used to test whether OxyR activates STnc1870 transcription from the predicted alternate site, while excluding the effects of STnc1870 repression. If OxyR did indeed bind this alternate site, it could explain why gtrA expression decreased after H₂O₂ addition – the OxyR protein may block gtrA expression in this case.

The predicted alternate OxyR site overlaps with the 4th GATC site of the gtr regulatory region. Should OxyR indeed be capable of binding here, would methylation of the GATC site interfere with binding? The potentially methylated base is marked bold and underlined in the consensus: ATAGxTxxxAxCTAT. OxyR may be able to tolerate methylation or mismatched bases of the lone T/A bases better than in the ATAG/CTAT elements (Zheng et al., 2001a). For example, the
phase varying S. Infantis_I gtr operon has mismatches at the following (underlined) positions in the OxyR C half site: AT\textbf{A}Gx\textbf{T}xxx\textbf{A}xC\textbf{T}AT.

One final comment concerning the possible alternative OxyR site was that transcription of STnc1870 in the absence of H$_2$O$_2$ treatment in the mutant strains (Path 946, 947, 960) was the same as in the wildtype strain (Path 891). This was an important finding – if the mutations had reduced the ability of OxyR to bind the gtr regulatory region and repress STnc1870, it would have increased the amount of STnc1870 transcribed in the absence of H$_2$O$_2$. In this case the relative increase of STnc1870 transcription could have been lower after H$_2$O$_2$ addition, making it difficult to make statements about the impact of the alternative OxyR site deletions.

It was noteworthy that after adding 1 mM H$_2$O$_2$ (as used in Kröger et al., 2013) to InSPI2 cultures of any of the strains used in this chapter, growth stopped and did not resume for at least two hours afterwards. Furthermore, ~80 % of cells in these cultures died. This effect was dependent on the amount of H$_2$O$_2$ added (for example, adding 10 μM H$_2$O$_2$ did not have a noticeable effect on the growth curve). In this regard, it could also be interesting to determine what dose of H$_2$O$_2$ is sufficient for STnc1870 induction.

The InSPI2 media was likely an important factor in the response to the 1 mM H$_2$O$_2$ shock. The same amount of H$_2$O$_2$ used on wildtype S. Typhimurium grown in LB media to an OD$_{600}$ of 0.3 did not affect growth (Hebrard et al., 2009). Peptides and other organic molecules present in LB may absorb the H$_2$O$_2$. InSPI2 media is minimal and defined, therefore the toxic effects of H$_2$O$_2$ could be much more pronounced. It may also be interesting to know whether the lower pH (5.8), or addition of certain metals (Mo, Se, Co, Cu, Fe, Mn) to InSPI2 media exacerbated the H$_2$O$_2$ shock or influenced STnc1870 transcription.

Could a generalised stress response cause STnc1870 induction? The high number of cells killed during experiments with H$_2$O$_2$ treatment indicates that the population would be under substantial stress and this could perhaps be a precondition for OxyR to induce STnc1870 transcription. If so, other conditions that cause high amounts of cell death and stress could induce STnc1870 transcription. However, other stress conditions tested by Kröger et al., 2013 such as nitrosative stress, osmotic shock, low pH shock or anaerobic shock did not induce STnc1870. Another possibility that cannot be excluded is that the population that survived the H$_2$O$_2$ treatment and transcribed STnc1870 had some exceptional characteristic that allowed it to
survive. The survival of this population was probably not dependent on transcription of STnc1870 – the alternate OxyR site deletion mutants (Path 946, 947, 960, see section 4.5) produced lower relative amounts of STnc1870 compared to the wildtype strain, but this subpopulation was equally able to survive the H$_2$O$_2$ shock.

The conditions of InSPI2 media and 1 mM H$_2$O$_2$ shock were intended to mimic the environment *Salmonella* encounters in the macrophage SCV. The amount of H$_2$O$_2$ present in the SCV of C57BL/6 murine macrophage is estimated to be ~10 μM (Aussel et al., 2011). Although this represents a 100-fold difference in amounts, it should be noted that H$_2$O$_2$ in the macrophage is supplied continuously, whereas the experiments in this chapter used a single dose. Also, the C57BL/6 mouse strain is highly susceptible to *Salmonella* infection due to lack of a functional Nramp1 protein (Roy and Malo, 2002). Nramp1 removes metal ions (Fe$^{2+}$, Mn$^{2+}$) from the SCV which the bacteria requires to detoxify H$_2$O$_2$ (Jabado et al., 2000; Loomis et al., 2014). Alternatively, Nramp1 could transport metal ions into the SCV which enhance production of reactive oxygen species through Fenton chemistry (Wessling-Resnick, 2015). A dose of 10 μM H$_2$O$_2$ is sufficient to induce oxidised OxyR (Aussel et al., 2011). Therefore the SCV environment could cause a persistent low level of STnc1870 transcription.

During the course of this work, RNA sequencing data obtained from *S*. Typhimurium infecting murine RAW264.7 macrophage was published (Srikumar et al., 2015). This same group had previously published the RNA sequencing data that showed STnc1870 production after growth in InSPI2 media with H$_2$O$_2$ shock. The newly published data showed that only background levels of STnc1870 were expressed in the macrophage. This is somewhat contradictory, as the experiments with InSPI2 and H$_2$O$_2$ were intended to mimic the macrophage environment. However, the RAW264.7 cell line does not express the Nramp1 protein (Govoni et al., 1999). Therefore, *Salmonella* infecting Nramp1 deficient RAW264.7 cells can grow easier and are likely to experience less oxidative stress than in cells with functional Nramp1. This reduced oxidative stress may be insufficient to induce STnc1870 transcription. It could be interesting to measure STnc1870 production in macrophage with functional Nramp1. Similarly, survival of STnc1870 deletion and overexpression mutants could be assessed in this environment.

Oxidative stress within macrophage with functional Nramp1 may be at a lower level than the 1 mM H$_2$O$_2$, but it is supplied persistently (Aussel et al., 2011). If STnc1870 is induced in macrophage with functional Nramp1, expression may not be as high as observed after the 1 mM H$_2$O$_2$ shock. *Salmonella* also experiences oxidative stress during the initial stages of infection.
when crossing the gut barrier and causing inflammation (Winter et al., 2010). Therefore, the macrophage environment may not necessarily be the only environment in which STnc1870 transcription could be induced.

Finally, it appeared that STnc1870 could be binding and reducing the expression of the SPI2 component ssaO. This effect appeared to be dependent on the 13 bp predicted by TargetRNA to be involved in the interaction between the two RNAs. To further confirm this result, it would be helpful to determine whether assembly of the SPI2 needle is inhibited by IPTG-induced STnc1870 overexpression. STnc1870 could cause reduced translation of SPI2 proteins by blocking ribosomes from accessing the mRNA, in addition to its potential role in causing ssaO mRNA degradation. If expression of the SPI2 needle is inhibited by STnc1870 overexpression, this would likely affect virulence as well (Westermann et al., 2016). For example, a strain constitutively expressing STnc1870 may be less capable of surviving in a macrophage infection model. This potential link between STnc1870 and ssaO will be discussed further in chapter 6.

However, the importance of the link to ssaO should not be overemphasised; the reduction observed after STnc1870 overexpression was consistent, but only 2-fold. It is quite possible that STnc1870 has a different primary target. A potential method to find such a target could make use of RNA sequencing following STnc1870 overexpression.

To summarise, the evidence presented in this chapter supports the following statements: Firstly, binding of OxyR in either the ON or OFF state represses STnc1870 transcription, as the STnc1870 -10 site overlaps with the OxyR B half site, which is occupied in both the ON and OFF state. Furthermore, addition of H$_2$O$_2$ causes OxyR to become oxidised and alter conformation, regardless of whether it is bound to the gtr regulatory region in the ON or OFF state.

As the 17 bp spacing of OxyR half sites in the gtr regulatory region is consistent with the reduced form of OxyR binding, oxidised OxyR could move to the alternative site upstream of STnc1870 (which has a 10 bp half site spacing more consistent with binding of oxidised OxyR, see Fig.4.10). In this alternative position, OxyR could stimulate transcription of STnc1870. Given that the alternative OxyR site overlaps with the gtrABC -10 site, expression of gtr could be blocked simultaneously, which would explain the reduced gtr expression measured after H$_2$O$_2$ treatment. Following transcription, the STnc1870 sRNA could bind the ssaO mRNA and
target it for degradation. As a result, the \textit{gtr} regulatory region could have a wider role in regulating expression of the SPI2 needle.
CHAPTER 5

INFLUENCE OF DNA SEQUENCE ON

$gtr$ PHASE VARIATION
5.1 INTRODUCTION

The work in this chapter focusses on sequence variations that were found in a number of \textit{gtr} regulatory regions of naturally occurring \textit{Salmonella} serovars. A minority of examined regulatory regions contained less than four GATC sites. It was thought that these sequence variations would affect phase variation, as previous work had shown the importance of GATC sites in this process. Methylation of GATC sites by Dam blocks OxyR binding. If the two GATC sequences furthest from the \textit{gtr} promoter are methylated, OxyR binds the OxyR B and C half sites and \textit{gtr} expression does not occur (OFF phase). If the other two GATC sequences closest to the \textit{gtr} promoter are methylated, OxyR binds the OxyR A and B half sites and \textit{gtr} expression is ON (see section 1.3.2 and Fig.1.1) (Broadbent et al., 2010). The absence of GATC sites in \textit{gtr} operons derived from clinically important strains such as \textit{S. Typhi} added a further incentive to determine their effect.

On a related topic, the differences between two methods used in this work to measure \textit{gtr} phase variation are examined. Previous work on \textit{gtr} phase variation relied on fusions of \textit{gtr} regulatory regions to the \textit{lacZ} reporter gene, which were inserted into a vector and subsequently integrated as a single copy into the \textit{Salmonella} chromosome at the lambda phage insertion site (Haldimann and Wanner, 2001). As part of this work, the \textit{lacZ} reporter gene was inserted downstream of the chromosomal \textit{gtrABC} genes, and therefore co-transcribed with the ‘native’ \textit{gtr} operon. The two methods gave different results for \textit{gtr} phase variation experiments.
5.2 THE S. TYPHI 2_II gtr

5.2.1 Diversity of gtr regulatory regions

The regulatory regions of 60 previously identified gtr operons were aligned to identify similarities (Davies et al., 2013) (Fig.5.1). Within this dataset, 40 gtr regulatory regions contained four GATC sites mimicking the LT2_I and P22 gtr operons. These 40 gtr operons were therefore considered likely to phase vary. The remaining 20 gtr regulatory regions contained between one and three GATC sites. Closer analysis revealed that those with three GATC sites (S. Typhi 2_II, S. Infantis_I and S. Gallinarum_I) shared high similarity with the LT2_I gtr regulatory region already known to phase vary (Broadbent et al., 2010) (Fig.5.2).

It was interesting that gtr regulatory regions with less than four GATC sites appeared to group in three separate clusters (as well as S. Gallinarum_I and S. Typhimurium LT2_II) (Fig.5.1). If the 60 analysed gtr regulatory regions evolved from a single ancestor with four GATC sites, this would suggest that loss of GATC sites by point mutations occurred independently on multiple occasions. However, the corresponding gtrC genes cluster in ten different families which cannot be assumed to have common ancestry due to low amino acid similarity between them (< 46 %) (Davies et al., 2013). Therefore it is difficult to make statements about the evolutionary relationship of gtr regulatory regions.

How did the ‘absent’ GATC sites affect gtr phase variation? If the sequence variations in these strains disabled gtr phase variation or altered the switch frequency, this would raise the question whether the lifestyles of these particular serovars selected for a different approach to O-antigen modification.

Of particular interest was the S. Typhi 2_II gtr regulatory region. Instead of a 4th GATC site (closest to the +1 site), this gtr regulatory region contained the sequence GAAA (= GAAA⁴) (Fig. 5.2). During previous work this regulatory region was inserted upstream of the lacZ reporter gene in a CRIM vector and integrated as a single copy into the S. Typhimurium chromosome (Haldimann and Wanner, 2001). After plating of the resulting strain on X-Gal media, only blue colonies grew, indicating constitutive expression (Edwin Kaptein) (Fig.5.4b).
A dataset of gtr regulatory regions (derived from Davies et al., 2013) was aligned by MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and an unrooted tree generated showing predicted relationships based on sequence similarity. The tree does not incorporate distance calculations. gtr regulatory regions without four GATC sites are highlighted in red.
Fig.5.2. Alignment of gtr regulatory regions

The above gtr regulatory regions were aligned by Clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). 120 bp preceding the predicted +1 sites were chosen for alignment. Homologous sequences are highlighted grey. GATC sites are bold and underlined. Point mutations of S. Infantis_I gtr and S. Typhi 2_II gtr are marked in red letters. OxyR half sites are annotated A, B or C with the OxyR half site binding consensus annotated for each. The -10 and +1 sites are annotated and underlined.
The absence of phase variation was intriguing, given the high similarities (98% sequence identity for gtrABC) between the S. Typhi 2_II gtr and the LT2_I gtr of S. Typhimurium – both operons were predicted to perform the same O-antigen modification (α 1-4 glycosylation of galactose) (Davies et al., 2013). Could there be an evolutionary reason explaining why this modification phase varied in S. Typhimurium, but not in S. Typhi?

S. Typhi is thought to have evolved from S. Typhimurium through mutations that disable certain genes, leaving it with 204 pseudogenes (Parkhill et al., 2001). However, the S. Typhi 2_II gtr operon does not contain any mutations that would indicate non-functionality (true for all S. Typhi sequences in GenBank as of 27/01/16).

A second gtr operon is present in the genome of S. Typhi, named Typhi 2_I gtr, which performs acetylation of the O-antigen at the rhamnose molecule (Erica Kintz). This gtr regulatory region was inserted into the CRIM vector and found to be capable of phase variation (Edwin Kaptein) (Fig.5.4a). If the constitutive expression of Typhi 2_II gtr was selected for by some unknown mechanism, the same did not apply to Typhi 2_I gtr.

An initial experiment aimed to determine whether the GAAA^4 sequence variation was the solitary cause for the absence of phase variation of Typhi 2_II gtr. If this was the case, replacing GAAA^4 with GATC would restore phase variation. If phase variation was not restored by this experiment, it would indicate that some other sequence variation was preventing this.

The Typhi 2_II gtr region had been previously inserted upstream of the lacZ reporter gene in a CRIM vector (Edwin Kaptein, Haldimann and Wanner, 2001) (Fig.5.3). This vector was point mutated by overlap extension PCR to replace the GAAA^4 sequence with a 4th GATC site (= GATC^4). The resulting vector was integrated as a single copy into the S. Typhimurium LT2 chromosome. This strain was chosen as a model organism, as all previous work on gtr made use of the LT2 strain, allowing comparisons to be made. The resulting strain was found to be capable of phase variation as indicated by the presence of blue and white colonies on X-Gal media (Fig.5.4c). This result indicated that the GAAA^4 sequence variation of Typhi 2_II gtr was responsible for the absence of phase variation.
Fig. 5.3. Diagram of the CRIM vector.

Annotations include *gtr* regulatory region in green, *lacZ* reporter gene (purple), chloramphenicol resistance marker (‘CAT marker’, orange), *attP* site (pink), origin of replication (‘R6Kg origin’, black) and Acc65I/PstI restriction sites used to insert the *gtr* regulatory region (blue). Graph to scale. Image created using Plasmapper (Dong et al., 2004).
Fig. 5.4. Colony phenotypes of S. Typhi gtr:\textit{lacZ} CRIM vector strains

A, Path 667, S. Typhi with Typhi 2_\text{I} gtr:\textit{lacZ} CRIM vector insertion. B, Path 636, S. Typhimurium LT2 with Typhi 2_\text{II} gtr:\textit{lacZ} fusion CRIM vector. C, Path 806, S. Typhimurium LT2 with Typhi 2_\text{II} GATC^4 mutant gtr:\textit{lacZ} CRIM vector. D, Path 833, S. Typhimurium LT2 with Typhi 2_\text{II} GATA^4 mutant gtr:\textit{lacZ} CRIM vector. Colonies were diluted and spread plated on M9 X-Gal media except for Path 667 which was plated on LB X-Gal media with aromatic amino acid supplements. Representative of two repeats.
5.2.2 Phase variation does not require four GATC sites

Why did adding an extra GATC site to Typhi 2_II gtr regulatory region restore phase variation? The extra GATC site would be a further target for Dam methylation. However, the T base inserted by the point mutation also improved the match with the predicted OxyR C half site (Fig. 5.2). This raised the question whether both of these point mutations were necessary to restore phase variation. Alternatively, a single bp change could be sufficient to restore phase variation.

To address this, it was decided to replace the GAAA sequence of Typhi 2_II gtr with GATA. This single base pair change would improve the OxyR C half site (similar to LT2_I gtr, see Fig.5.2), but without adding an extra GATC methylation target. If phase variation was possible in this strain, it would indicate that the wildtype Typhi 2_II gtr did not phase vary because the GAAA mutation prevented OxyR binding, not because a GATC methylation target was missing.

A further motivation for this experiment came from analysis of a further gtr regulatory region with only three GATC sites: S. Gallinarum_I contained a GATA sequence variation instead of a 4th GATC site (Fig.5.2). If the S. Typhi 2_II gtr GATA mutant was capable of phase variation, it could also indicate whether or not phase variation was possible in S. Gallinarum_I gtr.

The S. Typhi 2_II gtr regulatory region lacZ fusion contained in a CRIM vector was altered by replacing the GAAA sequence with GATA. The resulting vector was integrated as a single copy into the S. Typhimurium LT2 chromosome. Plating the resulting strain on X-Gal media produced blue and white colonies, showing that the Typhi 2_II gtr GATA mutant was capable of phase variation (Fig.5.4d). Therefore, the absence of phase variation in Typhi 2_II gtr was caused by the sequence variation GAAA preventing OxyR binding, as opposed to preventing methylation at this site.
5.2.3 Switch frequency differences between three and four GATC gtr regulatory regions

The insight that three GATC sites were sufficient for phase variation if OxyR binding was still possible in all three half sites, raised the question why the majority of studied gtr operons contain 4 GATC sites. Is there a difference between having three or four GATC sites? To answer this, the frequency at which OFF colonies switch to the ON phenotype and vice versa was measured for both the Typhi 2_II gtr GATC⁴ and GATA⁴ mutants. Single ON or OFF colonies were resuspended, diluted and spread plated on X-Gal media to determine the proportion that switched colony phenotype. The results showed that the GATA⁴ mutant (i.e. GATA instead of a 4th GATC site closest to the gtr promoter) had an ON to OFF switch frequency that was on average 2.7 – fold higher than the GATC⁴ mutant (i.e. 2.7 times more colonies switching from ON to OFF). The OFF to ON switch frequency displayed an even greater difference: this was 8.5 times higher on average in the GATA⁴ mutant than in the GATC⁴ mutant (Table 5.1). The GATA⁴ mutant therefore had a higher switch rate.

5.3 THE S. INFANTIS_I gtr

5.3.1 The S. Infantis_I gtr also contains three GATC sites

A further gtr operon investigated was the S. Infantis_I gtr: in this case the regulatory region contained the sequence variation TATC¹ instead of the first GATC site (furthest from the +1 site) (Fig.5.2). This regulatory region had been previously inserted into a CRIM vector upstream of the lacZ gene. A S. Typhimurium LT2 strain with a single copy insertion of this CRIM vector grew as blue and white colonies indicating that the S. Infantis_I gtr regulatory region was capable of phase variation (Davies et al., 2013) (Fig.5.5a).

Why was phase variation possible in S. Infantis_I gtr despite only three GATC sequences being present? The Typhi 2_II gtr also contained only three GATC sites, but did not phase vary. However, unlike the S. Typhi 2_II gtr, the TATC¹ sequence variation did not alter the sequence of the OxyR A half site it overlapped with, any more than a GATC site would. Considering the information gained from the Typhi 2_II gtr GATA⁴ mutant, it seemed likely that the reason phase variation occurred in this strain was because OxyR was capable of binding the OxyR A
Table 5.1. Switch frequencies and \textit{lacZ} expression levels of \textit{S. Typhi} \textit{gtr}:\textit{lacZ} CRIM vector strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>\textit{gtr} region</th>
<th>\textit{Lac} phenotype</th>
<th>Miller Units$^a$ (SD)</th>
<th>ON $\rightarrow$ OFF switch frequency</th>
<th>OFF $\rightarrow$ ON switch frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 637</td>
<td>\textit{S. Typhi} 2_I</td>
<td>Lac$^-$/Lac$^+$</td>
<td>1116 (42)</td>
<td>2.5 x $10^{-3}$</td>
<td>2.3 x $10^{-3}$</td>
</tr>
<tr>
<td>Path 636</td>
<td>\textit{S. Typhi} 2_II wt (GAAA$^4$)</td>
<td>Lac$^+$</td>
<td>738 (46)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Path 806</td>
<td>\textit{S. Typhi} 2_II (GATC$^4$)</td>
<td>Lac$^-$/Lac$^+$</td>
<td>619 (12)</td>
<td>6.3 x $10^{-3}$</td>
<td>1.9 x $10^{-3}$</td>
</tr>
<tr>
<td>Path 833</td>
<td>\textit{S. Typhi} 2_II (GATA$^4$)</td>
<td>Lac$^-$/Lac$^+$</td>
<td>250 (11)</td>
<td>1.8 x $10^{-3}$</td>
<td>1.9 x $10^{-2}$</td>
</tr>
</tbody>
</table>

\textit{a.} Miller Units are given as averages of 4 technical repeats, with the standard deviation (SD) in brackets. Path 637, Path 806 and Path 833 results were calculated for 100\% ON cultures (originally 86\%, 64\% and 65\% ON, respectively).
Fig. 5.5. Colony phenotypes of *S. Infantis_I* *gtr:*lacZ fusion CRIM vector strains

**A**, Path 438, *S. Typhimurium* LT2 with *S. Infantis_I* *gtr:*lacZ (TATC\(^1\)) CRIM vector insertion. **B**, Path 893, *S. Typhimurium* LT2 with *S. Infantis_I* TAAC\(^1\) mutant *gtr:*lacZ fusion CRIM vector. **C**, Path 924, *S. Typhimurium* LT2 with *S. Infantis_I* GATC\(^1\) mutant *gtr:*lacZ CRIM vector. Colonies were diluted and spread plated on M9 X-Gal media. Representative of two repeats.
half site. If this was true, altering the TATC\textsuperscript{1} sequence by removing bases that formed part of the OxyR A half site should abrogate phase variation.

The TATC\textsuperscript{1} sequence variation of the \textit{S. Infantis_I} \textit{gtr} regulatory region (fused to \textit{lacZ}) contained in a CRIM vector was changed to TAAC\textsuperscript{1} (Steven Watson). Doing so removed a T base that formed part of the OxyR A half site. Single copy insertion of the point mutated CRIM vector into \textit{S. Typhimurium} LT2 and plating on X-Gal media showed that all colonies grew white (Fig.5.5b). A \(\beta\)-galactosidase assay showed that this strain had very low \textit{lacZ} expression of 3 Miller Units, whereas the wildtype ancestor had 932 Miller Units (Table 5.2). This result indicated not only that phase variation had ceased, but also that OxyR could be repressing expression of the \textit{lacZ} reporter by persistently binding \textit{gtr} in the OFF position (at the B and C half sites). It was concluded that the TATC\textsuperscript{1} sequence variation did not prevent phase variation because it still allowed OxyR binding at the A half site.

### 5.3.2 The TATC\textsuperscript{1} sequence variation affects phase variation

Did the TATC\textsuperscript{1} sequence variation of \textit{S. Infantis_I} \textit{gtr} alter the switch frequency compared to a \textit{gtr} regulatory region containing four GATC sequences? The GATA\textsuperscript{4} mutation in \textit{S. Typhi_2_II} \textit{gtr} certainly altered the switch rate compared to the same \textit{gtr} with four GATC sites. If the same situation applied to \textit{S. Infantis_I}, changing the TATC\textsuperscript{1} sequence to GATC\textsuperscript{1} should produce a different switch rate.

The \textit{S. Infantis_I} \textit{gtr} regulatory region contained in a CRIM vector was point mutated by changing the TATC\textsuperscript{1} sequence to GATC\textsuperscript{1} (Steven Watson). Integrating the resulting vector as a single copy into the \textit{S. Typhimurium} LT2 chromosome produced blue and white colonies on X-Gal media (Fig. 5.5c). Phase variation was clearly still possible with a fourth GATC site.

The ON to OFF switch frequency of the GATC\textsuperscript{1} mutant strain showed a minor difference compared to the wildtype strain (averages of \(5.8 \times 10^{-3}\) and \(6.4 \times 10^{-3}\), respectively). However, the OFF to ON frequency of the GATC\textsuperscript{1} mutant was 12.2 – fold lower compared to the wildtype strain (averages of \(5.30 \times 10^{-4}\) and \(6.45 \times 10^{-3}\), respectively) (Table 5.2). The TATC\textsuperscript{1} sequence variation therefore did alter the switch frequency of \textit{S. Infantis_I} \textit{gtr}.
Table 5.2. Switch frequencies and *lacZ* expression levels of *S. Infantis_I* *gtr*:*lacZ* CRIM vector strains

<table>
<thead>
<tr>
<th>Strain</th>
<th><em>gtr</em> region (relevant sequence)</th>
<th>Lac phenotype</th>
<th>Miller Units&lt;sup&gt;b&lt;/sup&gt; (SD)</th>
<th>ON → OFF switch frequency</th>
<th>OFF → ON switch frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 444&lt;sup&gt;a&lt;/sup&gt;</td>
<td><em>S. Infantis_I</em> wt (TATC&lt;sup&gt;i&lt;/sup&gt;)</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;/Lac&lt;sup&gt;−&lt;/sup&gt;</td>
<td>932 (27)</td>
<td>6.0 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>5.3 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.8 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>7.6 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
</tr>
<tr>
<td>Path 893</td>
<td><em>S. Infantis_I</em> (TAAC&lt;sup&gt;i&lt;/sup&gt;)</td>
<td>Lac&lt;sup&gt;−&lt;/sup&gt;</td>
<td>3 (0.04)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Path 924</td>
<td><em>S. Infantis_I</em> (GATC&lt;sup&gt;i&lt;/sup&gt;)</td>
<td>Lac&lt;sup&gt;+&lt;/sup&gt;/Lac&lt;sup&gt;−&lt;/sup&gt;</td>
<td>1290 (10)</td>
<td>4.1 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3.7 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.1 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>6.9 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data from Davies et al., 2013.

<sup>b</sup> Miller Units are given as averages of 4 technical repeats, with the standard deviation in brackets. Path 924 results were calculated for 100 % ON cultures (originally 88 % ON).
5.4 DIFFERENCES BETWEEN *gtr:*lacZ AND *gtrABC::lacZ* REPORTER FUSIONS

As part of experiments to measure expression of *gtr* genes, the *lacZ* gene was inserted downstream of the LT2_I *gtrABC* operon on the *S. Typhimurium* LT2 chromosome by lambdaRed recombination (Datsenko and Wanner, 2000). The *lacZ* gene was separated from the *gtr* operon by 20 bp which contained an additional ribosome binding site. Transcription of the *gtr* operon resulted in co-transcription of the *lacZ* gene. The LT2_I *gtr* regulatory region contains four GATC sites and phase varies (Fig.5.2). The resulting strain (Path 859) grew as blue and white colonies on X-Gal media. The same procedure was performed on the ST4/74_I *gtr* operon (which is identical to LT2_I *gtr*) of the *S. Typhimurium* ST4/74 strain (= Path 891).

It was decided to compare *lacZ* expression levels and switch frequencies of the *gtrABC::lacZ* fusion strains with a previously produced CRIM vector containing the LT2_I *gtr* regulatory region fused to *lacZ* (Broadbent et al., 2010). This CRIM vector had been inserted as a single copy into the chromosome of *S. Typhimurium* LT2. The primary aim of the experiments was to confirm that the LT2_I *gtrABC::lacZ* fusion strain behaved similarly to the LT2_I *gtr* CRIM vector strain. This could not be taken for granted, as the *gtr* operon and insertion site for the CRIM vector are located in different areas of the chromosome and orientated in different directions relative to the origin of replication. Transcription of the *gtrABC::lacZ* operon occurs towards the origin of replication, and in the opposite direction for the *gtr:*lacZ fusion in the CRIM vector. The att B site used for insertion of CRIM vectors in the *S. Typhimurium* LT2 chromosome is at position 851647 – 851669, whereas the LT2_I *gtr* operon is encoded from 614807 – 617722. The sites are therefore displaced by ~233 kb.

β-galactosidase assays found that *lacZ* expression levels of the *gtrABC::lacZ* fusion strains (Path 859, Path 891) were lower than in the CRIM vector strain (Path 84) (Table 5.3). A possible explanation was that the *lacZ* gene in the *gtrABC::lacZ* fusions was separated from the promoter region by the 2.918 kilobase sequence of the *gtr* operon, which may have reduced the amount of transcription occurring. In the CRIM vector, the *gtr* promoter region was fused directly to the *lacZ* gene. It was important to note this difference in *lacZ* expression, as it could affect the switch frequency calculation: lower *lacZ* expression could cause the number of OFF colonies to be overestimated, which would give a higher ON to OFF switch frequency.
Table 5.3. Switch frequencies and *lacZ* expression levels of *S.* Typhimurium *gtr:*lacZ CRIM vector strains and *gtrABC::lacZ* fusion strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>gtr reporter fusion</th>
<th>Miller Units(^d) (SD)</th>
<th>ON → OFF switch frequency</th>
<th>OFF → ON switch frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Path 84(^{a,b})</td>
<td>LT2_I *gtr:*lacZ CRIM</td>
<td>Lac(^+)/Lac(^-) 1236 (140)</td>
<td>2.1 x 10(^{-2})</td>
<td>2.0 x 10(^{-3})</td>
</tr>
<tr>
<td>Path 859(^c)</td>
<td>LT2_I <em>gtrABC::lacZ</em></td>
<td>Lac(^+)/Lac(^-) 130 (6)</td>
<td>4.7 x 10(^{-3})</td>
<td>1.5 x 10(^{-3})</td>
</tr>
<tr>
<td>Path 891(^c)</td>
<td>ST4/74_I <em>gtrABC::lacZ</em></td>
<td>Lac(^+)/Lac(^-) 128 (2)</td>
<td>6.8 x 10(^{-3})</td>
<td>8.9 x 10(^{-4})</td>
</tr>
</tbody>
</table>

\(^a\) Data from Broadbent et al., 2010.

\(^b\) *gtr:*lacZ fusion CRIM vector insertion

\(^c\) *gtrABC::lacZ* chromosomal operon fusion

\(^d\) Miller Units are given as averages of 4 technical repeats, with the standard deviation in brackets. Path 84, Path 859 and Path 891 results were calculated for 100% ON cultures (originally 72%, 77% and 86% ON, respectively).
Switch frequency analysis was performed on the \textit{gtrABC::lacZ} fusion strains (Path 859, Path 891). It was found that the ON to OFF frequency was lower (fewer colonies switching OFF) in the \textit{gtrABC::lacZ} fusions (averages of $4.8 \times 10^3$ for LT2 and $3.1 \times 10^3$ for ST4/74) than in the CRIM vector strain (average of $2.2 \times 10^2$, Broadbent et al., 2010) (Table 5.3). This difference could not be explained by the lower $\beta$-galactosidase expression of the \textit{gtrABC::lacZ} fusion strains as it meant that more colonies were retaining an ON phenotype compared to the CRIM vector strain. Instead, it pointed to a different regulatory effect being involved.

Finally it should be mentioned that the ST4/74 \textit{gtrABC::lacZ} fusion strain also showed a lower OFF to ON switch rate compared to the CRIM vector strain (averages of $7.5 \times 10^4$ and $1.9 \times 10^3$, respectively). As mentioned, this discrepancy could be explained by lower expression of the \textit{lacZ} gene. However, this difference did not seem to be as pronounced in the LT2 \textit{gtrABC::lacZ} fusion strain which showed an OFF to ON frequency of $1.8 \times 10^3$ on average (Table 5.3). Given that the LT2 and ST4/74 strains showed equal amounts of $\beta$-galactosidase expression, it seems possible that a further effect could be involved in the ST4/74 strain.

\section*{5.5 DISCUSSION}

\subsection*{5.5.1 \textit{gtr} sequence variation in naturally occurring serovars}

The results in this chapter demonstrate how phase variation can be influenced by naturally occurring sequence variation (and possibly by the position and orientation of the \textit{gtr} operon in the chromosome). A benefit of \textit{gtr} phase variation is the increased population heterogeneity, which creates a variety of O-antigen phenotypes, even among a population derived from a single cell. This raises the question why \textit{S. Typhi} 2-II \textit{gtr} is incapable of phase variation. Is there an advantage to losing phase variation?

Given that \textit{S. Typhi} is thought to have evolved from \textit{S. Typhimurium}, it is reasonable to assume that the \textit{S. Typhi} 2-II \textit{gtr} is derived from LT2-I \textit{gtr}, as opposed to evolving independently (both operons perform the same modification and display DNA sequence homology of 98 %) (Parkhill et al., 2001). Therefore, it seems plausible that the GAAA\textsuperscript{4} sequence variation that prevents \textit{S. Typhi} 2-II \textit{gtr} phase variation was acquired during this evolution. Was this variation positively selected for, or simply not selected against? As the variation removes the potential
benefit of population heterogeneity it could point to another incentive for retaining it. Should that be the case, it seems to only apply to this particular gtr operon in S. Typhi – the other gtr operon present in the genome, S. Typhi 2_I is fully capable of phase variation. Could the S. Typhi 2_II gtr O-antigen glycosylation be of such benefit to S. Typhi, that phase variation is abandoned in favour of constitutive expression? During the course of this work, results obtained in the van der Woude laboratory showed that constitutive gtr glycosylation of the S. Typhi O-antigen led to a survival advantage in human serum (personal communication by Erica Kintz 2015, in preparation). This result could explain why the S. Typhi 2_II gtr operon evolved to be expressed constitutively.

With regard to such a potential advantage, the different lifestyles of S. Typhi and S. Typhimurium should be considered. While S. Typhimurium may cause self-limiting gastro-enteritis in a broad host range, S. Typhi causes systemic, persistent infections only in humans. The reasons for this host restriction are not fully understood, although they may be connected to differences in recruitment of neutrophil cells to the intestinal lining following infection (reviewed in Spanò, 2014).

It was interesting that the GATA^4 mutation in S. Typhi 2_II gtr still allowed phase variation, although at a different switch frequency. This particular mutation was also found in S. Gallinarum_I gtr, which has the same sequence instead of a 4th GATC site. Therefore the S. Gallinarum_I gtr may have a higher switch rate as well, although this would need to be confirmed separately, as there are some other sequence differences. For example, the OxyR B half sites differ by 3 bp (Fig.5.2). The mismatching bp are not part of the established OxyR binding site consensus and are therefore unlikely to affect the switch frequency.

The S. Gallinarum serovar is host-restricted to chickens, although it evolved from the broad-host range S. Enteritidis serovar (Thomson et al., 2008). S. Enteritidis hosts two gtr operons, both of which contain four GATC sites and are therefore likely to phase vary. Parallels could be drawn between these two serovars and S. Typhi and S. Typhimurium – the host-restricted serovars contain gtr operons with sequence variations that remove GATC sites, whereas the broad host-range serovars they evolved from contain gtr operons with four GATC sites and are predicted to phase vary. The gtr sequence variations could have been acquired during the evolution to host-restriction. Perhaps a higher gtr switch rate could be advantageous in certain environments.
However, host-restricted serovars are not the only strains with sequence variations that impact gtr phase variation: S. Infantis_I for example, infects both chickens and humans (Dunowska et al., 2007, Murakami et al., 1999). The exact function of the S. Infantis_I gtr is unknown although it appears to produce visible shifts in TSDS-PAGE separations of LPS molecules, indicating that it could be adding a molecule such as glucose (Davies et al., 2013).

5.5.2 Effect of sequence variation on gtr phase variation

Based on the results in this chapter (and chapter 4) the following statements can be made about the effect of sequence variation on gtr phase variation: firstly, alteration of a single GATC site in a way that removes the Dam target sequence but without changing the overlapping OxyR half site increases the phase variation switch frequency. The absence of a GATC site may increase the chances of OxyR binding the overlapping OxyR half site. This observation was made with both the S. Typhi 2_II GATA\(^4\) mutant and S. Infantis_I TATC\(^1\) (wildtype) gtr compared to the same gtr regulatory regions with 4 GATC sites.

Second, alteration of a single GATC site in a manner that reduces the match of the overlapping OxyR half site with the OxyR consensus binding site abrogates gtr phase variation. This is evidenced by the S. Typhi 2_II gtr wildtype (GAAA\(^4\)), in which a T base missing from the OxyR consensus was responsible for the locked ON phenotype. Similarly, the S. Infantis_I gtr TAAC\(^1\) mutant displayed a locked OFF phenotype. In both cases, the absence of a single T base reduced the match between the overlapping OxyR half site with the established OxyR binding consensus by one bp (to seven out of ten bp match). OxyR binding involves four sites which are bound with intermediate affinity (Toledano et al., 1994). The absent T bases in this work were sufficient to cause loss of phase variation in S. Typhi 2_II or S. Infantis_I gtr, which could be explained by a reduced overall affinity of OxyR to the mutated half sites.

Third, altering both GATC sites in a single OxyR half site without reducing the match with the OxyR half site binding consensus leads to loss of phase variation. This was evidenced by Path 931, in which alteration of GATC\(^3\) and GATC\(^4\) of the OxyR C half site (to GATA\(^3\) and TATC\(^4\), respectively) in ST4/74_I gtr resulted in a locked OFF phenotype. In this case OxyR likely bound the B and C half sites persistently, due to absence of Dam methylation (see chapter 4). Furthermore, changing GATC\(^1\) and GATC\(^2\) of LT2_I gtr to CATC\(^1\) and GATG\(^2\) caused a locked ON phenotype (Broadbent et al., 2010). Based on this and the first statement (see above), it
seems that OxyR affinity to a particular half site increases successively with the removal of GATC sites, provided that the match with the consensus binding site is not reduced. These statements apply to the gtr operons examined in this work. It should not be assumed that they apply equally to other gtr operons, as sequence variation outside the GATC sites could also have an impact.

The S. Typhi 2_II gtr GATA^4 mutation removed a GATC site in the OxyR C half site, but without altering the OxyR binding consensus. This may have increased the affinity of OxyR to the C half site (which is occupied by OxyR during the OFF phase), and therefore caused a 2.7-fold higher ON to OFF switch rate (compared to the S. Typhi 2_II gtr GATC^4 mutant). However, the 8.5-fold increase in OFF to ON switch frequency was unexpected. The OxyR A site had not been altered, but OxyR seemed to have an increased affinity towards it, as indicated by the elevated OFF to ON switch frequency. In short, the GATA^4 mutation increased both the OFF to ON and the ON to OFF switch frequency.

Furthermore, in the case of S. Infantis_I gtr, the TATC^1 sequence variation appeared to increase only the OFF to ON switch frequency (compared to the S. Infantis_I GATC^1 gtr mutant). In this case, TATC^1 replaced a GATC site in the OxyR A half site, and also did not alter the OxyR binding consensus. This may have caused OxyR to have increased affinity for the OxyR A site, causing an increase in the OFF to ON switch. However, unlike the S. Typhi 2_II gtr GATA^4 mutant, the TATC^1 sequence variation only affected the OFF to ON switch frequency.

Comparing S. Infantis_I gtr and S. Typhi 2_II gtr was therefore difficult, as the absence of single GATC sites appeared to have different effects on them. In particular, removal of single GATC sites, whithout altering the overlapping OxyR half binding sites did not have identical effects on the switch frequency. A number of other sequence variations in both gtr regulatory regions could provide an explanation. For example, the S. Typhi 2_II gtr OxyR A and C half sites contain 8 out of 10 bp of the OxyR binding consensus sequence, while the B half site contains 9 out of 10 bp. On the other hand, in S. Infantis_I gtr, the OxyR A half site contains an 8 out of 10 bp match, the B half site 10 out of 10 bp and the C half site 7 out of 10 bp (Fig. 5.2).

The S. Infantis_I gtr GATC^1 mutant is biased towards the OFF phase (10.6-fold lower OFF to ON switch frequency than ON to OFF), indicating that OxyR preferentially binds the B and C half sites. This seems paradoxical, given that the B and C half sites collectively have a worse
match with the OxyR binding consensus sequence (17 out of 20 bp), compared to the A and C half sites (18 out of 20 bp).

Sequence variations away from GATC sites, but within the OxyR half binding sites are known to affect phase variation: the LT2_I gtr regulatory region is biased towards the OFF phase and contains an OxyR B half site with a 9 out of 10 bp match to the OxyR binding consensus. The P22 gtr OxyR B half site matches the consensus perfectly (10 out of 10 bp) and is biased towards the ON phase. Changing the P22 OxyR B half site to imitate the LT2_I gtr B half site with 9 out of 10 bp caused an OFF bias. Alternatively, changing the LT2_I B half site by improving the OxyR binding site consensus match to 10 out of 10 bp reduced the bias towards the OFF phase (Sarah Broadbent).

Other potentially important sequence variations concern bp that are included in OxyR half sites but are not part of the established consensus OxyR binding sequence. For instance, in the sequence ATCGgT, the lower case g makes a positive contribution towards the average sequence conservation of an OxyR binding site (Zheng et al., 2001a). This motif is present in the S. Typhi 2_II gtr OxyR A half site, but not in the S. Infantis_I gtr OxyR A half site. In contrast, in the motif AgCTAT, the lower case g makes a negative contribution towards the average sequence conservation of an OxyR binding site. This sequence is present in the OxyR B half sites of both S. Infantis_I and S. Typhi 2_II gtr. The individual impact of these sequence variations (if any) would need to be determined separately and initially in the same model system such as LT2_I gtr, as this is probably the best understood.

Using X-ray crystallography, Horton et al., (2015) showed that E. coli Dam (92 % amino acid identity compared to S. Typhimurium LT2 Dam) can also bind to non-GATC sequences such as GTTTA, or incomplete GATC sites like ATC. By binding these sequences, Dam could repress transcription, or affect methylation of GATC sites in the proximity. These sequences are present in the phase varying agn43 regulatory region. The sequences are also present in the OxyR C half site between GATC³ and GATC⁴ (gatcGTTTATATCgatc, upper case letters) of the LT2_I, S. Typhi 2_II and S. Gallinarum_I gtr regulatory regions, among others. The P22 or S. Infantis_I gtr regulatory regions do not contain these sequences.

The GATA⁴ mutation introduced in S. Typhi 2_II gtr also improved the match of the -35 site (TCGATA) with the consensus sequence (TTGACA) compared to the wildtype (Madigan et al.,
2005). This did not appear to increase transcription: maximum β-galactosidase expression of the S. Typhi 2-II gtr GATA^4 strain was 250 Miller Units, compared to 738 in the wildtype gtr: lacZ fusion. The higher switch frequency of the S. Typhi 2-II gtr GATA^4 strain could have caused an overestimation of the number of switched ON colonies, resulting in a lower number of Miller Units being calculated.

5.5.3 Effect of chromosomal location and orientation on gtr phase variation

The chromosomal fusions of LT2-I gtrABC:: lacZ (Path 859, Path 891) seemed to indicate a possible effect of location or orientation on the chromosome on gtr switch frequency. DNA replication is presumably the only situation under which gtr can switch phases, as DNA methylation can be altered at this point (Broadbent et al., 2010). The competition between OxyR and Dam for binding newly synthesised DNA, and factors that influence this process are discussed below.

DNA-bound proteins such as OxyR are removed by helicases during replication, although head-on collisions with RNA polymerase slow the progress of the DNA replication fork (Merrikh et al., 2012). Ribosomal RNA genes are mostly (~ 70 % in E. coli) transcribed co-directionally with the replication fork. However, inversion of a ribosomal RNA gene delays the replication fork (Boubakri et al., 2010).

As mentioned, the gtrABC:: lacZ fusions were orientated towards the origin of replication. The LT2-I gtr: lacZ fusion contained in CRIM vector (Path 84) was inserted in the opposite direction on the chromosome and ~233 kb downstream of the gtrABC operon. Therefore transcription of gtrABC:: lacZ would encounter the replication fork head-on, whereas gtr: lacZ transcription would be co-directional with the replication fork. Transcription from the gtrABC:: lacZ operon fusion is therefore more likely to delay the replication fork than the CRIM vector based gtr: lacZ fusion. Dam is processive and methylates GATC sequences shortly, but not immediately after DNA replication (Pollak et al., 2014; Urig et al., 2002; Wion and Casadesús, 2006). Delaying the replication fork could therefore also lead to a delay in Dam methylation of gtr regulatory regions, which could in turn impact OxyR binding, although this have only a minor effect on gtr switch frequency.
A more pronounced effect could be caused by the order in which particular GATC sites are methylated in a gtr regulatory region. During DNA replication, the OxyR A half site of the CRIM vector based LT2_I gtr: lacZ fusion strain would encounter the replication fork before the B and C half sites. In this case, the switch frequency is biased towards the OFF phase (ON to OFF switch frequency was 11.9-fold higher than OFF to ON). Therefore the GATC sequences in the A half site have a higher chance of being methylated, which would lead to OxyR preferentially binding the B and C half sites causing the gtr OFF phase. This could suggest that Dam has a higher chance of accessing GATC sites in a newly synthesised OxyR half site than OxyR has of binding the new site.

In contrast, in the LT2_I gtrABC::lacZ operon fusion strain, the OxyR C half site would be replicated first. In this case, if Dam does indeed have a higher chance of accessing GATC sites in a newly synthesised OxyR half site before OxyR can bind, GATC sequences in the C half site would more likely be methylated. OxyR would then preferentially bind the A and B half sites leading to gtr having a higher likelihood of being switched ON. An ON bias was not measured in this strain, although the OFF bias was less pronounced (OFF to ON switch frequency was 2.6-fold higher than OFF to ON) than the CRIM vector based LT2_I gtr: lacZ fusion strain. Other factors, such as the potential alternative Dam sites (which are only present in the C site) or collision between transcription and the replication fork could affect the switch frequency bias as well. Furthermore, transcription of lacZ is likely to be lower in this strain due to being placed at the end of the gtrABC operon – so if there is an ON bias, it may not be easily detected due to lower expression of the β-galactosidase reporter.

The potential influence of chromosomal orientation provides a new perspective on the results obtained with the S. Infantis_I and S. Typhi 2_II gtr: lacZ fusions. Specifically, In S. Infantis_I gtr, the replication fork would hit the A half site first, containing the TATC^1 sequence variation. As Dam cannot methylate this sequence, OxyR could be able to bind the A and B half sites preferentially. This may cause the increased OFF to ON switch frequency compared to the S. Infantis_I gtr GATC^1 mutant.

The S. Typhi 2_II gtr GATA^4 mutant is harder to explain given that switch frequency increased for both OFF to ON and ON to OFF compared to the S. Typhi 2_II GATC^4 mutant. The replication fork encounters the OxyR C half site containing the GATA^4 mutation last, after passing though the A and B half sites. OxyR would have to compete with Dam to bind the A and B half sites. For unknown reasons, OxyR appears to bind the A and B half sites with equal
chance as the B and C half sites in S. Typhi 2_Il GATA^4 gtr. The presence of the potential alternative Dam target sequence in the OxyR C half site may contribute to this.

A further example of chromosomal orientation causing changes in switch frequency concerns the *E. coli* *agn43* gene which also phase varies through Dam methylation of GATC sites and OxyR binding. Three GATC sites overlap with a single OxyR binding site and the *agn43* transcriptional start site. OxyR binding halts transcription of *agn43* and GATC methylation blocks OxyR binding (Haagmans and Van Der Woude, 2000; Wallecha et al., 2002).

Insertion of three extra OxyR sites 466 bp upstream of a transcriptional fusion of the *agn43* regulatory region to the *lacZ* gene caused a locked OFF phenotype (Kaminska and van der Woude, 2010). In this case, the replication fork passed through the auxiliary OxyR sites before encountering the *agn43* regulatory region. DNA replication would have caused removal of OxyR from the auxiliary sites and increased its localised concentration. The increased amounts of OxyR may have been able to outcompete Dam for binding the *agn43* regulatory region after replication. In a further experiment, the entire *agn43: lacZ* fusion and the auxiliary OxyR sites were inverted on the chromosome. This way, replication fork encountered the *agn43* regulatory region before the auxiliary OxyR sites. As a result, phase variation was restored, with a bias towards the OFF phase. This example illustrates how shifts in the competition between OxyR and Dam can cause phase variation bias. The potential alternative Dam binding sites in the LT2_I gtr OxyR C site could have a similar effect on phase variation.

A further question concerns the effect of chromosomal location on gtr transcription. It is not unprecedented for gene regulation to be affected by position in the chromosome, as some areas may contain a lower G/C content that is preferentially bound by regulators such as H-NS (Brambilla and Sclavi, 2015). The horizontally acquired LT2_I gtrABC operon is part of the SPI16 pathogenicity island, with a G/C content of 39.8% (Vernikos and Parkhill, 2006). The G/C content of the *Salmonella* Typhimurium genome is 52.2%. H-NS reduces transcription by roughly one third from genes with an average of 46.8% G/C content (Navarre et al., 2006). The LT2_I gtrABC operon is therefore within the range that could be targeted by H-NS for repression. In contrast, the G/C content of the CRIM vector containing the LT2_I gtr: lacZ fusion is 50.1%, and is therefore unlikely to be a target for H-NS repression.
A possible method to test how chromosome location and orientation affects gtr regulation would be to produce gtr: lacZ fusions and insert them in varying orientations at different positions throughout the Salmonella chromosome. Alternatively, the gtr: lacZ fusion in the CRIM vector could be inverted to test how orientation affects the switch frequency.

5.5.4 Potential future experiments

A further observation concerned the use of reporter fusions of gtr regulatory regions to the lacZ gene. These provided a convenient tool for studying phase variation and the effects of sequence variation. The limitations of the method became clear during measurement of strains with higher switch frequencies (e.g. S. Typhi 2-II gtr GATA mutant), due to the reliance on assessment of colony morphology: switch frequency calculations assumed that a blue colony on X-Gal media was derived from a switched ON cell, and a white colony from an OFF cell. If the switch frequency of a strain is above ~1 x 10^{-2}, it may be difficult to distinguish separate colony phenotypes, as an ON cell will produce a colony with a large amount of OFF cells and vice versa. A strain with a high switch frequency could produce a uniform ON colony phenotype and thus mistakenly be labelled as non-phase varying.

As an example, in a dam` knockout strain, the LT2_1 gtr: lacZ fusion did not show phase variation, but rather constitutive low level expression (Broadbent et al., 2010). In this strain, OxyR should be able to access all three half sites in the gtr regulatory region because none of the GATC sites can be methylated by Dam. Removal of a single GATC methylation target, while allowing OxyR binding, increased the switch frequency of the S. Typhi 2-II gtr GATA mutant. In the dam` mutant, none of the GATC sites can be methylated, but OxyR can still bind the overlapping OxyR half sites. Perhaps phase variation does occur in the dam` mutant, but the switch frequency is too high for detection by the gtr: lacZ fusion reporter. A further possibility to study this without performing a dam` mutation (which could have wide-ranging side effects on the cell), would be to point mutate all GATC sites in a gtr regulatory region, but without altering the OxyR half sites.

Observing phase variation in such a rapidly switching system may have to make use of single cell assays, rather than measuring the aggregate effect on colonies. For example, a gtr regulatory region fused to a fluorescent reporter might allow detection of ON and OFF cells. Potentially interesting gtr regions to examine could contain point mutations that remove one,
two (in the same or separate half sites), three, or all GATC sites, as well as a dam- knockout strain. If the objective was simply to demonstrate that phase variation occurs without measuring the switch frequency, the LPS molecules could be examined – a rapidly switching population would contain both modified and unmodified O-antigen molecules, which may produce a distinctive pattern on TSDS-PAGE gels.

In summary, the work in this chapter demonstrated how naturally occurring sequence variation can have substantial effects on gtr phase variation. Removing single GATC sites without altering the overlapping OxyR half site increased switch frequency. On the other hand, removing single GATC sites as well as altering the overlapping OxyR site abrogated phase variation. Chromosomal location of gtr could also impact phase variation. Establishing a wider model that ties these effects together and applies equally to different gtr operons is difficult with the currently available data. It could be more prudent to focus further research on a single gtr operon such as LT2_I. If the role of sequence variation (other than GATC sites) and chromosomal location can be defined in this system, perhaps those insights can be extended to other gtr operons.
CHAPTER 6
DISCUSSION
6.1 INTRODUCTION

Previous work determined the mechanism of gtr phase variation (Broadbent et al., 2010). Phage resistance was identified as a biological role for gtr (Kim and Ryu, 2012; Kintz et al., 2015). gtr operons showed evidence of horizontal gene transfer (Davies et al., 2013). A further possible role for gtr concerned long term intestinal persistence in mice (Bogomolnaya et al., 2008).

The work presented here aimed to identify factors that influence gtr phase variation and expression, in order to further define the biological role of the gtr operon. It was found that naturally occurring sequence variation can have extensive effects on the switch frequency of gtr operons. These results provided new insights into how the process of phase variation can be fine-tuned, often by single bp changes.

Furthermore, conditions designed to mimic the macrophage SCV reduced gtr expression and induced expression of the short RNA STnc1870. This work identified a new role for OxyR, as both the repressor and possible activator of STnc1870 transcription. Finally, the SPI2 encoded ssaO mRNA was identified as a potential target of STnc1870, thereby linking gtr regulation to Salmonella’s intracellular lifestyle. The following chapter focusses on the wider implications of these results and potential future experiments.

6.2 INFLUENCES ON gtr EXPRESSION

Expression of gtr was analysed after growth in different conditions. S. Typhimurium entering stationary phase growth showed reduced expression of LT2_I gtr operon genes in qRT-PCR experiments, compared to exponential phase. Transcription of the gtr operon was also found to be independent of the stress response sigma factor $\sigma^{38}$. The exact cause of stationary phase downregulation of gtr expression remains to be determined, although it could simply be part of a general slowing of metabolism as the cells enter stationary phase and encounter nutrient limitation.

The S. Typhimurium LPS synthesis genes of the rfb cluster show reduced expression during stationary phase compared to exponential phase (Kröger et al., 2013; Schnaitman and Klena, 1993). In E. coli, LPS biosynthesis is highest during exponential growth and discontinues
S. Typhimurium LPS biosynthesis increases during growth from exponential to stationary phase (Bravo et al., 2008). Based on this, and because gtr transcription is rpoS independent, it is reasonable to assume that both the LPS biosynthesis genes and the gtr operon are transcribed by the ‘housekeeping’ RNA polymerase sigma factor $\sigma^{70}$ (RpoD). Therefore, transcription of gtr and the LPS biosynthesis genes would be dependent on cellular levels of $\sigma^{70}$. As mentioned, the proportional amount of $\sigma^{70}$ is reduced as the cell enters stationary phase and rpoD transcription decreases (Battesti et al., 2011; Kröger et al., 2013, see chapter 3). Other conditions that reduce transcription of rpoD include ‘shocks’ such as sudden transfer to anaerobic conditions or pH = 3 media (Kröger et al., 2013). Reduced expression of RpoD could in turn cause reduced expression of gtr. gtr expression could be examined under these conditions using the gtrC: lacZ fusion strain and qRT-PCR, to determine the influence of other stress factors.

Experiments with plasmids expressing the Erv1p sulfhydryl oxidase and DsbC isomerase did not indicate that oxidised OxyR influenced gtr expression or phase variation. This data contradicted later qRT-PCR results obtained using InSPI2 media that showed gtr downregulation and STnc1870 upregulation in response to H$_2$O$_2$ treatment. Upregulation of STnc1870 in response to H$_2$O$_2$ treatment was only achieved in strains with wildtype OxyR – a mutant strain with persistently reduced C199S OxyR could not upregulate STnc1870 in response to H$_2$O$_2$ treatment (see section 6.5).

However, a number of other differences between the experiments make comparisons difficult. For instance, the Erv1p experiments used M9 minimal growth media (pH = 7), as opposed to InSPI2 (pH = 5.8). The reporter systems also differed: for the Erv1p experiments, a single copy, chromosomally inserted plasmid carrying a fusion of the gtr regulatory region to the lacZ gene was used. The qRT-PCR experiments measured transcription of the chromosomal gtr operon. Given that the lacZ reporter system is less exact (due to reliance on protein production for detection and because reduced transcription may not be detected if the protein is not degraded)
and part of an artificial construct, the qRT-PCR results are likely to be more reliable and better suited for interpretations.

It may be worthwhile to repeat the Erv1p experiments using InSPI2 media. A strain carrying the Erv1p/DsbC plasmid and grtC:lacZ fusion could be grown in culture before splitting it and adding arabinose to one half to induce expression of Erv1p and DsbC. RNA extracted from both cultures could be used in qRT-PCR to compare relative amounts of grt expression. Unchanged grt expression would indicate that OxyR bound to the grt regulatory region cannot be oxidised by Erv1p and DsbC. Other experiments could be performed in vitro using the grt regulatory region DNA and testing whether GATC sites can be methylated by Dam in the presence of OxyR and the Erv1p and DsbC. Based on the work in chapter 4, if OxyR is oxidised by Erv1p and DsbC, it may not be able to bind the grt regulatory region at the A, B or C half sites. In this case, the overlapping GATC sites should be available for methylation by Dam. Methylated GATC sites can then be detected by restriction digestion (Broadbent et al., 2010). The endonuclease DpnI for example cuts exclusively at methylated GATC sites.

6.3 THE grt REGULATORY REGION PRODUCES A SHORT RNA MOLECULE

qRT-PCR experiments confirmed results of Kröger et al., 2013, which showed a short RNA (STnc1870) being produced from the grt regulatory region after growth in InSPI2 media combined with a 1 mM H₂O₂ shock. STnc1870 expression could be induced in cultures containing majorities of either grt ON or grt OFF cells. However, grtA expression was reduced in all cultures following H₂O₂ treatment, compared to untreated cultures.

Further work showed that STnc1870 transcription did not increase after H₂O₂ treatment in a strain expressing the mutant C199S OxyR protein, which cannot be oxidised or alter conformation in response to oxidative stress (Toledano et al., 1994). Finally, a strain with point mutations of 12 bp predicted to form an alternative OxyR binding site in the upstream region of the STnc1870 gene showed lower proportional increases in STnc1870 transcription after H₂O₂ treatment. Overexpression of STnc1870 from the pLAC22 plasmid reduced expression of the ssaO mRNA, which encodes a component of the SPI2 secretion needle.
These results were used to establish an expanded model of gtr regulation: in the absence of oxidative stress, gtr phase variation occurs through OxyR binding and GATC methylation in the upstream region of the gtr operon as established in Broadbent et al., 2010 (Fig.6.1). Growth in InSPI2 media and addition of a H$_2$O$_2$ shock may cause OxyR to change to the oxidised conformation and relieve repression of STnc1870 by moving to an alternative binding site. This process appears to be independent of the ON or OFF state of gtr. Oxidised OxyR bound to the alternative site could stimulate transcription of the STnc1870 sRNA. STnc1870 could then bind and downregulate expression of ssaO, a component of the SPI2 secretion needle.

The finding that STnc1870 overexpression caused reduced ssaO expression (and by extension could inhibit formation of the SPI2 needle) raised the following question: how could this benefit Salmonella? There appeared to be a contradiction, given that expression of the SPI2 needle is required for survival in the macrophage (Figueira and Holden, 2012). STnc1870 expression is also predicted to be highest in the macrophage. Therefore, STnc1870 inhibition of SPI2 needle formation would occur most while in the macrophage.

Why would a sRNA induced by the macrophage environment inhibit formation of the SPI2 needle that ensures Salmonella survival in the same environment? If SPI2 expression was inhibited too much, Salmonella may be killed. It should be noted that STnc1870 expression in the macrophage is unlikely to be as high as measured after the 1 mM H$_2$O$_2$ shock in qRT-PCR experiments. The concentration of H$_2$O$_2$ in the macrophage has been estimated to be approximately 10 μM (Aussel et al., 2011). Addition of 10 μM H$_2$O$_2$ to cultures grown in InSPI2 media did not inhibit growth. Presumably, this amount of H$_2$O$_2$ was rapidly detoxified by the cells. In this case, OxyR would have only become oxidised for a brief period, and only a low level of STnc1870 could be expressed before repression by OxyR was reinstated (however, STnc1870 expression was not measured under this condition). Expression of STnc1870 in the macrophage would therefore most likely be at a persistent low level.

Even after IPTG-induced overexpression of STnc1870, the maximum reduction of ssaO expression was 2 – fold. This could have a significant impact on formation of the SPI2 needle, particularly if translation is also inhibited by STnc1870. An example of such a mechanism is the OxyS sRNA, which inhibits translation of the RpoS stress response sigma factor (Battesti et al., 2011). However, as mentioned, expression of STnc1870 in the macrophage is likely to be much lower, and could therefore have a much smaller effect on SPI2 needle expression.
Other sRNAs are known to act as regulators of SPI virulence genes. One example in *S. Typhimurium* is PinT, an 80 bp sRNA induced by the low pH-sensing PhoP regulatory system (see chapter 3.4) (Westermann et al., 2016). PinT inhibits SPI2 genes (by binding the *ssrB* mRNA, which is in turn a global regulator of SPI2 genes (Worley et al., 2000)) as well as expression of the SPI1 effectors SopE and SopE2. SopE and SopE2 activate the host cell transcription factor STAT3 in order to form the specialised intracellular compartment that *Salmonella* occupies (Hannemann et al., 2013).

Interestingly, SPI2 effectors inhibit STAT3 activation. PinT appears to fine-tune expression of SPI1 and SPI2 and thereby promotes the transition to *Salmonella*’s intracellular lifestyle. Human epithelial HeLa cells infected with a Δ*pinT* deletion strain also produced higher levels of pro-inflammatory IL-8 compared to cells infected with wildtype *Salmonella* (Westermann et al., 2016). STnc1870 could perform a function similar to PinT by adjusting SPI2 expression. If formation of the SPI2 needle is inhibited by STnc1870, this could lead to increased activation of STAT3 which would promote formation of *Salmonella*’s intracellular compartment. However, inhibition of SPI2 cannot be excessive as this would lead to bacterial killing. A precise balance would be needed.

Data published by Bogomolnaya et al., 2008 showed that a *S. Typhimurium* Δ*gtrA* deletion strain had reduced replication during infection of J774 murine macrophage (derived from *Salmonella* susceptible BALB/c mice) and Caco-2 epithelial cells (compared to a wildtype strain). However, a Δ*gtrC* deletion strain was not defective for intracellular replication. This data indicated that the ability to produce the *gtr* O-antigen modification was not required for intracellular replication. The growth defect of the Δ*gtrA* deletion strain could perhaps be related to STnc1870. The predicted alternative OxyR site of STnc1870 overlaps with the *gtrA* -35, -10 and +1 sites. The *gtrA* deletion was performed by inserting a kanamycin resistance cassette (*kan^R^*). Depending on where the *kan^R^* cassette was inserted and in what orientation, it may have interfered with STnc1870 regulation (the insertion site was not identified by the authors). The interference could explain the lack of intracellular replication if STnc1870 is indeed required for regulation of SPI2.

Other data that points towards the role of STnc1870 was published by Chaudhuri et al., 2013 used transposon mutation libraries to infect chickens, pigs and cows and then analysed which
Fig. 6.1. Model for regulation of the *gtr* operon

OxyR half sites are marked in red and GATC sites are annotated. Methylated GATC sites are in red and the A base is underlined. The *gtr* promoter region is marked in blue and annotated, along with the STnc1870 promoter, in red. RNA polymerase and OxyR are in grey and annotated. Graph not to scale.

In the absence of oxidative stress, *gtr* phase variation occurs with reduced OxyR as described in Broadbent et al., 2010. Growth in InSPI2 media and addition of a H₂O₂ shock may cause OxyR to change to the oxidised conformation and relieve repression of STnc1870 by moving to an alternative binding site. This process appears to be independent of the ON or OFF state of *gtr*. Oxidised OxyR bound to the alternative site could stimulate transcription of the STnc1870 sRNA. STnc1870 could then bind and downregulate expression of *ssaO*, a component of the SPI2 secretion needle.
mutants could be recovered from the hosts by sequencing the populations before and after infection. It was found that mutants with insertions in the gtr regulatory region were significantly attenuated for infection of chickens and pigs (fitness scores of -3.4 and -5.7, p < 0.01 and p < 0.001, respectively). This particular insertion was located 38 bp upstream of the gtrA start codon thereby disrupting the gtr regulatory region and would likely inhibit both STnc1870 and gtr transcription. The attenuation of the mutant could therefore be due to loss of STnc1870 or gtr expression, or even both. However, other transposon insertions in the gtr operon did not cause significant attenuation, with one exception: an insertion in the gtrB gene caused significant attenuation in calves and pigs (fitness scores of -2.4 and -2.3 respectively, p < 0.001). On the other hand, transposon insertions in gtrA and gtrC did not cause significant attenuation. Before drawing conclusions, the virulence of S. Typhimurium strains with deletions of STnc1870, or alternatively strains that overexpress STnc1870 would need to be tested in animal models. Specifically, S. Typhimurium susceptible mice could be used to establish a systemic infection model. Given that STnc1870 was highly induced in InsPl2 media with H2O2 treatment, it seems likely that this sRNA is involved in the intracellular lifecycle of Salmonella. For the same reason, the survival of STnc1870 deletion or overexpression mutants in human or murine macrophage should be assessed. Additionally, RNA sequencing of strains artificially overexpressing STnc1870 should be used to identify other potential targets of STNc1870.

Further work should also aim to confirm whether oxidised OxyR can bind the potential alternative site in the gtr regulatory region and stimulate STnc1870 transcription. Either a DNase footprinting assay or an electro-mobility shift assay with purified OxyR and the relevant DNA sequence could be used to determine this.

### 6.4 THE S. TYPHI 2-II gtr CONTAINS A SEQUENCE SIMILAR TO STnc1870

The finding that ssaO could be a potential target for STnc1870 binding was surprising as it indicated a wider role for the gtr operon. Another interesting aspect was that the S. Typhi 2-II gtr (which performs the same O-antigen modification as the LT2_I gtr) operon had a sequence similar to STnc1870 in its regulatory region. An alignment of 300 bp of the regulatory regions showed a sequence identity of 87 %. A notable difference was a 20 bp sequence present in LT2_I gtr, but absent from S. Typhi 2-II gtr (Fig.6.2). The significance of this finding, if any, remains to be determined. However, if STnc1870 does in fact regulate the expression of the SPI2 needle, the sequence present in S. Typhi may not be capable of doing so (see below).
The *S. Typhi* 2_II *gtr* sequence that aligned with STnc1870 was entered into the TargetRNA prediction program (Kery et al., 2014). The *ssaO* mRNA was not among the predicted targets. A further program, RNAfold was used to predict the secondary structures of both STnc1870 and the aligned *S. Typhi* sequence (Gruber et al., 2008; Lorenz et al., 2011). The sequence differences produced clearly different secondary structures (Fig. 6.3). STnc1870 contained two repeats of the following sequence: GCTTTGCAT, which formed a stem loop in the RNAfold structure prediction. In *S. Typhi* 2_II *gtr* only one repeat is present and a stem loop is not predicted to form in this region.

This contrast drew parallels to the sequence variation that abolished phase variation of *S. Typhi* 2_II *gtr*. As mentioned previously (chapter 5), if *S. Typhi* evolved from *S. Typhimurium*, perhaps this process include a change in the function of the *gtr* operon. Was the absence of STnc1870 selected for along with the absence of phase variation? Given that there appears to be a link between *gtr* and *ssaO*, a component of the SPI2 needle required for intracellular survival of *Salmonella*, this could be an exciting example of *Salmonella* host adaptation by gene repurposing or silencing. This case is similar to the SPI2 effector SseJ, which is a critical virulence factor required by *S. Typhimurium* to generate the SCV environment. In *S. Typhi*, SseJ is a pseudogene. *S. Typhi* complemented with a plasmid expressing the *S. Typhimurium* *sseJ* gene had reduced cytotoxicity and proliferated to a higher level in human epithelial cells, compared to the wildtype strain (Trombert et al., 2010).

### 6.5 OxyR REGULATION OF *gtr* AND STnc1870

The *gtr* regulatory region was found to be a divergent promoter; the STnc1870 and *gtrA* genes are orientated in opposite directions, but their regulation is linked. H₂O₂ treatment reduced *gtrA* and increased STnc1870 expression in a switch regulated by OxyR. Another example of a divergent promoter is the *E. coli* OxyR regulatory region itself. The same regulatory region controls expression of both the *oxyR* mRNA and the *oxyS* sRNA. The -35 sites of the two genes are adjacent but orientated in opposite directions (Altuvia et al., 1997). OxyS is a regulator of the oxidative stress response and downregulates genes such as *rpoS* (Battesti et al., 2011). As in the case of *gtrA*/STnc1870, the regulation of *oxyRS* is influenced by oxidative stress. Both the reduced and oxidised form of OxyR can bind the *oxyRS* promoter and repress transcription of *oxyR* (Toledano et al., 1994). Oxidised OxyR causes upregulation of OxyS and downregulation of OxyR (Altuvia et al., 1997; Choi et al., 2001). The regulation of *gtrA* and STnc1870 appears
Fig. 6.2. Alignment of the *S. Typhi* 2_II and *S. Typhimurium* LT2_I *gtr* regulatory sequences

Sequences were aligned by Clustal Omega. The STnc1870 sequence is underlined. Red arrow indicates STnc1870 transcript (in full). The STnc1870 sequence that is predicted to interact with the *ssaO* mRNA is highlighted grey.
Fig. 6.3. Predicted secondary structures of STnc1870 sRNA (A) and the corresponding sequence in S. Typhi 2_II gtr (B)

RNA sequences are given in single-letter code. Colours represent base pairing probabilities. The region of STnc1870 predicted by TargetRNA to interact with ssaO, and corresponding S. Typhi 2_II gtr sequence are highlighted by grey boxes. Structure predictions and images produced using RNAfold (Gruber et al., 2008; Lorenz et al., 2011).
to be based on a similar mechanism whereby the switch from reduced to oxidised OxyR leads to transcription of a different gene from the same regulatory region.

A characteristic that distinguishes gtr from oxyRS is phase variation. Previously, it was assumed that the ON or OFF state established by OxyR in the gtr regulatory region was maintained until genome replication commenced. The reduced form of OxyR was also found to be sufficient for gtr phase variation (Broadbent et al., 2010). However the contribution of oxidised OxyR, if any was not understood. In this work, it was found that oxidation of OxyR was required to relieve repression of STnc1870 after H₂O₂ treatment. In contrast, in a C199S OxyR mutant strain STnc1870 transcription showed a very minor increase after H₂O₂ treatment, indicating that repression was maintained in this strain. These results and the observation that the spacing of the OxyR half sites in the gtr regulatory region is consistent with binding of the reduced form of OxyR (Broadbent et al., 2010) led to the following conclusion: the reduced form of OxyR binds the gtr regulatory region leading to phase variation and repression of STnc1870. However, oxidised OxyR cannot bind and therefore repression of STnc1870 is relieved. The H₂O₂ shock that induced STnc1870 expression killed ~80% of the cells. The treated culture did not increase in optical density for ~ 2 hours after H₂O₂ addition. Plate counts showed the same percentage of ON colonies compared to an untreated control culture (Table 4.1). Based on these results, it seems unlikely that the H₂O₂ shock affected gtr phase variation in a heritable manner. However, gtrA downregulation coincided with high expression of STnc1870.

The regulation of gtr and STnc1870 could be the first example of OxyR binding causing repression (of STnc1870) and phase variation (of gtr) in the reduced form, but following oxidation relieving repression (of STnc1870) by vacating the A, B or C half sites. The E. coli agn43 gene phase varies by GATC methylation and OxyR binding similar to gtr (Wallecha et al., 2002). However, both the reduced and oxidised forms of OxyR are capable of repressing agn43 transcription from unmethylated DNA in vitro. Furthermore, addition of 0.2 mM H₂O₂ to cultures grown in M9 minimal media did not affect expression or phase variation of a reporter fusion of the agn43 regulatory region to lacZ. Both a single dose of H₂O₂ and 15 repeated doses in 6 minute intervals were tested (Wallecha et al., 2003).

Another system that uses GATC methylation and OxyR binding for phase variation is the opvAB operon which regulates O-antigen chain length of S. Typhimurium. Mutant strains with constitutive opvAB expression have very short O-antigens (3-8 subunit repeats) and reduced survival in murine macrophage (Cota et al., 2012). It would therefore be detrimental to
Salmonella for opvAB expression to be in the ON phase during macrophage infection. Binding of OxyR is required for opvAB expression (Cota et al., 2015a). As in gtr and agn43 regulation, the reduced form of OxyR is sufficient for phase variation to occur. The spacing of the OxyR half sites is consistent with binding of reduced OxyR. If oxidised OxyR was unable to bind the opvAB regulatory region, transcription would not occur. Perhaps this mechanism prevents opvAB transcription during macrophage infection. Again, this could be tested using InSPI2 media with H2O2 treatment.

6.6 INFLUENCE OF DNA SEQUENCE ON gtr PHASE VARIATION

The regulatory regions of S. Typhi 2_II, S. Infantis_I and S. Gallinarum_I gtr all contained three GATC sites. The absence of a 4th GATC site was predicted to alter phase variation, compared to the ‘model’ S. Typhimurium LT2_I gtr, which had four GATC sites. This prediction was correct; the Typhi 2_II gtr regulatory region showed no phase variation at all, whereas the wildtype S. Infantis_I gtr regulatory region displayed a higher switch rate compared to a point mutated S. Infantis_I gtr with a 4th GATC site introduced. A further question is whether sequence variation that affects gtr phase variation by altering OxyR binding sites also influences STnc1870 regulation. In particular, if OxyR binding of the A, B and C half sites is either improved or inhibited, this could reduce or increase repression of STnc1870.

The S. Gallinarum_I gtr regulatory region was not tested independently. However, a S. Typhi 2_II gtr regulatory region was produced with a GATA point mutation instead of a 4th GATC site, which mimicked the sequence variation found in S. Gallinarum_I gtr. This point mutated Typhi 2_II gtr also displayed a higher switch frequency, again compared to another Typhi 2_II gtr mutant with 4 GATC sites.

Although the S. Typhi 2_II and S. Gallinarum_I gtr regulatory regions are quite similar, there are some key differences between the OxyR B half sites. For this reason, it should not be assumed that the results achieved with the S. Typhi 2_II GATA point mutant apply equally to S. Gallinarum_I gtr. An important insight gained in this research was that single base-pair changes in the gtr regulatory regions can have extensive effects on phase variation. Changing a base-pair that forms a part of a GATC site and the OxyR binding site consensus was sufficient to abolish phase variation of S. Infantis_I gtr. For this reason, it would be ideal to measure the switch
frequency of *S. Gallinarum_I gtr* separately, and determine whether it could be affected by introduction of a 4th GATC site.

In general, it was confirmed that naturally occurring sequence variation of *gtr* operons can have extensive effects on phase variation. This was particularly interesting in the case of *S. Typhi 2_II gtr* – the lack of a 4th GATC site is likely to cause constitutive expression of the *gtr* operon and by extension, constitutive glycosylation of the O-antigen. Constitutive O-antigen glycosylation could provide a currently unknown selective advantage to *S. Typhi*. This advantage could be connected to the ability of *S. Typhi* to systemically infect and persist in humans. This environment may be sufficiently distinct from the lifestyle of non-host adapted serovars such as *S. Typhimurium*. As a result, the *S. Typhi 2_II gtr* operon could have evolved to perform a function other than phage resistance and population heterogeneity. This would mean that the absence of a 4th GATC site in the *S. Typhi 2_II gtr* regulatory region was acquired by positive selection.

What selective advantage could a *gtr* O-antigen modification provide to a host adapted *Salmonella* serovar? As mentioned, research into *S. Typhi* is hindered by the lack of an animal model (due to the human host restriction). However, the *S. Gallinarum* serovar, which is host-restricted to chickens and causes a typhoidal infection, could provide a model system to explore this question (Thomson et al., 2008). If the GATA sequence variation of *S. Gallinarum_I gtr* does cause a higher switch frequency as predicted, how does this help the strain during infection? Perhaps this serovar is currently evolving towards constitutive O-antigen modification. Alternatively, a higher switch frequency by itself could provide a selective advantage.

An experiment could compare survival of chickens following challenge with *S. Gallinarum* strains carrying either the phase varying wildtype *gtr* operon, or a point mutated constitutively expressing *gtr* operon. A deletion strain should be used as a control. If all three strains produce equal results, this would indicate that the *S. Gallinarum_I gtr* is not a required virulence factor. In this case, the benefit of *gtr* modification would likely be tied to the environment outside the host. On the other hand, if a selective advantage is provided by *S. Gallinarum_I gtr*, the constitutively expressing mutant strain could be more virulent than both the wildtype and deletion mutant.
A chicken infection model is probably not ideal to study human typhoid disease. However, if a role for *S. Gallinarum* I gtr is discovered in this system, it might indicate which aspects of *S. Typhi* 2 II gtr to study further. It should be mentioned that the gtrC proteins of *S. Gallinarum* I and *S. Typhi* 2 II cluster in the same family and are therefore likely to perform the same O-antigen modification (Davies et al., 2013). The O-antigen subunits of *S. Gallinarum* and *S. Typhi* are identical, consisting of galactose-rhamnose-mannose repeats with tyvelose added to the mannose sugar. Glycosylation of the galactose sugar by *S. Gallinarum* I gtr prevented binding of antibodies that targeted the unmodified O-antigen (Brooks et al., 2008).

Could constitutive gtr modification be a disadvantage in certain circumstances? The majority of gtr operons are predicted to phase vary (see chapter 5). A possible disadvantage of abolished phase variation relates to phage resistance. Phase variation allows a population to escape a phage targeting a particular O-antigen structure (Kim and Ryu, 2012; Kintz et al., 2015). Absence of gtr phase variation could therefore leave a population more vulnerable to such an attack.

A simple experiment to demonstrate the disadvantage in losing phase variation could make use of *S. Typhimurium* and the SPC35 phage, which targets the unmodified O-antigen as a co-receptor. The *S. Typhimurium* LT2_I gtr could be point mutated to match the *S. Typhi* 2 II gtr and produce constitutive gtr expression. Initially this strain should show resistance to infection by SPC35 phage. The wildtype phase varying LT2_I gtr operon is known to provide transient defence against SPC35 phage infection (Kim and Ryu, 2012). However, a phage could eventually evolve that would target the modified O-antigen, which the population would not be able to escape from. Alternatively, if a population did survive, it may contain mutations that either disable the gtr operon or restore phase variation.

The above experiment would not identify the advantage of losing phase variation, but it would demonstrate the potential disadvantage involved. Phage typically drive evolution of a population towards higher diversity (Jessup and Forde, 2008). Therefore, *S. Typhi* is either not confronted with as large a variety of phage as *S. Typhimurium* (possibly due to the host-restricted nature of *S. Typhi*), or there is some other benefit provided by constitutive expression of *S. Typhi* 2 II gtr that outweighs the loss of phage resistance.
A wider question that remains is how gtr phase variation relates to *Salmonella* virulence. A number of other bacterial LPS modifications are required for virulence. For example, *Shigella flexneri* glycosylates the O-antigen to expose a type III secretion needle and infect cells (Cunliffe, 2003; West et al., 2005). The PhoP/Q and PmrA/B systems of *S. Typhimurium* add phosphoethanolamine to the lipid A component of the LPS molecule to resist antimicrobial peptides (Kato et al., 2012; Navarre et al., 2005). Neither of these systems phase vary. *Helicobacter pylori* performs fucosylation of the O-antigen to mimic Lewis antigens on the surface of human cells, which aid immune evasion by suppression of the T\_H1-cell response (Bergman et al., 2016). This system phase varies by slipped strand mispairing (Moran, 2008; Wang et al., 2000).

Which of the systems mentioned above is most comparable to gtr? LT2\_I gtr does not appear to be an essential virulence factor, unlike *Shigella flexneri* O-antigen glycosylation for instance (see section 6.3, Chaudhuri et al., 2013). Due to phase variation, gtr might have more in common with *Helicobacter pylori* O-antigen fucosylation. However, the constitutive expression of *S. Typhi* 2\_II gtr indicates that gtr modifications could evolve to become a virulence factor, in which case phase variation might be detrimental.

gtr may therefore be a system that primarily provides population heterogeneity through phase variation, which could affect the development of glycoconjugate vaccines for *Salmonella* (Micoli et al., 2014). O-antigen modification by gtr is therefore not necessarily a virulence factor. For instance, clinical *S. Typhimurium* isolates from Kenya showed no clear correlation between the degree of O-antigen glycosylation and either clinical presentation or resistance to killing by pooled serum from healthy adults (Onsare et al., 2015). However, gtr could transition into a role as an essential virulence factor, depending on whether a particular modification provides a specific benefit.

### 6.7 CONCLUSIONS

The work presented here achieved the intended aim of identifying factors that influence gtr phase variation and expression. The findings indicated a biological role for LT2\_I gtr beyond O-antigen modification and phage resistance. Firstly, the STnc1870 sRNA links the LT2\_I gtr operon to regulation of the SPI2 needle during macrophage infection. Future research should focus on either confirming this connection or finding other potential targets of STnc1870. The
absence of STnc1870 from S. Typhi should also be examined in this context. In addition, the regulation of STnc1870 demonstrated a novel mechanism for OxyR regulation: if reduced OxyR binds the regulatory region, gtr expression can phase vary and STnc1870 is repressed. If OxyR becomes oxidised STnc1870 repression is relieved. This combination of OxyR mediated phase variation coupled to oxidative stress responsive transcription could be a unique example thus far.

Secondly, work on sequence variation showed that single bp changes can abrogate phase variation or alter the switch frequency. This raised new questions about the possible advantages of losing phase variation, and whether this is related to the lifestyle of the serovar and the evolution of host restriction. In particular, what is the benefit of constitutive expression of S. Typhi 2_II gtr? This question is related to the sequence in S. Typhi 2_II gtr with 87% identity to STnc1870 in S. Typhimurium LT2_I gtr. These differences may have been acquired as part of the adaptation to the human host restricted lifestyle.
ABBREVIATIONS

+1  Transcription start site
-10/-35  RNA polymerase binding sites (for $\sigma$ factor)
Amp$^R$  Ampicillin Resistance
att  Attachment or integration site for bacteriophage
bp  Base pairs
BLAST  Basic Local Alignment Search Tool
cAMP  cyclic Adenosine Monophosphate
CAP  Catabolite Activator Protein
Cm$^R$  Chloramphenicol resistance
Cm$^S$  Chloramphenicol sensitivity
CRIM  Conditional Replication, Integration and Modular plasmid system
Dam  DNA adenine methyltransferase
EDTA  Ethylenediaminetetraacetic acid
g  gravitational force
GATC  Dam methylation target site on DNA (G, A, T, C)
GDP  Guanosine diphosphate
GFP  Green Fluorescent Protein
GTP  Guanosine triphosphate
gtr  glycosyltransferase
gtrABC  glycosyltransferase operon ($gtrA$, $gtrB$, $gtrC$)
iNTS  invasive Non-Typhoidal *Salmonella*
IPTG  Isopropyl-$\beta$-D-thiogalactoside
kb  Kilo base pairs
Kan$^R$  Kanamycin Resistance
lacZ  $\beta$-D-galactosidase gene
LB  Lysogeny Broth
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOS</td>
<td>Lipooligosaccharide</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MilliQ water</td>
<td>Ultrapure water, 18 MΩ</td>
</tr>
<tr>
<td>NADPH oxidase</td>
<td>Nicotine Amide Dinucleotide Phosphate oxidase</td>
</tr>
<tr>
<td>Nramp1</td>
<td>Natural resistance-associated macrophage protein 1</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;, OD&lt;sub&gt;420&lt;/sub&gt;</td>
<td>Optical density at 600, 420 nm</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-nitrophenyl-β-galactoside</td>
</tr>
<tr>
<td>OxyR</td>
<td>Oxidative stress response regulator</td>
</tr>
<tr>
<td>OxyR C199S</td>
<td>OxyR variant (cysteine 199 replaced with serine)</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative Real time PCR</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
</tr>
<tr>
<td>RO water</td>
<td>Reverse Osmosis water</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SCV</td>
<td><em>Salmonella</em> Containing Vacuole</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal growth with Catabolite repression</td>
</tr>
<tr>
<td>SOE-PCR</td>
<td>Site directed mutagenesis by Overlap Extension PCR</td>
</tr>
<tr>
<td>SPI1/2/16</td>
<td><em>Salmonella</em> Pathogenicity Island 1/2/16</td>
</tr>
<tr>
<td>sRNA</td>
<td>bacterial small RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris Acetate EDTA</td>
</tr>
<tr>
<td>Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Tetracycline Resistance</td>
</tr>
<tr>
<td>Tet&lt;sup&gt;S&lt;/sup&gt;</td>
<td>Tetracycline Sensitivity</td>
</tr>
<tr>
<td>tetRA</td>
<td>Tetracycline resistance cassette</td>
</tr>
<tr>
<td>Tris</td>
<td>Trisaminomethane</td>
</tr>
<tr>
<td>TSDS-PAGE</td>
<td>Tricine Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>v/v</td>
<td>volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
</tbody>
</table>
X-Gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
REFERENCES


Balbontín, R., Villagra, N., Pardos de la Gándara, M., Mora, G., Figueroa-Bossi, N., Bossi, L., 2015. Expression of IroN, the Salmochelin siderophore receptor, requires mRNA activation by


Promega Corporation [WWW Document], n.d. URL http://www.promega.co.uk/ (accessed 2.7.16).


194


