MECHANISMS OF TRANSCRIPTION MODULATION BY LOW CONCENTRATIONS OF RIFAMPICIN IN *SALMONELLA TYPHIMURIUM*

by

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Abstract.

Screening of a *S. typhimurium* promoter-*luxCDABE* fusion library revealed that the antibiotic rifampicin modulated transcription of a subset of the fusions. This thesis examines promoter sequences of three down-modulated (*fliA*, *flgK*, *invF*) and three up-modulated (*spvA*, *traS*, STM3595) promoters in an effort to elucidate the mechanisms by which sub-minimal inhibitory concentrations of rifampicin modulate transcription.

Reverse transcriptase polymerase chain reaction was used as a second method to examine expression in response to rifampicin and the results were consistent with reporter data. Transcription modulation was abolished in a strain carrying a rifampicin resistance mutation in the gene for the beta subunit of RNA polymerase suggesting that rifampicin effects are mediated by its binding to RNA polymerase. Transcription modulation of the selected genes was observed after cells were treated with several antimicrobials; the effects on transcription differed from those of rifampicin.

DNA deletion studies of two promoters for genes involved in flagellar biosynthesis, P_{fliA} and P_{flgK}, showed that ~50 bp fragments containing the σ^{28}-dependent promoters were sufficient to observe rifampicin mediated down-modulation in culture. For P_{invF}, a promoter specific activator binding site was required for down-modulation. *In vitro* transcription from P_{invF} and P_{flgK} was hypersensitive to rifampicin when compared to a control. The results indicate that the selectivity of rifampicin mediated down-modulation reflects variation in the direct interaction of rifampicin with different promoter:RNA polymerase complexes.

In contrast to the down-modulated promoters, factors others than rifampicin and RNA polymerase holoenzyme may be involved in up-modulation. Transcription up-modulation from the promoters for STM3595 and *traS* could not be shown *in vitro. In vivo,* time course assays of rifampicin induced transcription up-modulation showed a lag period between rifampicin addition and induction. Use of spent media containing rifampicin was not able to shorten the lag phase, suggesting that an intracellular rather than a secreted factor was involved. DNA deletion studies suggested that nucleotides downstream of the transcription start site were involved with up-modulation. A potential intrinsic terminator was found in the untranslated region of the STM3595 transcript, suggesting that transcription attenuation may be involved in rifampicin mediated up-modulation.

Preface.

Chapter 3.1 includes work published in Yim, G., de la Cruz, F., Spiegelman, G. B. & Davies, J. (2006). Transcription modulation of *Salmonella enterica* serovar Typhimurium promoters by sub-MIC levels of rifampicin. Journal of Bacteriology 188:7988-7991. I conducted all research and wrote the corresponding manuscript.

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ATP	adenosine 5’-triphosphate
bp	base pair
BSA	bovine serum albumin
CTD	carboxy terminal domain
CTP	cytidine 5’-triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetra-acetic acid
FACS	Fluorescence Activated Cell Sorting
GTP	guanosine 5’-triphosphate
iNTP	initiating NTP
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB	Luria-Bertani
LBA	Luria-Bertani agar
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid
NTD	amino terminal domain
NTP	nucleoside triphosphate
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
ppGpp	guanosine tetraphosphate

RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNAP	RNA polymerase
RT-PCR	reverse transcriptase polymerase chain reaction
SDS	sodium dodecyl sulfate
SPI	*Salmonella* pathogenicity island
TBE	tris-borate EDTA
TE	tris-EDTA
UTP	uridine 5'-triphosphate

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Dedication.

I would like to dedicate this dissertation to my family, my husband Alan and both the past and current members of the Davies and Spiegelman labs.

Chapter 1. Introduction.

1.1. Antibiotics.

Antibiotics are molecules that kill or inhibit the growth of microbes. The word itself means "against life." In the past 50 years, antibiotics have been critical to the control of many infectious diseases. Generally, antibiotics work by blocking crucial processes in microbial cells. Hence, antibiotics have been classified according to their structure and mode of action. Several examples of such classes are the rifamycin, beta-lactam, macrolide and aminoglycoside antibiotics. Rifamycins bind and inhibit bacterial DNA-dependent RNA polymerase (RNAP) (Campbell *et al.*, 2001). Beta-lactams weaken the cell wall by inhibiting the transpeptidation of peptide strands within the bacterial peptidoglycan layer (Wickus & Strominger, 1972). Macrolide and aminoglycoside antibiotics inhibit protein synthesis. Macrolides block the elongation of the nascent peptide chain by binding to the 50S subunit of the ribosome (Tenson *et al.*, 2003) while aminoglycosides bind to the A site of the 30S subunit (Moazed & Noller, 1987; Purohit & Stern, 1994).

Kohanski *et al*. has recently suggested a common mechanism of bacterial cell death in response to bactericidal antibiotics (Kohanski *et al.*, 2007). Although the concept of antibiotic mode of action is well understood, the property by which an antibiotic is either lethal or inhibitory is unknown. Also unclear are the events following binding of the antimicrobial to its primary target, such as the consequences of protein synthesis inhibition, which lead to cell death. This is akin to disconnecting a tube or a wire in a car and the car stops running; one suspects key components are damaged but the exact process by which the car stopped is unknown. In *E. coli,* bactericidal but not bacteriostatic antibiotics were shown to induce hydroxyl radical formation.

In turn, hydroxyl radicals increased the rate of bacterial cell death. Deleterious effects of hydroxyl radicals could be ameliorated by the addition of thiourea and 2,2'-dipyridyl, agents which prevent the formation of radicals in bacteria (Kohanski *et al.*, 2007).

Although the first antibiotic isolated for therapeutic use, penicillin G, is produced by a fungus, the majority of antibiotics have been isolated from bacteria of the genus *Streptomyces*. The first well characterized antibiotic isolated from a streptomycete was streptomycin. Selman Waksman, the discoverer of streptomycin, defined "true" antibiotics as natural products made by the metabolism of microorganisms that possess antimicrobial activity at low concentrations (Bryskier, 2005). Quinolones block DNA synthesis by targeting DNA gyrase and topoisomerase, enzymes responsible for DNA supercoiling and deconcatenation of interlinked daughter chromosomes following DNA replication (DiGate & Marians, 1988; Kato *et al.*, 1990), respectively. Since quinolones are synthetic and not derived from microbes, by Waksman's definition, the quinolones are not antibiotics but antimicrobials; however, they perform antibiotic functions in therapy.

Antimicrobial is a general term for natural, synthetic or semi-synthetic inhibitory small molecules. Natural products are isolated from cultures of microorganisms. Synthetic compounds are chemically derived and semi-synthetic products are modified (chemically or enzymatically) derivatives of natural products. Antimicrobials will either kill (bactericidal) or inhibit growth (bacteriostatic) of microbes. Most antibiotics are identified by screening growth medium of microorganisms for antibacterial activity. Beta-lactams and aminoglycosides are generally considered bactericidal while macrolides and tetracyclines are considered bacteriostatic

(Kohanski *et al.*, 2007). For some antibiotics, such as rifampicin, bactericidal or bacteriostatic action on the cell is concentration dependent (Canadian Pharmaceutical Association, 2008).

Bacteria produce a plethora of diverse small molecules with many functions in nature. Some of these small molecules have been isolated for clinical use as antibiotics. Small molecule biosynthesis is a complex process. Many small molecule biosynthetic gene clusters are often in excess of 100 kb, encoding large multi-subunit enzyme complexes: non-ribosomal peptide synthases, polyketide synthases or hybrids thereof. Peptides synthesized by non-ribosomal peptide synthases have been found to contain over 100 unusual amino acids such as γ-aminobutyrate, β-alanine and δ-aminoadipate (Walsh, 2003). Genome analysis of a representative streptomycete, *Streptomyces coelicolor*, indicated the presence of 21 large biosynthetic clusters, each encoding proteins required for the synthesis of a specific molecule or a group of chemically related small molecules (Bentley *et al.*, 2002). Three *S. coelicolor* clusters, including the well-studied polyketide antibiotic actinorhodin, had previously been identified to produce molecules having antibacterial properties (Bentley *et al.*, 2002). Other predicted or known products of the encoded clusters include siderophores, pigments and lipids, as well as uncharacterized molecules (Bentley *et al.*, 2002).

The role of small molecules in bacterial cell-cell signaling has been extensively studied as mediators of quorum sensing. Quorum sensing systems allow gene regulation in response to cell density. Examples of the "quorum" or cell density sensing by homoserine lactones in Gram-negative bacteria and peptides in Gram-positive bacteria have been widely documented. These signals can be passed within (Latifi *et al.*, 1995) or between species (Surette *et al.*, 1999).

Extensive work in this field has invigorated interest in bacterial cell-cell signaling (Novick & Geisinger, 2008; Parsek & Greenberg, 2000; Waters & Bassler, 2005; Williams & Camara, 2009).

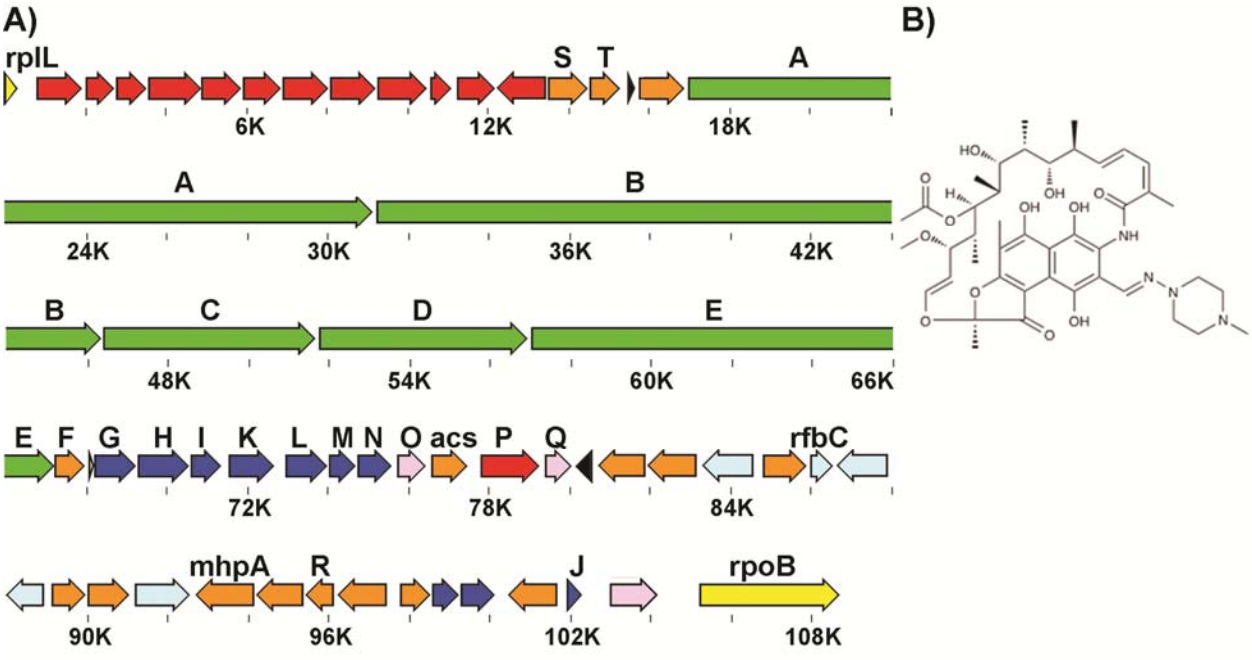

Figure 1.1. The *rif* cluster and flanking genes *rplL* and *rpoB* of *Amycolatopsis mediterranei* and the structure of rifampicin. A. Genes encode the following functions: transport (red), post-polyketide synthase modification (orange), unknown (black), polyketide synthase (green), precursor synthesis (dark blue), regulation (pink) and sugar synthesis (light blue). Figure adapted from (Floss & Yu, 2005). B. The structure of rifampicin.

Once antibiotics are isolated for commercial use, a few naming conventions are loosely followed. The suffix "mycin" (as opposed to "micin") is usually reserved for compounds isolated from *Streptomyces*. Derivatives of a natural product usually have the same ending as the original compound name (*i.e.* penicillin, ampicillin, oxacillin, carbenicillin, *etc.*) (Bryskier,

2005). Compounds have been named after the genus or species of the producer organisms, chemical characteristics of the antibiotic, places or even movie stars! For example, kanamycin was named after its producer *S. kanamyceticus* (Bentley & Bennett, 2009), bicyclomycin has a bicyclic structure (Bentley & Bennett, 2009), the producer organism of kasugamycin was first isolated from soil at the Kasuga shrine in Nara, Japan (Umezawa, 1967)) and melinamycin was named after the Greek actress and politician Melina Mercouri (J. Davies, personal communication).

Rifampicin is a clinically useful semi-synthetic compound derived from rifamycin B. Rifamycin B is produced by fermentation of *Amycolatopsis mediterranei*. Rifamycin B is subsequently modified to synthesize the final product rifampicin. The rifamycin B biosynthetic gene cluster is ~100 kb (Figure 1.1) and encodes a hybrid polyketide-non-ribosomal peptide synthase (Floss & Yu, 2005). Rifampicin inhibits growth of many Gram-positive bacteria, particularly *Mycobacterium*. It is used in the treatment of *M. tuberculosis, M. leprae* and other AIDS-associated mycobacterial infections. It is estimated that one third of the human population, primarily in Africa and South-East Asia, are infected with tuberculosis (World Health Organization, 2010). Interestingly, in May 2004, the Food & Drug Administration approved rifaximin, a derivative of rifampicin that is not absorbed by the body, for the treatment of traveller's diarrhea caused by non-invasive strains of *Escherichia coli* (Adachi & DuPont, 2006).

What are the roles of antibiotics in nature? Antibiotics have been found to have different effects at high concentrations versus the effects at concentrations lower than the drug's minimal inhibitory concentration (MIC, this term in discussed later in section 4.8). Clinically, antibiotics

are used in a concentrated and purified form to treat bacterial infections of humans. In the past decade, recognition of a second functionality of antibiotics has emerged, the modulation of bacterial gene transcription at sub-lethal dosages (Cheung *et al.*, 2003; Evers *et al.*, 2001; Goh *et al.*, 2002; VanBogelen & Neidhardt, 1990). Antibiotic modulation of bacterial transcription raises the question of whether the natural purpose of antibiotic production by microbes is to inhibit growth of neighboring microbes for inter-microbial competition. It is likely that the concentrations of antibiotics found in the soil or in the environment are significantly lower than those encountered in clinical settings and would not aid in intermicrobial competition. Antibiotics are a type of naturally derived small molecule which have been isolated for inhibition of microbial growth. Other small molecules have been shown to have a diverse array of biological activities such as antiviral, antifungal, antitumor and immunosuppressive (Chadwick & Whelan, 1992; Demain & Fang, 2000).

1.2. Modulation of bacterial transcription by antimicrobials.

Antibiotics and other small molecules have been observed to change bacterial transcription and protein expression patterns. Studies on the global effects of sub-lethal concentrations of various antimicrobials on different bacteria have enjoyed increasing popularity in the recent years (for a comprehensive review see (Davies *et al.*, 2006)). For example, *E. coli* proteome studies have shown that various protein synthesis inhibitors induce expression of genes in a manner similar to a mild or strong heat shock (puromycin, kanamycin and streptomycin) or cold shock (chloramphenicol, erythromycin, fusidic acid, spiramycin, tetracycline) (VanBogelen & Neidhardt, 1990). In *Pseudomonas aeruginosa*, phenazines help maintain redox homeostasis by acting as electron acceptors (Price-Whelan *et al.*, 2006). Quinolones stimulate expression of

integrase genes and integron recombination which may lead to antimicrobial induced acquisition of antibiotic resistance cassette or a reshuffling of cassettes (Guerin *et al.*, 2009).

In addition to genes involved in metabolism and stress, antibiotics have been found to regulate the expression of virulence genes. Studies with enterohaemorrhagic *E. coli* have shown that expression of prophage genes, prophage encoded toxin genes and toxin release are increased by quinolones and trimethoprim (Herold *et al.*, 2005; Kimmitt *et al.*, 2000; Matsushiro *et al.*, 1999; Zhang *et al.*, 2000). Aminoglycosides induce biofilm formation via the bacterial second messenger cyclic di-GMP (Hoffman *et al.*, 2005). In the Gram-positive *Staphylococcus aureus* and *Streptococcus pyogenes*, sublethal concentrations of antibiotics have been shown to suppress virulence factor synthesis by acting at the transcription and translation levels (Bernardo *et al.*, 2004; Gemmell & Ford, 2002; Herbert *et al.*, 2001). In *Salmonella enterica* serovar Typhimurium, *S. typhimurium*, cationic microbial peptides have been shown to affect virulence gene expression (Bader *et al.*, 2003). A well studied precedent for the use of antibiotics to repress virulence function rather than growth inhibition is the use of macrolide antibiotics (such as erythromycin and azithromycin) in diffuse panbronchiolitis and cystic fibrosis infections. Macrolides do not reduce the bacterial load but inhibit the expression of virulence determinants and also have immunomodulatory effects (for a comprehensive review, see (Tateda *et al.*, 2007)). The examples of transcription and protein expression changes elicited by sub-MIC antibiotics on different bacteria continually grow in number.

1.3. Mode of action determination by reporter strain panels.

Gene or protein expression patterns of bacteria treated by antibiotics have been used as a means to group inhibitory small molecules of unknown structure and function with antimicrobials with known modes of action. Compounds with similar expression profiles have similar modes of action. A compound with a mode of action that differs from the reference compounds would putatively have a different expression profile. Early studies showed that different macrolide-lincosamide-streptogramin antibiotics, with slightly different binding sites on the ribosome, could also be differentiated by their transcription profiles (Tsui *et al.*, 2004). On a larger scale, reference libraries of transcriptome or proteome expression profiles from bacteria treated with different classes of antibiotics have been collected (Freiberg & Brotz-Oesterhelt, 2005). The modes of action of several small molecules have been identified or reclassified using expression profiles (Freiberg & Brotz-Oesterhelt, 2005; Hutter *et al.*, 2004). Urban *et al.* (2007) identified a small number of promoters, chosen for their responsiveness to reference compounds, to create a panel of reporters that can be used in a similar fashion to the genome-scale expression patterns. These promoters can be either directly related to the compound mode of action, *i.e* the *fabHB* promoter to detect fatty acid biosynthesis inhibitors or can be promoters unrelated to the target of growth inhibition (*i.e.* involved in motility, virulence, metabolism) (Mesak *et al.*, 2010) but are nonetheless responsive to the various reference compounds. These studies have used either lethal dosages of antibiotic for short exposure times (minutes) or sub-MICs for longer times (hours). These mode of action panels were employed to identify modes of action of unknown compounds present in small quantities in the supernatant of potential antibiotic producer organisms (Mesak *et al.*, 2010); these compounds would likely be overlooked by traditional screening methods using growth inhibition of tester organisms to identify potential compounds.

1.4. Bacterial RNA polymerase.

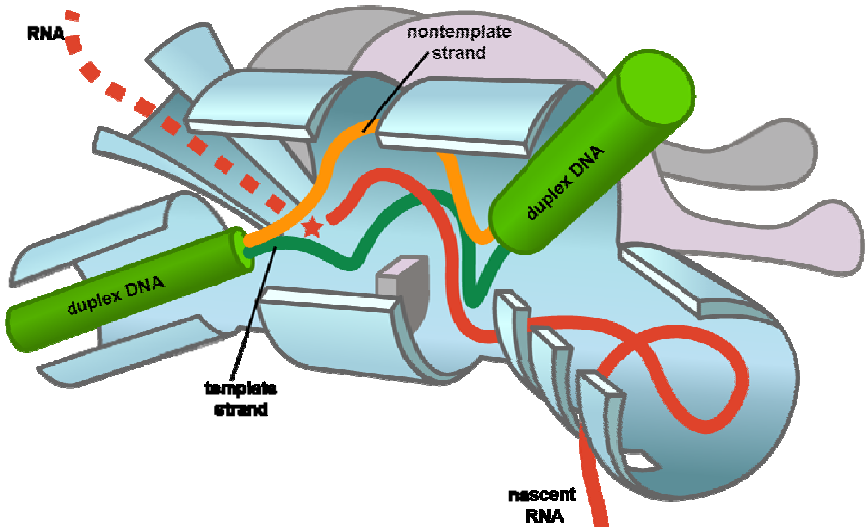

Figure 1.2 Structural overview of bacterial RNA polymerase. A schematic representation of the *T. thermophilus* elongating complex. The main channel secures the RNA-DNA hybrid in the hybrid-binding site (center). β domains clamp the downstream DNA in the DNA duplex-binding site (left). The β zipper, Zn finger, and lid along with β-flap form the RNA exit channel (right). The catalytic site (star) is located at the bottom of the secondary channel (top left). Figure adapted from Nudler (2009).

The enzyme responsible for RNA synthesis in the cell is RNAP. In the best studied organism, *E. coli*, the RNAP core enzyme is competent for transcription elongation and is composed of five subunits, ββ'$\alpha_2\omega$ (Browning & Busby, 2004) (Figure 1.2). The 3.3 Å crystal structure of *Thermus aquaticus* RNAP revealed a crab claw-like structure (Zhang *et al.*, 1999). The active site which binds the DNA:RNA complex is formed by the β and β' subunits (Korzheva *et al.*, 2000). A Mg^{2+}ion is bound at the beginning of the secondary channel through which NTP diffuses into the active site (Zhang *et al.*, 1999). The 5' end of the RNA transcript exits the

active site via the main DNA/RNA channel (Korzheva *et al.*, 2000). Each α subunit has two independently folded domains, the αCTD (carboxy terminal domain) and the αNTD (amino terminal domain) (Blatter *et al.*, 1994). Dimerized αNTD directs ββ' assembly while αCTD is a DNA binding module important for interactions with promoters and transcription factors (Gourse *et al.*, 2000).

To begin transcription at a specific promoter sequence, the core enzyme requires a σ factor to form the holoenzyme, $\sigma\beta\beta'\alpha_2\omega$. In *E. coli* and many other species including *S. typhimurium*, σ^{70} is the main σ factor allowing RNAP to recognize the majority of promoters in the genome (Paget & Helmann, 2003). In *Bacillus subtilis* and many other organisms, σ^{70} is referred to as σ^{A}. *E. coli* has seven known σ factors: σ^{70}, σ^{28}, σ^{32}, σ^{S}, σ^{E}, σ^{FecI} and σ^{54}. Similarly, *S. typhimurium* has five known alternative sigma factors, σ^{28}, σ^{32}, σ^{S}, σ^{E} and σ^{54} (Bang *et al.*, 2005). *S. coelicolor* has 63 known sigma factors (Paget & Helmann, 2003). In bacteria, there are two families of σ factors, σ^{54} and σ^{70}. The members of the σ^{70} family have four conserved domains as determined by sequence conservation in the σ^{70} family (Paget & Helmann, 2003). In *E. coli*, σ^{28}, σ^{32}, σ^{S}, σ^{E} and σ^{FecI} are members of the σ^{70} family. The σ^{70} family recognizes –10 and –35 hexamers while the σ^{54} family recognizes –12 and –22 elements (Buck *et al.*, 2000). Within bacteria, only regions two and four are well conserved in the σ^{70} family (Paget & Helmann, 2003). The *Salmonella* σ^{28} is a pared-down version of its σ^{70} protein, containing only three of the four conserved domains (Schaubach & Dombroski, 1999).

Five DNA elements contribute to promoter recognition by RNAP:σ^{70}. They are the -10 hexamer, -35 hexamer, extended -10, UP element and discriminator. The relative contribution of each element to promoter strength varies with the promoter (Browning & Busby, 2004). The consensus sequences are as follows: -10 (TATAAT), -35 (TTGACA), extended -10 (TGTG), UP elements (AAAWWTWTTTTNNNAAANNN where W = A or T and N is any base) and discriminator (GGG) (Haugen *et al.*, 2008). The -10 hexamer is centered at -10 where +1 is the corresponding transcription start site. The -35 region is centered around -35, the extended -10 is located immediately upstream of the -10 hexamer, UP elements typically appear from -57 to -38 and the discriminator is directly downstream of the -10 from -6 to -4. Specific regions of σ^{70} bind to the -35, extended -10, -10 and discriminator, while the αCTD binds UP elements (Haugen *et al.*, 2008). Consensus sequences for other sigma factors are not as well defined and can be quite different from the σ^{70} consensus. For example, *E.coli* and *S. typhimurium* σ^{28} (flagellar) consensus sequences are TAAAGTTT (-35) and GCCGATAA (-10) (Ide *et al.*, 1999). An extended -10, UP element and discriminator have not been described for σ^{28}.

While natural promoters in which all elements exactly match all the consensus sequences do not exist (Browning & Busby, 2004), natural promoters have been altered to have a higher percent identity to consensus. Mutations causing higher nucleotide similarity to consensus and increased promoter strength are often referred to as "up" mutations while those changing sequences away from consensus and decreasing promoter strength are referred to as "down" mutations. Examples of classical "strong" promoters with "up" mutations are as follows: the *lacUV5* promoter (-35, TTTACA, -10 TAT**AA**T) has two up mutations (bold) compared to the wildtype *lac* promote*r* (-35 TTTACA, -10 TAT**GT**T) (Fuller, 1982). The *tac* promoter has consensus -10

and -35 elements and was derived by combining the -10 element of the *trp* promoter and the -35 element of the *lacUV5* promoter, creating a promoter which was stronger than either of its progenitors (de Boer *et al.*, 1983).

1.4.1. Stages of transcription.

Transcription has been functionally separated into three parts: initiation, elongation and termination. The kinetics of initiation has been studied in detail but many questions still remain. During the process of initiation, RNAP and DNA will transition through a series of isomerizations that reflect structural changes in both RNAP and DNA. Figure 1.3 shows a simple schematic of transcription initiation. Some promoters have additional closed and open complexes that have been referred to as RP_{C1}, RP_{C2}, RP_{O1} and RP_{O2} (Record *et al.*, 1996). Closed complex is characterized by specific binding of RNAP to promoter DNA without separation of the DNA strands. The open complex is formed by melting of the double stranded DNA template in the -10 region of the promoter. The transition from open to initiated complex occurs as the RNAP polymerizes NTPs. Once a 7 – 12 nucleotide transcript forms, RNAP forms a processive elongation complex. At some promoters, short RNAs (abortive transcripts) are released and the RNAP returns to an open complex to begin again (Record *et al.*, 1996). The conversion from initiated complex to elongating complex involves promoter escape and clearance. The rate of chain elongation is approximately 50-100 nucleotides/s (Roberts *et al.*, 2008). Termination occurs when the nascent RNA transcript is released from RNAP and the DNA template. It can be mediated by the protein Rho, the protein Mfd or by intrinsic terminator sequences (regions are characterized by a hairpin followed by a run of U residues) (Roberts *et al.*, 2008). In some transcription units, terminator sequences can be found in the untranslated

region, between the core promoter elements and the start codon for translation. These sequences can cause attenuation, which is discussed below.

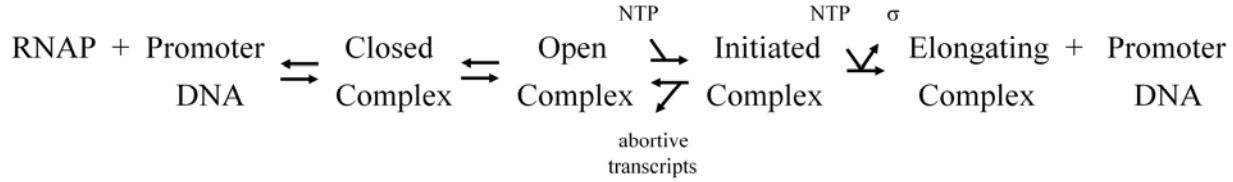

Figure 1.3. A model for the multi-step mechanism of transcription initiation. Figure modified from (Record *et al.*, 1996)).

1.4.2 Transcription regulation at initiation.

Transcription factors down or up-regulate transcription by influencing RNAP. Regulatory factors will often affect transcription by stabilizing or destabilizing certain forms of the RNAP:DNA complex, such as open complex, or help recruit RNAP to specific promoters. Many transcription factors bind DNA specifically at or upstream of the core promoter elements (Browning & Busby, 2004). Some notable exceptions to this include the transcription factor DksA which potentiates transcription of rRNA promoters by binding in the RNAP secondary channel (Paul *et al.*, 2004). Some global regulators have dual roles as activators and repressors, their action as either repressors or activators depends on their binding sites. For example, CRP and FNR have repressor binding sites located around the +1 and have upstream activator binding sites (Gralla & Collado-Vides, 1996).

1.4.3 Regulation of transcription elongation, termination, antitermination and translational attenuation.

Although most mechanisms of transcription regulation studied to date involve regulation of transcription initiation, there are a number of well documented mechanisms that involve regulation of transcription elongation and termination. The RNAP transitions into an elongating complex when RNAP has released σ and has cleared the promoter region of the DNA; at this time the nascent RNA is approximately 7-12 nucleotides long (Record *et al.*, 1996). Obstacles to elongation include DNA lesions and DNA binding proteins such as σ^{70} which can bind to -10-like sequences (Ring *et al.*, 1996). These obstacles can cause stalling and a subsequent buildup of elongating complexes (Ring *et al.*, 1996). Transcription elongation factors such as NusA, NusG, GreA and GreB are known to modulate the rate of elongation by stimulating or inhibiting paused elongating complexes (Borukhov *et al.*, 2005; Burns *et al.*, 1998; Lewis *et al.*, 2008). Gre proteins can also enhance elongation by inhibiting abortive initiation complexes (Vassylyeva *et al.*, 2007). When transcribing rRNA, elongating complexes form antitermination complexes which are highly sensitive to intrinsic and extrinsic pause and termination signals (Lewis *et al.*, 2008).

Attenuation has been broadly defined as modulation of gene expression that influences aspects of transcription or translation but does not influence transcription initiation (Kolter & Yanofsky, 1982); others have described it as repression involving neither repressors nor activators (Turnbough & Switzer, 2008). Transcription attenuation is hallmarked by intrinsic terminators and a leader region. The first well-studied attenuation controlled transcript was the *E.coli trp* operon. The leader region is defined as the RNA from the transcription start site to the start of protein coding region and in the *trp* operon, this region contains a short peptide encoding ORF

with its own ribosome binding site. In general, attenuation involves the following mechanism (Turnbough & Switzer, 2008). When transcription occurs without pausing or slowing, the transcript will form an intrinsic terminator (hairpin structure) in the leader region of the nascent RNA causing transcription to terminate. When the elongating complex has slowed or paused, the ribosome which is translating the leader peptide follows quickly behind the elongating complex (transcription translation coupling), blocking the formation of the hairpin in the transcript and allowing transcription to continue without termination.

Examples of transcripts regulated by attenutation include *pyrBI* (Turnbough & Switzer, 2008), *cat* (Alexieva *et al.*, 1988) and *ermC* (Mayford & Weisblum, 1989). The *pyrBI* operon encodes aspartate carbamylase, an enzyme which catalyzes the first step in the de novo synthesis of pyrimidine nucleotides. Attenuation control of *pyrBI* transcription coordinates the level of aspartate carbamylase synthesis to the intracellular levels of UTP. In the presence of high levels of UTP, ~98% of transcription terminates (Turnbough & Switzer, 2008). *E.coli pyrBI* has a 158 bp leader region which includes a 44 codon ORF, multiple clusters of U residues (UTP sensitive) and an intrinsic terminator 23 bp before the *pyrB* structural gene. When UTP levels are low, RNAP will stall at U-rich pause sites, allowing transcription translation coupling, prevention of terminator formation and transcription through the *pyrBI* operon. If UTP levels are high, RNAP will not pause and no coupling occurs. The terminator will form in the mRNA and terminate transcription, preventing transcription of the *pyrBI* genes (Turnbough & Switzer, 2008).

Attenuation can also control the level of translation. Translation of antibiotic resistance genes such as *cat* and *erm*, which confer chloramphenicol and erythromycin resistance to cells,

respectively, is regulated by attenuation. The leader region of the *catA86* gene of *B. subtilis* requires a minimum 84 bp (Alexieva *et al.*, 1988). The leader region has a nine codon ORF and a stemloop in the mRNA that when formed, sequesters the ribosome binding site required for translation of the *cat-86* structural gene (Alexieva *et al.*, 1988). If chloramphenicol binds to and stalls the ribosome, stalling at discrete sites in the leader peptides causes destabilization of the mRNA secondary structure and allows translation of the downstream resistance determinant (Alexieva *et al.*, 1988).

1.5. Rifampicin:RNA polymerase interactions.

1.5.1. Mode of action.

The biochemical target for rifampicin in the bacterial cell is the β subunit of bacterial RNAP and is encoded by *rpoB*. The most highly cited *in vitro* study on rifampicin mode of action was published by McClure & Cech in 1978. They suggested that rifampicin inhibited transcription by sterically blocking elongation of nascent RNA. The crystal structure of rifampicin complexed with *T. aquaticus* RNAP was solved and supported the classical model for rifampicin mode of action (Campbell *et al.*, 2001). A rarely cited study suggested that rifampicin may inhibit transcription by destabilizing the binding of intermediate oligonucleotides to the active RNAP:DNA complex (Schulz & Zillig, 1981). In 2005, a study using several rifamycin antibiotics challenged the classical model for the mode of action of rifampicin suggesting that rifamycins do not work solely by steric hindrance but also by inducing allosteric changes in RNAP that inhibit its catalytic activity (Artsimovitch *et al.*, 2005). Subsequently, this newly proposed mode of action of rifampicin was challenged by Feklistov *et al*. (2008). At present, the

only known mode of action for rifampicin remains the one suggested by McClure and Cech (1978).

1.5.2. Rifampicin resistance.

Rifampicin resistance can be conferred by mutations of the drug target, by drug inactivation or by efflux of the antibiotic. Resistance mutations usually occur in *rpoB* (Musser, 1995). This predominant site for resistance conferring mutations was early genetic evidence for RNAP as the target of rifampicin. Some environmentally and clinically isolated bacteria possess rifampicin modifying enzymes, such as those encoded by *arr* which inactivate rifampicin by ADP-ribosylation; these proteins seem to more clinically important in non-mycobacterial strains (Floss & Yu, 2005). Bacterial rifampicin resistance arises quickly during therapy. In an effort to slow the emergence of resistance in the treatment of *M. tuberculosis*, rifampicin is used in combination along with isoniazid, pyrazinamide and ethambutol (first-line drugs) (Musser, 1995). Widespread rifampicin resistance is of great clinical concern, as there are many cases of both multidrug resistance (resistant to isoniazid and rifampicin) and extensively drug resistant (resistant to rifampicin, isoniazid and second line drugs) *M. tuberculosis* (World Health Organization, 2010). This global problem has raised interest in the study of the mode of action of rifampicin, its derivatives (rifapentin, rifabutin, *etc.*), as well as further derivatization to create more drugs (Artsimovitch *et al.*, 2005).

Since RNAP is a component of the basic cellular machinery, studies have examined the phenotypes of different *rpoB* mutants. In *E.coli*, particular phenotypes appear to be associated with different clusters of mutations in the *rpoB* gene. Phenotypes include altered transcription

termination, temperature sensitivity and uracil susceptibility (Jin *et al.*, 1988; Jin & Gross, 1989). These studies elucidated the pleiotropic effects resulting from alterations to cellular machinery and functional specialization of certain areas of the RNAP β subunit.

1.6. Pathogenicity and virulence of *S. typhimurium*.

Salmonella enterica serovar Tyhpimurium (*S. typhimurium*) is a facultative intracellular pathogen which lives a variety of pathogenic lifestyles. Salmonellosis is usually caused by the ingestion of contaminated food or water. *S. typhimurium* infection is usually limited to gastroenteritis (unable to invade intestinal epithelium) in humans but causes a systemic (typhoid like) infection in several strains of mice (Holden, 2002). *S. enterica* serovar Typhi causes typhoid fever, a systemic infection in humans but not in nonprimate mammals (Holden, 2002). Thus, mice infected with *S. typhimurium* are often used as an animal model for human typhoid fever (Holden, 2002). During systemic infection (*S. typhimurium* infection of mice or *S. typhi* infection of humans), bacteria travel from the stomach and colonize the intestinal epithelial cells. At the intestinal wall, injection of proteins using the type III secretion system induces epithelial cells to take up *Salmonella* (Galan, 1999). *Salmonella* disseminates to local mesenteric lymph nodes where it invades and survives in phagocytic cells. *Salmonella* then travels to extraintestinal sites, *i.e.* spleen and liver, resulting in a systemic infection. This thesis focuses on two *S. typhimurium* virulence genes *invF* and *spvA*. Genes such as *invF*, related to enteropathogenic infection, allow *Salmonella* to invade host cells. Genes such as *ssrAB* and *spvABCD* are related to systemic infection and allow *S. typhimurium* to survive and replicate within epithelial and macrophage cells inside a unique membrane-bound vacuole (Holden, 2002).

The majority of virulence factors present in *Salmonella* species are encoded within *Salmonella* Pathogenicity Islands (SPIs) or on the virulence plasmid (Hensel, 2004). Thus far, 21 SPIs distributed throughout the various serovars have been identified (Blondel *et al.*, 2009; Hensel, 2004). The SPIs were likely acquired through horizontal gene transfer (Groisman & Ochman, 1996). The *Salmonella* virulence plasmids vary in size between the serovars and can restore virulence to plasmid cured *Salmonella* strains (Gulig *et al.*, 1993). Examples of genes on the *Salmonella* virulence plasmids are the *Salmonella* Plasmid Virulence, *spv*, genes (Chu *et al.*, 1999).

1.6.1. The virulence regulons.

Virulence genes in *S. typhimurium* can be grouped into 1) those involved with the enteropathogenic phase of infection (invasion of intestinal cells) or 2) those involved in the systemic phase of infection. The SPI-1 genes and their associated effector proteins are central to the enteropathogenic phase of infection. The primary regulator for SPI-1 expression is HilA, a SPI-1 encoded protein in the OmpR/ToxR family (Bajaj *et al.*, 1996). HilA expression is influenced by a multitude of proteins: CsrAB, Fis, Fad, FliZ, OmpR/EnvZ, HilC, HilD, PhoPQ, PhoBR, Lon protease, Ams, HupB and Hha (Teplitski *et al.*, 2003). HilA has been shown to activate two multi-gene operons encoded on SPI-1. The first genes of these operons are *prgH* and *invF,* respectively (Lostroh *et al.*, 2000). InvF in turn positively regulates SPI-1 encoded effectors which are secreted by the SPI-1 encoded type III secretion system (Eichelberg & Galan, 1999). The *siiA* operon, located in SPI-4, is required for the enteropathogenic phase of infection and is also HilA dependent (Thijs *et al.*, 2007). There are at least seven SPI-1 effectors encoded by genes located outside SPI-1: SopA, SopD, SopE, SopE2, SlrP, SspH1 and SopB (SigD)

(Raffatellu *et al.*, 2005). Their transcription is thought to be directly activated by InvF and therefore indirectly by HilA (Eichelberg & Galan, 1999).

Known regulators and genes that are involved in the systemic phase of infection include *spvABCD* and the genes encoded in SPI-2. SPI-2 genes are essential for systemic infection in mice. They are expressed upon entry into host cells and are required for survival in macrophages (Hensel *et al.*, 1998). The regulator for SPI-2 is likely *ssrAB* which encodes a two-component regulatory system (Feng *et al.*, 2004). The *spv* genes are also involved in systemic infection and survival in macrophages (Libby *et al.*, 2000; Matsui *et al.*, 2001), but it is unclear if their regulation is related to SsrAB.

1.7. Flagellar regulon.

The genes responsible for flagella formation and motility in *E. coli* and *S. typhimurium* are transcriptionally organized into classes that form a hierarchy (Soutourina & Bertin, 2003). Products of the class I genes are required for transcription of class II genes and products of class II genes are required for transcription of class III genes. This thesis focuses on two flagellar genes, *fliA* (the flagellar sigma factor, class II) and *flgK* (hook associated protein, class III). The flagellar regulon consists of >50 genes and >17 operons. Class I genes encode *flhD* and *flhC*. $FlhD_2C_2$ activates the σ^{70} dependent class II genes (Liu & Matsumura, 1994). Class II genes encode proteins that compose the flagellar export system, the basal body proteins, σ^{28} (*fliA*) and FlgM, the anti-σ^{28} factor (Kutsukake & Iino, 1994; Kutsukake, 1997; Ohnishi *et al.*, 1992). Transcription of class III genes is σ^{28} dependent and is inhibited by FlgM. FlgM sequesters σ^{28} until the basal body of the flagella is complete, at which time FlgM is exported out of the cell via

the flagellar export system (Hughes *et al.*, 1993). Subsequently, the transcription of the class III genes, encoding the flagellar filament, hook-associated, motor function and chemotaxis proteins, proceeds and the flagella are completed.

1.8. Thesis objective.

Davies *et al.* demonstrated transcription modulation in response to several antibiotic classes in *S. typhimurium* 14028 using a 6528-clone library constructed using a *luxCDABE* promoter trap vector (Goh *et al.*, 2002; Tsui *et al.*, 2004; Yim *et al.*, 2011). Using this library of promoter-reporter clones, it was shown that sub-MIC levels of the antibiotic rifampicin, dramatically up- and down-modulated transcription from many reporter strains (Table 1.1). *S. typhimurium* genes involved with host cell invasion and motility were putatively found to be down-modulated by rifampicin and those involved with carbon metabolism or virulence were putatively up-modulated by rifampicin (Table 1.1). The following *in vivo* and *in vitro* experiments were conducted to elucidate the mechanism by which transcription modulation by rifampicin occurs.

i) To characterize the rifampicin response, the expression of promoter-reporter fusions were studied under various liquid and solid media growth conditions. To determine whether the target of rifampicin binding leading to transcription modulation by rifampicin was RNAP, the wild type strain and a rifampicin resistant strain were examined for their responses to rifampicin. To examine the specificity of the rifampicin response, strains harboring reporters for six rifampicin responsive promoters were exposed to rifampicin, various other antibiotics and oxidative stress. To characterize the timing of the rifampicin response, expression of the promoter fusions was measured after rifampicin was added at different times during culture growth. To determine

whether a compound accumulated intracellularly or was released into the supernatant to mediate the rifampicin response, experiments employing spent media and rifampicin pretreated cells were conducted.

ii) Two types of experiments (reporter constructs and RT-PCR) were used to confirm the identity of rifampicin responsive genes. Since the original *lux* reporter library used to screen for rifampicin responsive genes was constructed by cloning partial *Sau*3A digested DNA, deletion analysis of presumptive promoters was conducted. Expression of fusions carrying various truncations of the original DNA fragments grown in the presence and absence of rifampicin was examined. Smaller deletions were used to map regions of DNA necessary for rifampicin mediated transcription effects. RT-PCR was used a secondary method to check expression levels of rifampicin modulated genes.

iii) *In vitro* transcription was used to determine whether it was possible to replicate transcription modulation by rifampicin with isolated components *in vitro*.

iv) Since several lines of evidence suggested that an unknown cellular factor was involved in rifampicin mediated up-modulation, a screen for mutants no longer displaying up-modulation by rifampicin was conducted to identify genes or RNAs involved in transcription up-modulation by rifampicin. A strain carrying a plasmid with *gfp* fused to a promoter which was strongly up-modulated by rifampicin was randomly mutagenized with *Tn*10*d*tet. Mutants showing loss of transcription modulation by rifampicin were isolated by FACS and further studied using the Victor II plate reader.

v) To identify possible nucleotide motifs involved in transcription modulation by rifampicin, nucleotide sequences for the six rifampicin responsive promoters identified in ii) were analyzed for motifs. Luminescence of *lux* reporters carrying promoter DNA with altered motifs were examined for their responses to rifampicin.

Table 1.1. Characteristics of rifampicin responsive promoters in *S. typhimurium* 14028.

Promoter Identification	Fold Repression (↓) or Activation (↑) by Rifampicin	Putative Function
STM2899/*invF*	↓ 200	virulence; invasion
STM1091/*sopB*	↓ 163	virulence; invasion
STM2066/*sopA*	↓ 115	virulence; invasion
STM4255-8/*unknown, ssb, siiAB*	↓ 13	virulence; invasion
STM1956/*fliA* (σ^F)	↓ 32	motility
STM1914/*flhBA*	↓ 26	motility
STM1183/*flgK*	↓ 18	motility
STM2199/*cirA*	↓ 4	iron metabolism
STM1328	↓ 25	unknown
*S*TM1248	↓ 19	unknown
pSLT102-3/*traST*	↑ 5	virulence
STM1154-55/*yceE,htrB*	↑ 4	virulence; systemic
pSLT041-39/*spvRAB*	↑ 4	virulence; systemic
STM4118/*yijP*	↑ 3	virulence
STM4454/*treB*	↑ 13	carbon metabolism
STM2445/*ucpA*	↑ 4	carbon metabolism
STM1597/*ydcW*	↑ 3	carbon metabolism
STM2473/*talA*	↑ 3	carbon metabolism
STM0425/*thiI*	↑ 8	RNA modification
STM3595	↑ 24	unknown
STM0389/*yaiA*	↑ 4	unknown
STM2287	↑ 3	unknown
STM2901 (control)	↓ 1.6	unknown

*modified from (Yim *et al.*, 2006)

Chapter 2. Methods and materials.

2.1. Bacterial strains, culture methods and reagents.

Luria-Bertani (LB) broth or agar (LBA) plates were used for growth and maintenance of strains. All media components were obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA). Liquid cultures were grown at 37 °C in shaking tubes while cultures in 96-well plates were grown without shaking. Media were supplemented with tetracycline (12.5 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), ampicillin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}) and rifampicin (5 μg ml^{-1}) as appropriate and unless otherwise indicated. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA) or from the laboratory collection. NTPs were obtained from Amersham Biosciences (Piscataway, NJ, USA); [α-^{32}P]GTP (111 TBq $mmol^{-1}$), [α-^{32}P]ATP (111 TBq $mmol^{-1}$) and [γ-^{32}P]ATP (222 TBq $mmol^{-1}$) were supplied by PerkinElmer Life Sciences (Waltham, MA, USA).

Table 2.1. List of strains and phages.

	Description	Source and/or reference
Strains		
14028	*S. typhimurium* wildtype strain	(Goh *et al.*, 2002)
DH10B	*E. coli* strain used for cloning	Invitrogen
BL21 star	*E. coli* strain used for protein expression	Invitrogen
BL21(DE3) pLysS	*E. coli* strain used for protein expression	Novagen
TOP10	*E. coli* strain used for protein expression	Invitrogen
GY10	Derivative of 14028, *pcnB*::*Tn*10*d*	This study
GY11	Derivative of 14028, *yciK*::*Tn*10*d*	This study
R306	Derivative of 14028, rifampicin resistant	(Yim *et al.*, 2006)
TT10423	*proAB*47 / F' *pro*(+) *lac*(+) *zzf*-1831::*Tn*10*d*tet	M.G. Surette
Phages		
P22 HT105/1-*int*	Salmonella phage	M.G. Surette
P22 H5	Salmonella phage	S. Ramey

Table 2.2 List of plasmids.

Plasmid	Description	Source
pCS26	Promoterless *luxCDABE* reporter, kan^r	(Goh *et al.*, 2002)
pCH112	*hilA* cloned into *pBAD/*Myc-*His*, amp^r	(Lostroh *et al.*, 2000)
pKH445	*fliA* cloned into pET15b, amp^r	(Chadsey *et al.*, 1998)
pAW44	template for *abrB* PCR	M. Strauch, U. of Maryland
pGY7	Derivative of pCS26; *fliA lux* library reporter	(Goh *et al.*, 2002)
pGY8	*Sau*3A *fliA* fragment of pGY7 inserted into pCS26	This study
pGY9	Derivative of pGY7, pCSFor/fliAP2 rev used for construction	This study
pGY10	Derivative of pGY7, fliAP1 for/fliAP1 rev used for construction	This study
pGY11	Derivative of pGY7, fliAP2 for/fliAP2 rev used for construction	This study
pGY12	Derivative of pCS26; *flgK lux* library reporter	(Goh *et al.*, 2002)
pGY13	Derivative of pGY12; flgKent2 for/flgKent rev used for construction	This study
pGY14	Derivative of pGY12; flgKent for/flgKent rev used for construction	This study
pGY15	Derivative of pGY12; flgKmin for/flgKmin rev used for construction	This study
pGY16	Derivative of pCS26; *invF lux* library reporter	(Goh *et al.*, 2002)
pGY17	Derivative of pGY16; invFent for/invFent rev used for construction	This study
pGY18	Derivative of pGY16; invFent for/invFmin rev used for construction	This study
pGY19	Derivative of pGY16; invFmin for/invFent rev used for construction	This study
pGY20	Derivative of pGY16; invFmin for/invFmin rev used for construction	This study
pGY21	Derivative of pCS26; STM2901 *lux* library reporter	(Goh *et al.*, 2002)
pGY22	Derivative of pCS26; *traS lux* library reporter	(Goh *et al.*, 2002)
pGY23	Derivative of pGY22; slyAent for/ slyAent rev used for construction	This study
pGY24	Derivative of pGY22; traTent for/ traTent rev used for construction	This study
pGY25	Derivative of pGY22; traSent for/ traSent rev used for construction	This study
pGY26	Derivative of pGY22; traSent for/ traSmin rev used for construction	This study

Plasmid	Description	Source
pGY27	Derivative of pGY22; traSmin for/ traSent rev used for construction	This study
pGY28	Derivative of pGY22; traSmin for/ traSmin rev used for construction	This study
pGY29	Derivative of pCS26; *spvA lux* library reporter	(Goh *et al.*, 2002)
pGY30	Derivative of pGY29; spvAent2 for/ spvAent rev used for construction	This study
pGY31	Derivative of pGY29; spvAent for/ spvAent rev used for construction	This study
pGY32	Derivative of pGY29; spvAent for/ spvAmin rev used for construction	This study
pGY33	Derivative of pGY29; spvAmin for/ spvAent rev used for construction	This study
pGY34	Derivative of pGY29; spvAmin for/ spvAmin rev used for construction	This study
pGY35	Derivative of pCS26; *STM3595 lux* library reporter	(Goh *et al.*, 2002)
pGY36	Derivative of pGY35; STM3595ent2 for/ STM3595ent rev used for construction	This study
pGY37	Derivative of pGY35; STM3595ent for/ STM3595ent rev used for construction	This study
pGY38	Derivative of pGY35; STM3595min for/ STM3595ent rev used for construction	This study
pGY39	Derivative of pGY35; STM3595ent for/ STM3595min rev used for construction	This study
pGY40	Derivative of pGY35; STM3595min for/ STM3595min rev used for construction	This study
pGY41	Derivative of pGY35; STM3595PE2 for/ STM3595ent rev used for construction	This study
pFPV25	Promoterless *gfp* reporter with a FACS optimized *gfp*	(Valdivia & Falkow, 1997)
pGY42	Derivative of pFPV25; STM3595::*gfp*mut3a	This study
pGY43	Derivative of pCS26; abrB for and abrB rev used for construction	This study
pGY44	Derivative of pGY11, fliAP2 C for/fliAP2 rev used for construction	This study
pGY45	Derivative of pGY11, fliAP2 for/fliAP2 GCC rev used for construction	This study
pGY46	Derivative of pGY11, fliAP2 C for/fliAP2 GCC rev used for construction	This study
pGY47	Derivative of pGY15; flgKmin C for/flgKmin rev used for construction	This study
pGY48	Derivative of pGY15; flgKmin for/flgKmin GCC rev used for construction	This study
pGY49	Derivative of pGY15; flgKmin C for/flgKmin GCC rev used for	This study

Plasmid	Description	Source
	construction	
pGY50	Derivative of pGY18; invFent for/invFmin GCC rev used for construction	This study
pGY51	Derivative of pGY19; invFmin C for/invFent rev used for construction	This study
pNK2880	contains the transposase gene	M.G.Surette
pLysS	Used for protein expression	Novagen

2.2. Isolation of rifampicin resistant mutants.

Overnight cultures of *S. typhimurium* 14028 were spun down and spread on LBA supplemented with 300 μg ml^{-1} of rifampicin and incubated at 37 °C for two days. Colonies were purified twice for single colonies on LBA supplemented with 300 μg ml^{-1} of rifampicin. To identify the mutation conferring rifampicin resistance, the following primer pairs were used to amplify regions of the *rpoB* gene: ST rpoBI sense/antisense and ST rpoBII sense/antisense. PCR products were nucleotide sequenced using the primers used for amplification (NAPS Unit, UBC, Canada). (The MIC for rifampicin when *S. typhimurium* 14028 is grown on LBA, as determined by Etest (AB Biodisk, Solna, Sweden) or by the microbroth dilution method, is ~12 μg/ml.)

2.3. Luminescence time courses at various times of rifampicin addition.

Overnight cultures of reporter strains were diluted 1:50 into 60 ml of LB supplemented with kanamycin and 15 ml aliquoted into four 50 ml flasks and were incubated with shaking at 37 °C. Rifampicin was added to a different flask every hour for the first two hours. 150 μl aliquots were removed from each flask every hour and luminescence (cps) and OD (595 nm) were measured in a Victor II Multi-label Counter (PerkinElmer, Waltham, MA, USA).

2.4. Pretreated cells and spent medium experiments.

Spent medium and pretreated cells were collected as depicted in Figure 3.5. An overnight culture of 14028 carrying the STM3595 reporter pGY37 was diluted 1:100 into two flasks containing 35 ml of LB supplemented with kanamycin and incubated with shaking at 37 °C for three hours. After one and three hours, $OD_{600\ nm}$ was measured in a Genesys 20 spectrophotometer (Thermo Electron, Milford, MA, USA). Rifampicin was added to one flask after one hour. Both flasks were incubated shaking at 37 °C for an additional two hours. Three hours after the initial dilution, cells from both flasks were spun down and 15 ml or 30 ml of supernatant/spent medium was removed from the rifampicin flask or LB flask, respectively. Excess medium was removed from centrifuge tubes and discarded. Pretreated cells were resuspended in fresh medium to an $OD_{600\ nm}$ of 0.02. Resuspended cells were aliquoted into two 15 ml cultures and rifampicin was added to one flask. 30 ml of LB spent medium was inoculated at 1:50 with the overnight culture and split into two 15 ml cultures and rifampicin was added to one flask. 15 ml of rifampicin supplemented spent medium was inoculated 1:50 with the overnight culture. Flasks with resuspended cells and spent medium were incubated shaking an additional two to three hours at 37 °C. 150 µl of culture was removed from flasks every 30 minutes and luminescence and $OD_{595\ nm}$ were measured in a Victor II Multi-label Counter.

2.5. Liquid media *lux* reporter assays with sub-cloned reporters.

Luminescence measurements were taken in 96-well sterile, white, clear-bottomed microtitre plates (Nalge Nunc, Rochester, NY). 150 µl of overnight cultures of reporter strains were aliquoted into 96 well plates and a 96-pin replicator (V&P Scientific, San Diego, CA) was used to inoculate microtitre plates containing LB kanamycin supplemented with or without rifampicin

(5.0 μg ml^{-1} or as otherwise indicated). For luminescence assays described in sections 3.1.2, 3.2, 3.3.3 and 3.5, plates were sealed with Mylar Plate Sealers (Thermo Electron) or a Breathable Sealing Membrane (#163340, Nalge Nunc, discontinued), incubated at 37 °C in a Victor II Multi-label Counter without shaking and luminescence measured every hour for 16-21 hours. Peak luminescence readings for replicates were used. For short assays described in section 3.6.2, inoculated plates covered with lids were incubated without shaking for three hours at 37 °C at which point lids were removed, luminescence and $OD_{595\ nm}$ read in the Victor II Multi-label Counter every 30 minutes for four additional hours. Using three different seed cultures, a minimum total of six sets of bacterial luminescence measurements were taken.

2.6. Reporter construction.

All plasmid constructs (Table 2.2) were cloned in *E. coli* DH10B (Invitrogen, Carlsbad, CA). With the exception of pGY8, derivatives of pCS26 were made by insertion of *BamH*I-*Xho*I digested PCR products into the *BamH*I-*Xho*I fragment of pCS26. Primers for DNA amplification are listed in Table 2.3. *S. typhimurium* genomic DNA was used as a template for all PCR reactions with two exceptions: for pGY9 construction pGY8 plasmid DNA was used as a PCR template and for pGY43 construction pAW44 was used as a template. pGY8 was constructed by cloning the *Sau*3A fragment of pGY7 containing the *fliA* gene into pCS26 digested with *Bam*HI. pGY42 was constructed by cloning the *Bam*HI digested product of PCR amplification of genomic DNA using STM3595ent for and STM3595ent rev into the *Bam*HI-*Sma*I (blunt end) fragment of pFPV25. Plasmid constructs were validated by nucleotide sequencing (Macrogen, Korea). Plasmids isolated from *E. coli* were electroporated into *S. typhimurium* 14028 by standard methods.

Table 2.3. List of oligonucleotides.

Oligonucleotide	Nucleotide Sequence 5' to 3'	Source
AAP	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG	Invitrogen
abrB for	**ACTCGAG**AAACAAAATGATTGACG	This study
abrB rev	**TGGATCC**TTACCTTCATAGCATAAC	This study
AUAP	GGCCACGCGTCGACTAGTAC	Invitrogen
flgKent for	**ACTCGAG**ATTCCGCGTCTATAGCTC	This study
flgKent rev	**AGGATCC**AAGCTGGACATGATGGTTC	This study
flgKent2 for	**TCTCGAG**TGGTGCGCAAAGCCATAC	This study
flgKmin for	**ACTCGAG**TTTTAAATTGCTCAAGTC	This study
flgKmin C for	**ACTCGAG**TTTTAAATTGCTCAAGTCCAAGT	This study
flgKmin rev	**AGGATCC**TCAATACTCGTTGTTATC	This study
flgKmin GCC rev	**AGGATCC**TCAGCCCTCGTTGTTATC	This study
fliAP1min for	**TCTCGAG**TTATTCCTTCGATAGAACCCTC	This study
fliAP1min rev	**TGGATCC**GTTATCGGCATGATTATC	This study
fliAP2min for	**TCTCGAG**GATAGAACCCTCTGTAGAAACG	This study
fliAP2min C for	**TCTCGAG**GATAGAACCCTCTGTAGAAAAGG	This study
fliAP2min rev	**TGGATCC**GCGTTAAATGAGTTATCG	This study
fliAP2min GCC rev	**TGGATCC**GCGGCCAATGAGTTATCG	This study
fliC for	GCTGTTGATACGCAGACCG	This study
fliC rev	GCTATTTCGCCGCCTAAG	This study
GSP1	CTGCGTCTTGAGTATTCTT	This study
GSP2	TCCATCTTTGCCCTACCGTATA	This study
invFent for	**ACTCGAG**GAATGCGTTTCAGGAATG	This study
invFent rev	**AGGATCC**GTCTGTATAAACCATGCTTC	This study
invFmin for	**TCTCGAG**TGATTGCATCAGGATTTT	This study
invFmin C for	**TCTCGAG**TGATTGCATCAGGATTTTGACA	This study
invFmin rev	**TGGATCC**AATATGTAAACAATACCG	This study
invFmin GCC rev	**TGGATCC**AGCCTGTAAACAATACCG	This study
P2only5'A	GCGAATTCAAACAAAATGATTGACG	M. Strauch, University of Maryland
pCSFor	TGGCAATTCCGACGTCTAAG	(Tsui *et al.*, 2004)
pCSRev	CACTAAATCATCACTTTCGG	(Tsui *et al.*, 2004)
PRI1	ACATGAAGGTCATCGATAGCAGGA	(Nichols *et al.*, 1998)
PRI3	AACAGTAATGGGCCAATAACACCG	(Nichols *et al.*, 1998)
PRI5	CCAAAATCATTAGGGGATTCATCA	This study
PRI6	CCAACGCTTTTCCCGAGATC	This study
ST rpoBI sense	GACAGATGGGTCGACTTGTCAGCG	This study
ST rpoBI antisense	AGGTGGTCGATATCATCCACTT	This study
ST rpoBII sense	TCGAAGGTTCCGGTATCCTGAGC	This study

Oligonucleotide	Nucleotide Sequence 5' to 3'	Source
ST rpoBII anti sense	GGGTACATCTCGTCTTCGTTAAC	This study
RT-16S-F	GAACTGTGAGACAGGTGCTG	This study
RT-16S-R	GCAACAAAGGATAAGGGTTG	This study
RT-fliA-F	TGTGGCAGCGTTATGTACC	This study
RT-fliA-R	GCTTGTAGCAGATCGTCCAG	This study
RT-invF-F1	GCAGGATTAGTGGACACGAC	(Choi *et al.*, 2007)
RT-invF-R1	TTTACGATCTTGCCAAATAGCG	(Choi *et al.*, 2007)
RT-STM2901 F2	CCGCAGTCATAGCACGATA	This study
RT-STM2901 R2	TTTGCCTGAGTTCTCCTTGA	This study
RT-STM3595 F	TCATCCTTATACCGCAGGTG	This study
RT-STM3595 R	GATCATTTCCGCCATCAAC	This study
S187	TGCAAGGCGATTAAG	M. Strauch
slyAent for	**TCTCGAG**GATCTTGTAAGGGCAATC	This study
slyAent rev	**TGGATCC**TCAGAACCTAGTGGCGAT	This study
spvAent for	**TCTCGAG**CTGAAGAAACTGAATAACGG	This study
spvAent rev	**TGGATCC**TTCGACCTGTGAAAGTGC	This study
spvAent2 for	**TCTCGAG**CCTTGTATTACCCGACCA	This study
spvAmin for	**CCTCGAG**CGATTCCGCACAGCAGAAAAATAGCAC	This study
spvAmin rev	**CGGATCC**GGCTTATATTGAGTTTATTT	This study
STM3595ent for	**GCTCGAG**TGTCCAGATAGAGAAACGG	This study
STM3595ent rev	**GGGATCC**AGAGATGACGCCAGTACAG	This study
STM3595ent2 for	**TCTCGAG**CAACAGTGGGAAGTCGCT	This study
STM3595min for	**CCTCGAG**CGTTGTGAAAGTATTGCA	This study
STM3595min rev	**TGGATCC**GTTGACGGTTTGATGATAC	This study
STM3595PE2 for	**CCTCGAG**TCAAACCGTCAACTGCGC	This study
traSent for	**ACTCGAG**CAGGTTGATAATATGGTC	This study
traSent rev	**AGGATCC**CAATAAACTGTACCACTG	This study
traSmin for	**ACTCGAG**ATAAAACAGAAGCACTATC	This study
traSmin rev	**TGGATCC**GCCTTTTCTTCATTATAT	This study
traTent for	**ACTCGAG**GAGTGGGTGAAATGTCAG	This study
traTent rev	**AGGATCC**GCCAGAGTGGAACTGACC	This study

2.7. Disk diffusion assays.

Overnight cultures were diluted 1:200 into 5 ml of 0.7% (w/v) agar overlays and poured over LBA plates. Antibiotic disks were placed on top of overlays. Antibiotic disks were premade (BD

Biosciences, Rockville, MD) or antibiotic solutions were spotted onto 6 mm filter paper discs (Advantec, Japan). Plates were incubated overnight at 37°C. Images of luminescence produced by reporters were taken with a LB980 camera (Berthold Technologies, Oakridge, TN) or a MF-Chembis (Berthold Technologies).

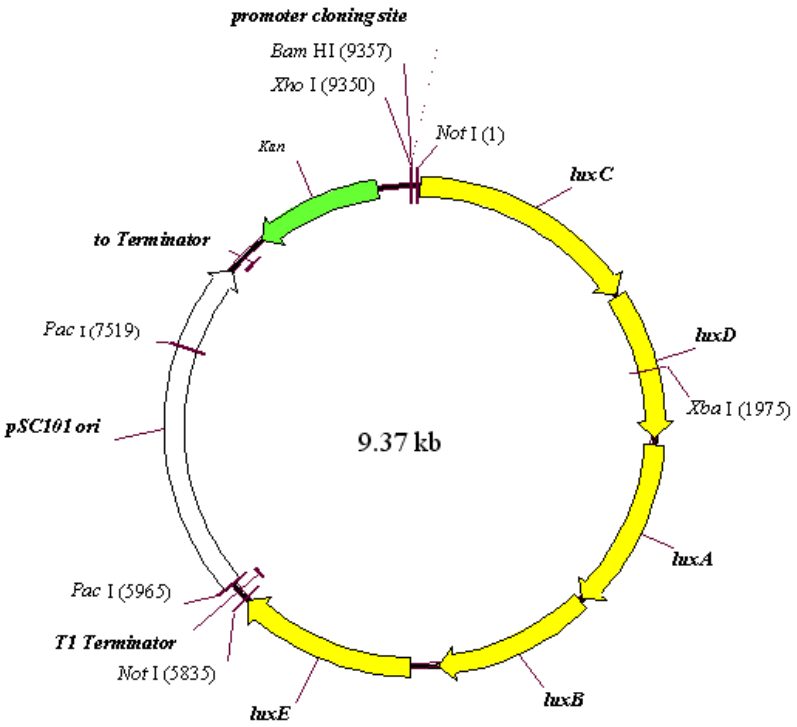

Figure 2.1. Promoterless-*lux* reporter plasmid pCS26. The LuxCDE proteins catalyze the conversion of fatty acids into a long chain aldehyde, RCHO, and the LuxAB proteins are the two subunits of the luciferase enzyme. The reaction is as follows (R represents a long carbon chain where 13 is the most efficient): $RCHO + FMNH_2 + 0_2 \rightarrow FMN + RCOOH + H_2O + light_{490\ nm}$ (Meighen, 1993). Due to the pSC101 origin of replication, it is likely that the plasmid is maintained at five copies per chromosome (Wadood *et al.*, 1997). No exogenous substrate is needed to produce light.

2.8. RNA isolation and RT-PCR.

Cultures were grown in the presence or absence of rifampicin (5.0 μg ml^{-1}) and treated with RNAprotect solution (Qiagen, Missassauga, ON). RNA was extracted with RNeasy columns (Qiagen), digested with DNase I and purified with RNeasy columns (Qiagen). Extracted RNA was amplified using a one-step SYBR Green Quantitative RT-PCR kit (Sigma, St. Louis, MO) in a MX3000P cycler (Stratagene, La Jolla, CA). The following primers were used for amplification of cDNA: RT-16S-F, RT-16S-R, RT-fliA-F, RT-fliA-R, RT-invF-F1, RT-invF-R1, RT-STM2901 F2 and RT-STM2901 R2. A minimum of three replicate cultures were used to prepare RNA samples and at least two separate amplifications were performed from each RNA sample. Relative quantities were calculated using the relative standard curve method. RNA amounts were normalized to 16S RNA. The effect of rifampicin was calculated using normalized values ($RNA_{no\ rif}/RNA_{rif}$).

2.9. Protein expression.

pKH445 was transformed into *E. coli* BL21(DE3) pLysS (Novagen, Gibbstown, NJ). Overnight cultures of transformants were diluted 1:10, incubated at 37 °C, induced with 1 mM IPTG when the culture reached an OD_{600} of 0.8 and grown for an additional 2 hours before harvesting. pCH112 was transformed into *E. coli* TOP10 (Invitrogen). Overnight cultures of transformants were diluted 1:100, incubated at 37 °C, induced with 0.02% (w/v) arabinose at an OD_{600} of 0.5 and grown for an additional 16 hrs at 22 °C before harvesting.

2.10. Protein purification.

RNAP was isolated from *E. coli* W3100 (28 g pellet) grown to mid-exponential phase by the University of Alabama at Birmingham Fermentation Facility. RNAP was isolated as described previously (Seredick & Spiegelman, 2007) except active fractions from the DNA-cellulose column were not concentrated. Active fractions were adjusted to a final concentration of 50% glycerol (v/v) and then stored at -80 °C for direct use. HilA was purified from TOP10/pCH112 after induction (as described in section 2.9) using a Ni-NTA resin (Qiagen) according to manufacturer's protocol for isolation of native proteins with an additional wash (Qiagen lysis buffer with 20 mM imidazole). HilA extracts were dialyzed into 1X transcription buffer (10 mM HEPES pH 8, 80 mM potassium acetate, 10 mM magnesium acetate, 0.1 mM DTT, 0.1 mM acetylated BSA) with 20% (v/v) glycerol. After dialysis, glycerol was added to a final concentration of 50% (v/v) and the protein concentration determined using the Bio-Rad protein assay using BSA as a standard (Mississauga, ON). After induction (as described in section 2.9), σ^{28} was purified from BL21(DE3)/pLysS/pKH445 under denaturing conditions (8 M urea) using a Ni-NTA resin (Qiagen) according to the manufacturer's protocol and reconstituted as described previously (Schaubach & Dombroski, 1999).

2.11. *In vitro* transcription assays.

Linear templates for *in vitro* transcription were made using PCR with the following primer/template combinations: invFent for, pCSrev / pGY17 (*invF*); P2only5'A, S187 / pAW44 (*abrB*); flgKent2 for, pCSrev / pGY13 (*flgK*); and fliC for, fliC rev / *S. typhimurium* 14028 genomic DNA (*fliC*). For transcription assays with *flgK* and *fliC*, template and 1.5 µl of reconstituted RNAP-σ^{28} were incubated in transcription buffer for 2 min at 37 °C. Transcription

was started by the addition of a mixture containing NTP, rifampicin (varying concentrations) and heparin to limit the transcription to a single round. For transcription assays with *invF* and *abrB*, template and HilA (or HilA dialysis buffer, respectively) were incubated for 2 min at 37 °C in transcription buffer, RNAP added and incubated for another 2 min at 37 °C. Transcriptions were then challenged with a mixture of NTP, rifampicin (varying concentrations) and heparin. See Figure 3.13 for a schematic of incubation times used. All reactions had a final volume of 10 µl and final concentrations were as follows unless otherwise indicated: 1X transcription buffer (10 mM HEPES pH 8, 10 mM magnesium acetate unless otherwise indicated, 80 mM potassium acetate, 0.1 mM DTT, 0.1 mg acetylated BSA ml^{-1}), 16 nM for all templates except *abrB* (6 nM), 75 nM RNAP, 3 µM σ^{28} (as appropriate), 0.3 µM HilA (as appropriate), 50 µg heparin ml^{-1}, 400 µM CTP, 400 µM UTP, 400 µM ATP, 5 µM GTP and 111 kBq [α-^{32}P] GTP or 400 µM GTP, 5 µM ATP and 111 kBq [α-^{32}P]ATP, as indicated. Transcripts were elongated for 5 min at 37 °C and then terminated by the addition of 5 µl of loading buffer (1.5X transcription buffer with 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol and 7 M urea). Prior to use in transcription assays, RNAP-σ^{28} was reconstituted by incubating in dilution buffer (10 mM HEPES pH 8, 10 mM magnesium acetate, 10% glycerol (v/v), 0.1 mM DTT, 0.1 mg ml^{-1} acetylated BSA) on ice for 10 min. In transcription assays with P_{invF} or P_{abrB}, RNAP was diluted 1:1 with dilution buffer before use. Transcripts were electrophoresed through 8% denaturing acrylamide gels and imaged using a Storage Phospor screen (Amersham Biosciences, Piscataway, NJ), scanned by a Typhoon 9400 (Amersham Biosciences) and quantified using ImageQuant 5.2 software (Amersham Biosciences).

2.12. 5’ RACE.

The transcription start of the STM3595 transcript was determined with the 5' RACE System (Invitrogen). RNA isolated from *S. typhimurium* 14028 carrying pGY37 grown in the presence of rifampicin was used as a template for cDNA synthesis with the gene specific primer, GSP1. The AAP/GSP2 primer pair was used in first round PCR. The AUAP/pCS rev primer pair was used in second round PCR. Two PCR products were visible after second round PCR. DNA was isolated with a Qiaquick Gel Extraction kit (Qiagen) and nucleotide sequenced using pCS rev (Macrogen). Only one amplicon had a sequence related to STM3595; the other amplicon was likely a product of non-specific priming during PCR.

2.13. Primer extension.

Oligonucleotides STM3595ent rev and traSminF rev were labeled using 22.2 MBq [γ-^{32}P]ATP and T4 polynucleotide kinase, 3’ phosphatase free (Roche, Indianapolis, IN) according to manufacturer’s instructions for direct phosphorylation. Sequencing ladders for primer extension gels were created using 5’ labelled oligonucleotide in a Sequitherm Excel II kit (Epicentre, Madison, WI) according to manufacturer’s instruction. Templates for the sequencing reactions were created by PCR. PCR products using 5’ labeled oligonucleotides were generated using *S. typhimurium* 14028 genomic DNA as template with either of the following primer combinations: STM3595ent for/STM3595ent rev or traSmin for/traSent rev. M-MLV reverse transcriptase (Promega, Madison, WI) and 5’ labeled oligonucleotides were used to make radiolabelled cDNA using either RNA isolated from *S. typhimurium* 14028 grown in the presence of rifampicin or RNA synthesized *in vitro*. RNA was isolated from culture as done in section 2.8 and RNA synthesized as in section 2.11, but on a larger scale. Products were

separated by electrophoresis through 8% denaturing acrylamide gel, dried and visualized by radiography using Bioflex EconoFilm (Interscience, Markham, ON).

2.14 *Tn*10*d* mutagenesis and Fluorescence Activated Cell Sorting.

Phage infections and lysates were produced as described in various P22 protocols (http://rothlab.ucdavis.edu/protocols). EGTA chelates Ca^{2+} and prevents reinfection by phage. Infected cells were grown in LB broth or agar supplemented with ampicillin and EGTA (pH 8, 10 mM) unless otherwise indicated.

S. typhimurium 14028 pNK2880 was infected with a P22HT *int* lysate made using *S. typhimurium* TT10423. Infected cells were plated on LBA supplemented with EGTA and tetracycline to create ~100,000 colonies/mutants. Colonies from plates were pooled and infected with P22 HT105/1-*int* creating a transducing phage library lysate. The lysate was used to infect *S. typhimurium* 14028 containing the STM3595::*gfp*mut3a reporter, pGY42. Infected cells were plated on LBA supplemented with EGTA, tetracycline and ampicillin to make approximately 40,000 14028/pGY42 *Tn*10*dTet* mutants. Colonies were pooled, resuspended (LB broth supplemented with ampicillin, EGTA and 20% glycerol (v/v)) and stored without growth at -80 °C.

Frozen stocks were diluted 1:20 into fresh media and grown overnight at 37 °C. Overnight cultures were diluted 1:30 and supplemented with or without rifampicin (2.5 μg ml^{-1}) to give a final optical density of 0.1 (600 nm, Genesys 20 Spectrophotometer, Thermo Scientific). Cells were grown for three hours and fluorescence at 530 nm (excitation at 488 nm) measured using an

Influx cell sorter (BD Biosciences). 10^7 events (cells and debris) representing a population of approximately ~ 40,000 *Tn*10*d* mutants were sorted. Particles with fluorescence intensities in the bottom 1% of the population were collected into 3 ml of diluted media (50% PBS and 50% media). 1 ml of the sorted particles was resorted. Particles with fluorescence intensities in the bottom 5% of the 3 x 10^4 particles sorted were collected in 300 µl of media and spread onto agar plates. After overnight growth, 150 colonies representing potential non-fluorescing mutants were visible. Colonies were restreaked twice on agar plates, single colonies were used to inoculate 150 µl broth in 96-well plates. A 96-well pin replicator (V&P Scientific) was used to stamp colonies into assay plates containing LB broth supplemented with ampillicin and rifampicin. These colonies were further analyzed for fluorescence using the Victor II plate reader (excitation at 485 nm, emission at 535 nm). Three of the 150 mutants analyzed by the Victor II plate reader had the desired phenotype in the presence of rifampicin, low expression of the STM3595::*gfp* reporter. Two different mutants were identified by nucleotide sequencing of the inverse PCR products (Figure 2.2). The two mutants were located in *pcnB* and *yciK*, respectively (Figure 3.24).

2.15 Inverse PCR methods.

Inverse PCR products were obtained from the *Tn*10*d* strains as described previously (Nichols *et al.*, 1998; Ochman *et al.*, 1988) with a few modifications: approximately 5 µg of genomic DNA were digested overnight at 37 °C with 25 U of *Hpa*II (Roche, Laval, Quebec) or *Rsa*I (New England Biolabs, Pickering, Ontario) according to manufacturer's instructions and inactivated by heating for 20 minutes at 70 °C. For circularization, 500 ng of the digested DNA was ligated using 2.5 U of T4 DNA Ligase (Invitrogen) in a total volume of 25 µl for 16 hrs at 12 °C as per

manufacturer's instruction. Ligation mix (2.5 µl) was used as template in a 25 µl PCR reaction with the primers pri1 and pri6. PCR product from the pri1/pri2 PCR reaction (2.5 µl) was used as template in a 25 µl PCR reaction with primers pri3 and pri5. Figure 2.2 shows the restriction sites and primer binding sites within the *Tn*10*d* sequence. The nucleotide sequence of the final PCR product was determined using pri3 or pri5.

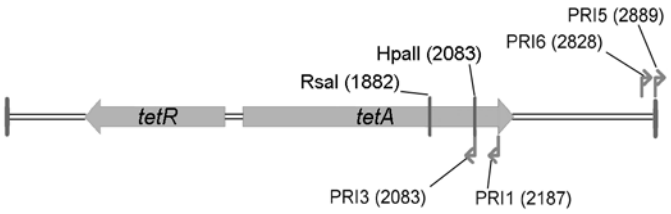

Figure 2.2. Schematic of *Tn*10*dtet* and inverse PCR relevant primers and restriction sites. Numbers in parentheses indicate relative nucleotide positions of primer binding sites (pri1, pri3, pri5 and pri6) and restriction endonuclease (*Hpa*II and *Rsa*I) recognition sequences. After digestion, DNA from restriction at a site 3' to the transposon, located in the genomic DNA, is ligated to DNA resulting from restriction at RsaI(1882) or HpaII(2083). The resulting piece of circular DNA is used as a template in PCR.

Chapter 3. Results.

3.1. *In vivo* characterization of transcription modulation by rifampicin.

Growth inhibition by rifampicin is caused by an inability of bacterial RNAP to produce full length RNA transcripts (Floss & Yu, 2005). The lowest concentration at which a given antibiotic inhibits growth of a given strain under the given conditions is the MIC. Using promoter::*lux* fusions, at concentrations below the MIC (sub-MIC) rifampicin both up-modulates and down-modulates transcription of various promoters (Table 1.1). Six promoters, three down-modulated promoters (*fliA*, *flgK* and *invF*) and three up-modulated promoters (*spvA*, *traS* and STM3595) are studied in this thesis.

3.1.1. RT-PCR.

In addition to promoter::*lux* fusion assays, RT-PCR carried out on RNA harvested from rifampicin treated *S. typhimurium* 14028 was used as secondary method to test gene expression in response to rifampicin. Relative amounts of transcript in the presence and absence of rifampicin was determined for two rifampicin down-modulated promoters, *fliA* and *invF*, and two rifampicin up-modulated promoters, STM3595 and *traS* (Fig 3.1). Primers for 16S and STM2901 annealed to the 3' end of the respective genes. Primers for *invF* and *fliA* annealed to the 5' end of the coding region. Primers for STM3595 were in the center of the protein coding region. 16S rRNA was used to normalize for input RNA as it was presumably unaffected by sub-MIC rifampicin. Since expression of STM2901 in 14028/pGY21 grown in liquid culture was found to be unaffected by rifampicin (Table 1.1, Fig 3.8), STM2901 transcripts were used as a negative control.

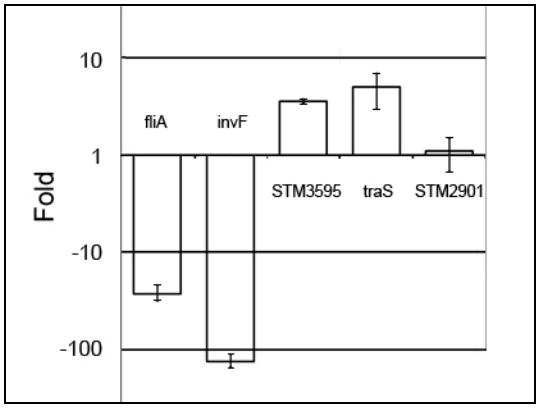

Figure 3.1. Fold decreases of *fliA* and *invF* and fold increases of STM3595 and *traS* transcripts in response to sub-MIC rifampicin. Relative RT-PCR levels of transcripts identified as rifampicin responsive in reporter assays. RNA levels were measured in the presence and absence of rifampicin. After normalizing levels using 16S RNA levels, ratios were obtained to determine fold change. Error bars indicate standard deviation of at least three experiments.

In terms of directionality and magnitude, gene expression changes measured by *lux* reporters and RT-PCR on RNA from rifampicin treated cultures showed similar responses to rifampicin. Using RT-PCR, *fliA* was decreased approximately 27-fold in the rifampicin treated sample (Fig 3.1) compared to a 32-fold decrease in luminescence using the *lux* reporter assay (Table 1.1). The other rifampicin down-modulated transcript *invF* was 130-fold less abundant in the rifampicin treated sample (Fig 3.1) compared to a 200-fold decrease found using the *lux* reporter assay (Table 1.1). The *traS* transcript was 5 times more abundant in the rifampicin treated sample (Fig 3.1) compared to a 5-fold increase in luminescence (Table 1.1). The STM3595 transcript had the most disparate results when comparing the luminescence assay to RT-PCR but was up-modulated by rifampicin using both methods. This transcript was found to be 4-fold

more abundant in the rifampicin treated sample (Fig 3.1) compared to a 24-fold up-modulation (Table 1.1). RT-PCR analyses of transcripts from cells treated with and without rifampicin were comparable to the magnitude of luminescence changes obtained from analogous reporter strains grown in the presence or absence of rifampicin.

3.1.2. Measurement of rifampicin mediated transcription modulation in rifampicin resistant strains.

It was theoretically possible that transcription modulation by rifampicin was due to interaction of rifampicin with one or more targets other than RNAP. To test whether transcription modulation by rifampicin and rifampicin mediated growth inhibition were both mediated by interaction with RNAP, a rifampicin resistant mutant was examined for transcription modulation by rifampicin at sub-MIC rifampicin. A spontaneous resistant mutant of *S. typhimurium* 14028, strain R306, was isolated by plating cells on media containing 300 μg ml^{-1} of rifampicin. By nucleotide sequencing, resistance in R306 was found to be conferred by a H526Y mutation (*E. coli* numbering) in the β subunit of RNAP. This mutation is in rifampicin resistance cluster I of the *rpoB* gene as named by Jin *et. al*. (1988).

Two rifampicin down-modulated promoters (*invF* and *fliA*), two rifampicin up-modulated promoters (STM3595 and *traS*) and a promoter unaffected by rifampicin when grown in liquid media (STM2901) were examined in a rifampicin resistant mutant. The basal level of expression from the rifampicin down-modulated promoters in R306 was within the error limits of repressed expression from the wild type exposed to rifampicin (not shown). The basal level of expression of rifampicin up-modulated promoters in the mutant was similar to the untreated levels of expression from the wild type (not shown). Transcription modulation of the four promoters

tested (STM3595, *traS*, *invF* and *fliA*) in response to rifampicin was abolished or greatly reduced in R306 (Fig 3.2). These data suggested that transcription modulation by rifampicin was mediated by rifampicin acting directly on RNAP rather than on secondary target.

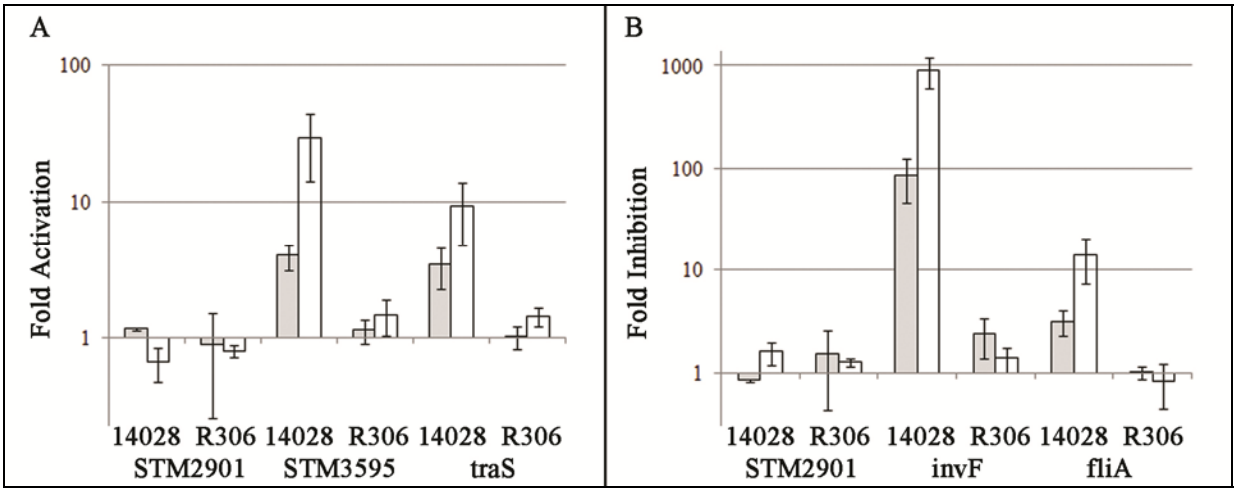

Figure 3.2. Comparison of luminescence produced by reporters carried by wildtype and rifampicin resistant strains. Strains were grown in 2.5 μg ml^{-1} (grey) or 5.0 μg ml^{-1} (white) of rifampicin. Fold activation was calculated by dividing the level of luminescence of rifampicin treated cells by the luminescence of cells grown in the absence of rifampicin and vice versa for fold inhibition. A. From left to right, reporter plasmids are pGY21, pGY35 and pGY22. B. From left to right, reporter plasmids are pGY21, pGY16 and pGY7.

3.1.3. Disk diffusion assays.

Sub-MIC antibiotics are known to have a plethora of effects on both bacterial transcription and translation. These effects might act through a common mechanism or through mechanisms unique to the antibiotic class. To examine whether other antibiotics had similar effects of transcription modulation and thus perhaps a similar mechanism of transcription modulation as

rifampicin, the response of strains carrying seven *lux* reporter fusions to various antibiotics and oxidative stressors was observed. Three rifampicin down-modulated promoters: *fliA*, *flgK* and *invF* (Fig 3.3), three rifampicin up-modulated promoters: *traS*, *spvA* and STM3595 (Fig 3.4) and one control promoter which is unaffected by rifampicin in liquid media (STM2901) were examined. The rifampicin resistant strain, R306, was also used as a host strain for these experiments.

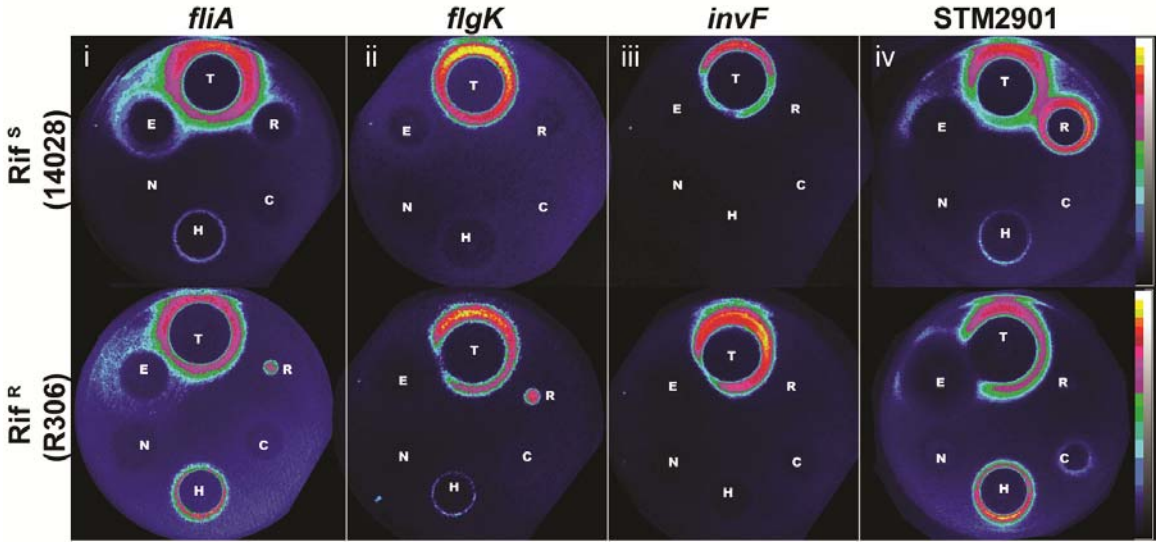

Figure 3.3. Disk diffusion assays with *fliA, flgK, invF* and STM2901 *lux* reporter strains. In liquid media *fliA*, *flgK* and *invF* are down-modulated by rifampicin. Luminescence images of *fliA* (pGY8, i), *flgK* (pGY13, ii), *invF* (pGY17, iii) and *STM2901* (pGY21, iv) promoter fusions in *S. typhimurium* 14028 (top row) or its rifampicin resistant derivative R306 (bottom row). Paper discs containing rifampicin (R, 30 µg), cumene peroxide (C, 5 µl of 1%), hydrogen peroxide (H, 5 µl of 3%), novobiocin (N, 30 µg), erythromycin (E, 300 µg) or tetracycline (T, 30 µg) are placed on top on the overlay. Luminescence intensity is converted to the color scale on the right, yellow being strong luminescence and dark blue being low luminescence.

The assay used to examine the expression in reporter strains was a combination disk diffusion and luminescence assay. Filter paper disks containing the compound of interest were placed on a bacterial agar overlay and cells were grown overnight. Compounds in the disks diffuse through the agar and as a result are concentrated near the disk and become more dilute farther away from the disk. Growth sensitivities to various compounds and antibiotics can be observed as zones of clearing where there is no growth (dark) and as bacterial lawns (light). The MIC of that particular compound occurs at the border of growth and no growth. For each antimicrobial shown, the diameter of the zone of clearing (zone of inhibition) was very similar between reporter strains; one representative plate is shown (Fig 3.4.iv). Luminescence images of the agar plates were recorded to monitor light production (Fig 3.3, Fig 3.4).

In liquid media, strains with *fliA*, *flgK* and *invF* reporters show rifampicin mediated down-modulation. In plate assays, these reporters showed no response to rifampicin (Fig. 3.3). However, it is difficult to detect down-modulation on solid media unless there is high basal luminescence. Thus, it is possible that rifampicin mediated down-modulation also occurs on solid media. Similar to the responses seen in liquid media, rifampicin induced a luminescence response from the *traS*, *spvA* and STM3595 (Figure 3.4). Interestingly, although unresponsive to rifampicin in liquid media, STM2901 was induced by rifampicin on solid media (Figure 3.4).

Promoters had varying response ranges to different antimicrobials. Outside the zone of growth inhibition, strains with *fliA*, *flgK*, *invF*, *traS* and *spvA lux* reporter fusions showed strong induction by tetracycline (Fig. 3.3, 3.4), a protein synthesis inhibitor that binds to the 30S ribosomal subunit. The *fliA* reporter strain was also weakly induced by erythromycin, a

macrolide which binds to the peptide exit tunnel of the ribosome, and by hydrogen peroxide but not to cumene peroxide (an organic peroxide) or novobiocin, a gyrase inhibitor (Fig. 3.3). The *invF* and STM3595 promoters were only induced by one compound tested, tetracycline and rifampicin, respectively, while the *fliA* promoter was induced by several compounds.

As expected, rifampicin produced a zone of inhibition in the overlay containing the wild type strain (Fig 3.4.iv, upper) but no zone of inhibition when R306 was used, as R306 is rifampicin resistant (Fig 3.4.iv, lower). For all other antibiotics, zones of inhibition were similar in 14028 and R306 regardless of the promoter-*lux* fusion used (Fig 3.4.iv).

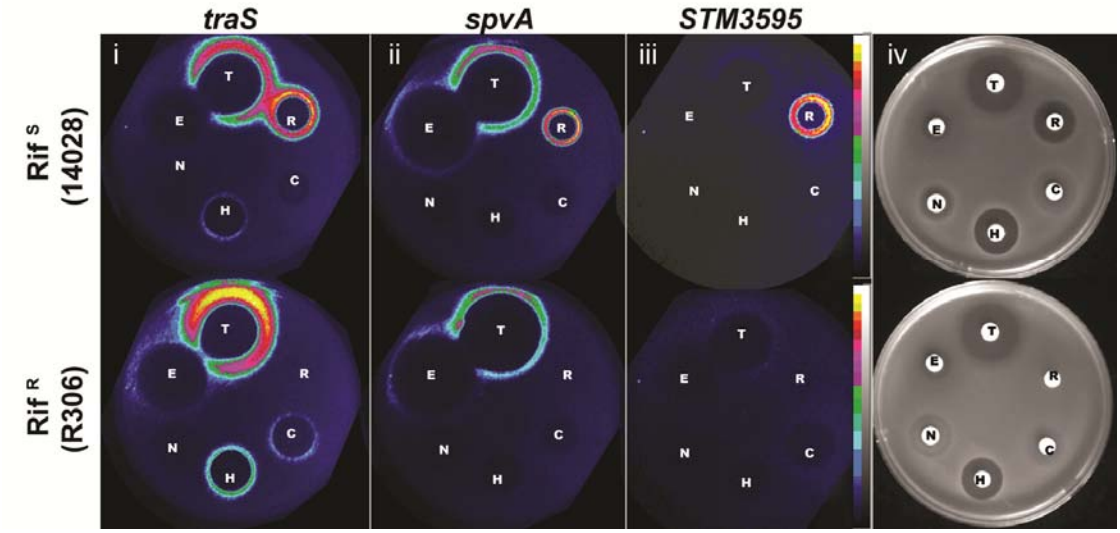

Figure 3.4. Disk diffusion assays with *traS*, *spvA* and STM3595 *lux* reporter strains. Luminescence images of *traS* (pGY25, i), *spvA* (pGY30, ii), STM3595 (pGY37, iii) and a photograph of *STM2901* (pGY21, iv) promoter fusions in *S. typhimurium* 14028 (top row) or its rifampicin resistant derivative R306 (bottom row). Compounds are labeled as in Fig 3.3.

R306 carrying the same reporters as the wild type strain was examined for responses to various compounds and stress induction (Fig.3.4). If a common mechanism was the basis of transcription modulation by tetracycline, erythromycin, hydrogen peroxide and rifampicin, rifampicin resistance should change the responses to the other compounds tested. Expression in the wildtype strain carrying the *traS*, *spvA* and STM3595 reporters showed strong up-modulation in response to rifampicin at ~9 mm away from the centre of the disk (Fig 3.4, upper row) but not close to the disk. At the same concentration range (~9 mm away from the centre of the disk), the response was abolished in the rifampicin resistant strains (Fig 3.4, lower row); although *fliA* and *flgK* reporter strains responded to rifampicin, these responses occurred at relatively high concentrations of rifampicin (at the center of the disk). Luminescence responses to other antibiotics and chemicals were largely unaffected by rifampicin resistance in the host (Fig 3.4). Rifampicin resistance only affected the response of rifampicin, supporting the notion that rifampicin has a different mechanism of transcription modulation compared to other active compounds tested.

3.1.4. Time courses.

To observe when transcription modulation by rifampicin occurs in the growth cycle of a batch culture, growth and luminescence of reporter cultures were monitored after stationary phase cultures were diluted into fresh media. For these experiments, rifampicin was added at different times post-inoculation (Fig 3.5). One promoter fusion, STM2901, that is affected less than two-fold by rifampicin, is shown as a control (Fig 3.5F). All reporter strains grew similarly, and the growth of one reporter is shown (Fig 3.5G). For the rifampicin down-modulated reporter, *fliA*,

the basal luminescence increased in the early exponential phase of growth, began to plateau during mid-exponential and declined in late exponential (Fig. 3.5A). For the rifampicin down-modulated reporter, *flgK*, the basal luminescence began to increase during mid-exponential and continued to increase for the duration of the assay (Fig. 3.5B). For the rifampicin down-modulated reporter, *invF*, the basal luminescence at the time of inoculation was relatively high, declined in the first hour of early exponential, began to increase in mid-exponential and continued to increase for the duration of the assay (Fig 3.5C). When rifampicin was added to cultures, the luminescence of down-modulated promoters showed similar patterns. When rifampicin was added before basal expression increased, rifampicin prevented the increase in expression levels (Fig 3.5A-C). If rifampicin was added after gene expression had already begun to increase, 2 hrs post inoculation for the *fliA* culture (Fig 3.5A), expression decreased. This suggested that for down-modulated promoters, rifampicin prevented expression around the time of addition.

In the case of the two rifampicin up-modulated promoters, *traS* and STM3595, the level of induced expression was higher when rifampicin was added earlier (Fig 3.5D, E). Furthermore, for the rifampicin up-modulated promoters, differential expression between treated and untreated cultures could not be seen until two to three hours after rifampicin addition. This type of lag was not observed in assays with the rifampicin down-modulated promoters (Fig. 3.5A-C). This lag suggested that induction of these promoters by rifampicin might require the accumulation of an unknown small molecule, protein or modification. This is addressed in the next section.

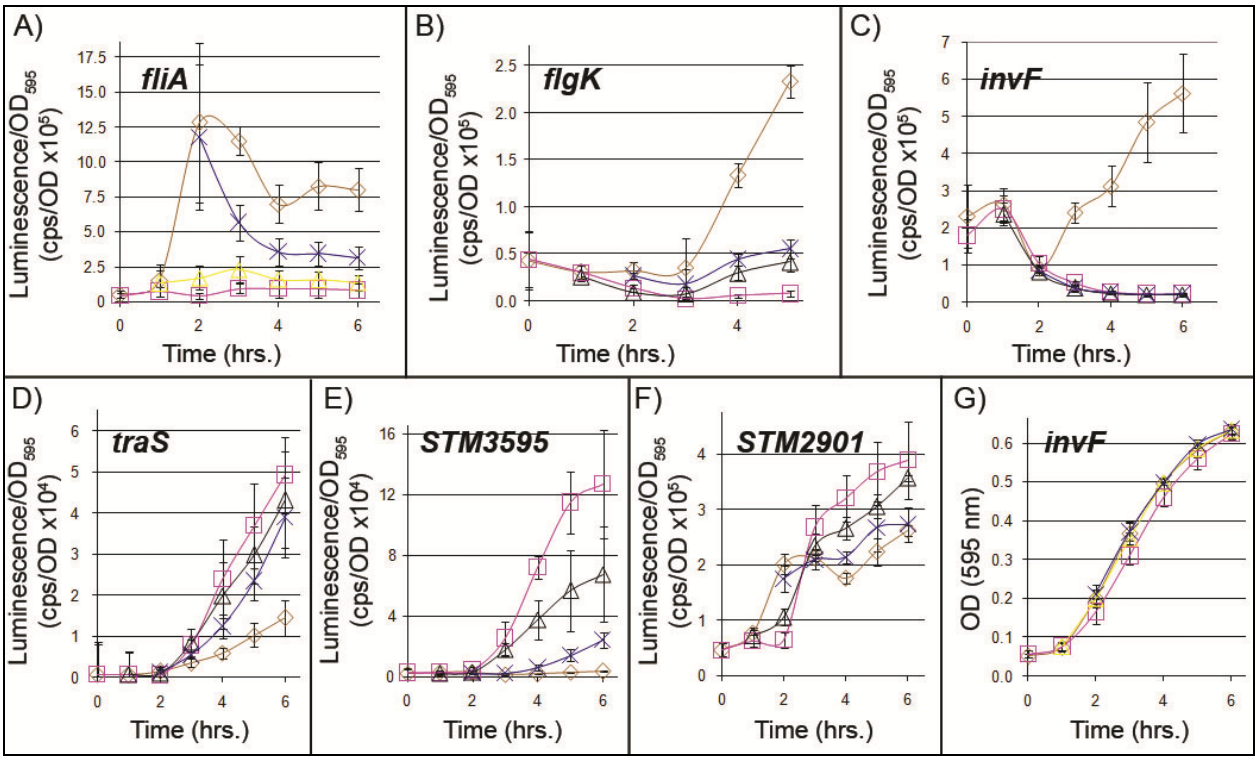

Figure 3.5. Time courses of rifampicin activation and inhibition. Luminescence (A-F) or growth (G) of strains carrying *fliA* (pGY7, A), *flgK* (pGY13, B), *invF* (pGY17, C,G), *traS* (pGY25, D), STM3595 (pGY37, E) or STM2901 (pGY21, F) *lux* reporters grown in the absence of rifampicin (diamond), or rifampicin added zero (square), one (triangle) or two hours (x) post-inoculation. Error bars indicate standard deviation of at least three experiments.

3.1.5. Pretreated cells and spent media experiments.

To determine whether accumulation of an unknown factor, either intracellularly or extracellularly, was involved with rifampicin mediated up-modulation, a series of rifampicin pretreatment and spent medium experiments were conducted. Luminescence and growth of the STM3595 reporter strain was examined as depicted in Figure 3.6. Untreated cells or rifampicin pretreated cells (Fig 3.6A, 3.7A) were grown in spent media or fresh media (Fig 3.6B-C, 3.7B-C). If a secreted, extracellular factor were involved in rifampicin mediated up-modulation,

one might expect to see stronger induction or induction occurring with a short lag period when untreated cells are grown in spent medium compared to fresh medium.

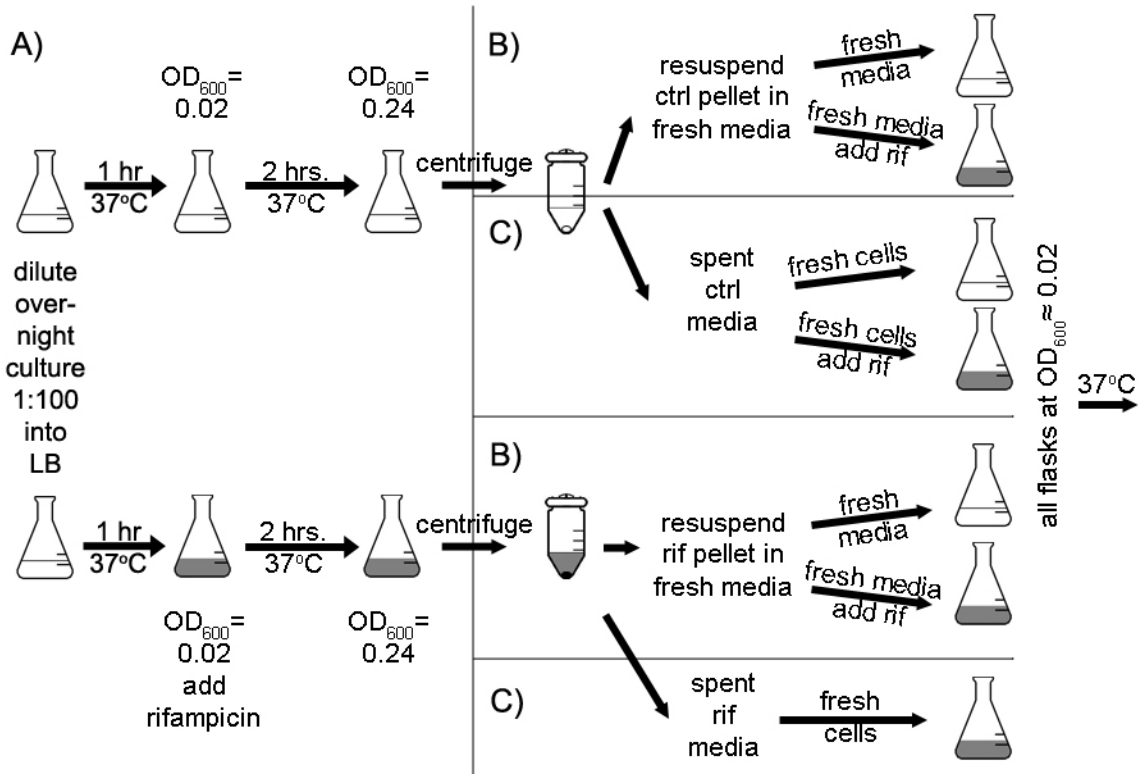

Figure 3.6. A schematic representation of spent medium experiments.

Luminescence of rifampicin pretreated cells grown in fresh medium (Fig 3.6B, 3.7B) had a higher starting luminescence but had a similar level of induction compared to control pretreated cells (Fig 3.7B). This higher starting luminescence was stable and may represent the accumulation of a factor or a modification of cellular components. Fresh cells grown in rifampicin spent media (rifampicin not removed) and fresh cells grown in control spent media supplemented with rifampicin were up-modulated similarly (Fig. 3.6C, 3.7C). Since growing

cells in spent media did not shorten the time of induction or amplify the fold increase, the data suggested that rifampicin mediated up-modulation does not involve a stable secreted factor. However, the data does not rule out accumulation of an intracellular factor.

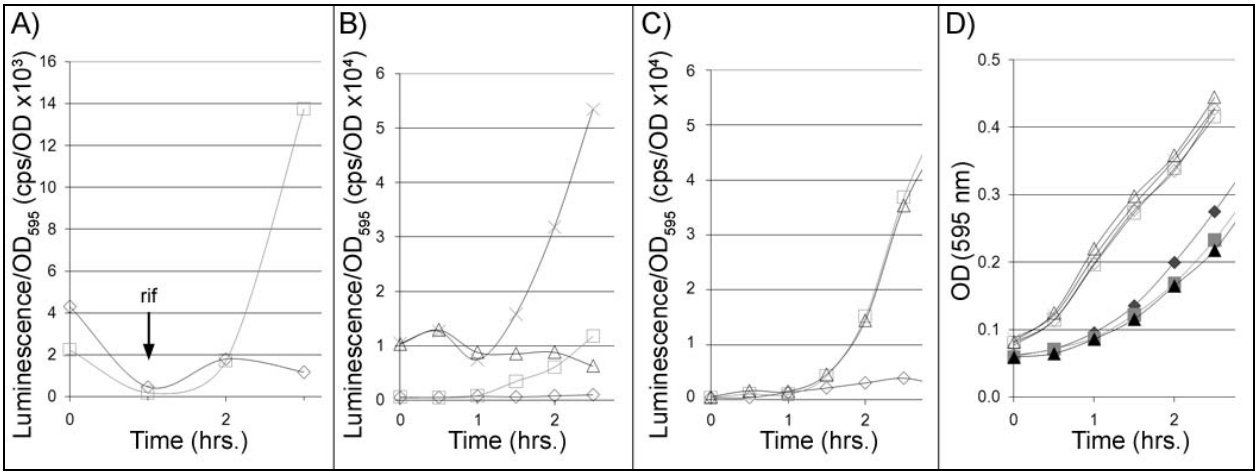

Figure 3.7. Spent media and pretreatment effects of rifampicin on STM3595 expression. A. Luminescence of 14028/pGY37 grown in the absence (diamond) or the presence of rifampicin (square). B. Luminescence of cells grown in the absence (diamond, square) or presence (triangle, x) of rifampicin resuspended and grown in fresh media without (diamond, triangle) or with rifampicin (square, x). Time of resuspension is shown as zero hours. C. Luminescence of fresh cells grown in spent media from cultures grown in the absence of rifampicin (diamond), presence of rifampicin (triangle), or spent media from cultures grown in the absence of rifampicin but supplemented with rifampicin when fresh cells were added (square). Time of reinoculation is shown as zero hours. D. Growth of cultures shown in B (open symbols) and in C (closed symbols). See Fig. 3.6 for a schematic with corresponding lettering.

3.2. Mapping DNA regions necessary for transcription modulation by rifampicin.

To more precisely identify the DNA fragments necessary for transcription modulation by rifampicin, six rifampicin responsive *lux* reporters were mapped by sub-cloning. DNA from

library plasmids was sub-cloned into pCS26 and luminescence of 14028 carrying these new constructs was measured. The three rifampicin repressed constructs chosen for examination contained promoters for the following genes: *fliA*, *flgK* and *invF*. The three rifampicin activated constructs chosen for examination contained the promoters for the following genes: STM3595, *spvRAB* and *traS*. These constructs were chosen as they displayed some of the strongest responses to rifampicin and were possibly important from a pathogenic perspective (Table 1.1). Several constructs had two fragments from different locations in the chromosome (Fig 3.8A, 3.9A) or had several genes with putative promoters.

In order to identify which fragment was involved in transcription modulation by rifampicin, sub-clones with approximately 300 bp (from 250 bp upstream to 50 bp downstream of the putative start codon) were constructed. Once the putative promoter was identified, smaller (~50 bp) fragments were cloned with the logic that if rifampicin acted directly on RNAP-promoter interactions alone, a fragment of this size should have a promoter sensitive to rifampicin. These fragments spanned nucleotides from approximately –45 to +5 relative to the published or determined +1 transcription start and would likely not contain complete binding sites for transcription factors. They should contain the -35 elements, -10 elements and +1 sequences and sufficient sequence to make specific contacts with RNAP. For each promoter, four to six reporter constructs which were derivatives of the 300 bp and 50 bp fragments were constructed. Luminescence of the strains carrying the variant constructs was monitored in the presence or absence of rifampicin. The data for these experiments is summarized in Figures 3.8 and 3.9.

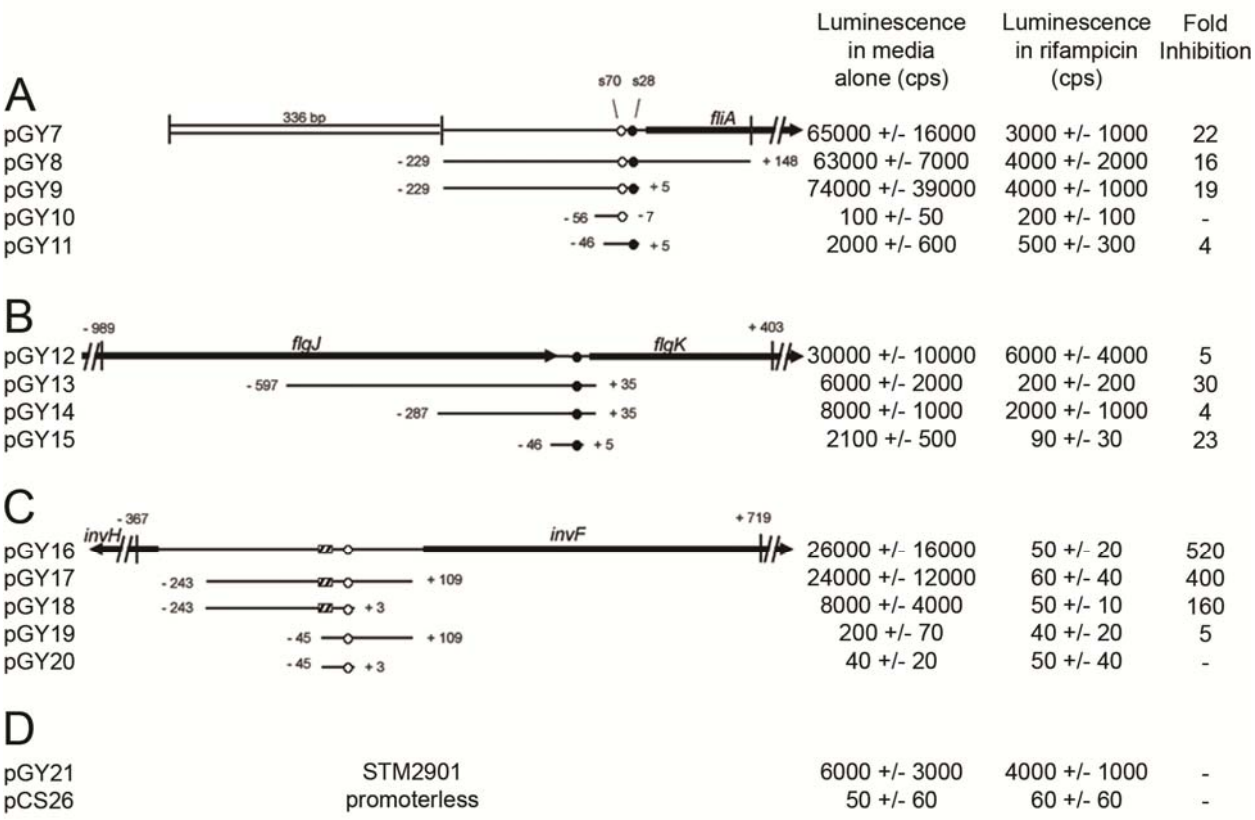

Figure 3.8. Mapping of rifampicin repressed promoters. Schematics of *fliA* (A), *flgK* (B) and *invF* (C) promoter region truncations and the luminescence of the strains carrying the respective promoter-*lux* reporter fusion. Open circles indicate σ^{70} dependent +1 transcription start sites while closed circles indicate σ^{28} dependent +1 transcription start sites (Ikebe *et al.*, 1999; Kutsukake & Ide, 1995; Lostroh *et al.*, 2000). pGY11 contains the σ^{70} dependent +1 but lacks the corresponding -35 element. Double lines indicate DNA from another region of the genome as compared to single lines in the same construct. The striped box indicates that all bases of the HilA binding site are present (Lostroh *et al.*, 2000).

The original reporter construct from the library with the *fliA* promoter (pGY7) had two pieces of DNA from different regions of the chromosome. To identify the region of DNA required for rifampicin mediated down-modulation, a 377 bp fragment carrying the presumed *fliA* promoter region was cloned into pCS26 to create plasmid pGY8. pGY9, a sub-clone of pGY8 was created by deleting 143 bp 3' from the σ^{70} and σ^{28} dependent promoters identified for the *fliA* gene

(Ikebe *et al.*, 1999). Strains carrying pGY8 and pGY9 had similar levels of *lux* expression and rifampicin hypersensitivity as the strain with the original library construct, pGY7 (Fig. 3.8A). The other 336 bp fragment had no detectable promoter activity. A strain carrying plasmid pGY10 that contained a 50 bp fragment with only the σ^{70}dependent *fliA* transcription start site and 46 bp upstream of this site showed no *lux* activity (Fig. 3.8A). In contrast, *lux* expression in a strain carrying a 51 bp fragment containing the σ^{28}dependent transcription start site, pGY11, showed hypersensitivity to rifampicin, albeit with lower basal levels of activity than the larger fragments (Fig 3.8A). This suggested that in the case of the *fli*A reporter, rifampicin mediated down-modulation is associated with transcription by the RNAP holoenzyme containing σ^{28}.

A 632 bp fragment with the *flgK* σ^{28}dependent promoter (Kutsukake & Ide, 1995) was sub-cloned into pCS26 creating plasmid pGY13. Lux expression in the strain carrying pGY13 showed higher fold induction to rifampicin than a strain with the original construct (pGY12) but also showed much lower basal levels (Fig 3.8B). Removal of 310 bp of DNA 5' to the transcription start site in pGY13, leaving 287 bp 5' to the start in pGY14 did not change basal expression but decreased hypersensitivity to rifampicin (Fig. 3.8B). Additional 3' and 5' truncations of the *flgK* promoter yielded a 51 bp fragment containing the σ^{28}dependent transcription start site and 45 bp 5' to that site (pGY15). Rifampicin repressed *lux* expression in the strain carrying was to a similar extent (fold change) as seen in the strain carrying the larger promoter fragment (pGY13, Fig. 3.8B).

A 352 bp fragment with the σ^{70}dependent promoter of *invF* was sub-cloned from the library construct (pGY16) to create plasmid pGY17. pGY17 showed similar basal expression levels and

fold hypersensitivity to rifampicin as the original library construct (Fig. 3.8C). 106 bp were deleted from pGY17, leaving 3 bp downstream of the *invF* +1 and creating plasmid pGY18. Transcription in strains carrying pGY18 retained rifampicin hypersensitivity but showed lower basal levels (Fig. 3.8C). A 198 bp truncation 5' to the *invF* transcription start site fragment was made which removed half of the HilA binding site (Lostroh *et al.*, 2000) (pGY19). The strain carrying pGY19 showed low basal *lux* expression and low rifampicin hypersensitivity (Fig. 3.8C) suggesting that the HilA binding site was either required for rifampicin mediated down-modulation of *invF* transcription or just required to increase basal luminescence so that down-modulation is detectable in this assay. Removal of 106 bp 3' to the transcription start leaving DNA from -45 to +3 relative to the HilA dependent transcription start site fused to the *lux* genes (pGY20) had no basal activity (Fig. 3.8C).

In summary, for both flagellar genes *fliA* and *flgK*, transcription from ~ 50 bp fragments containing the transcription start sites of the σ^{28}dependent promoters exhibited rifampicin mediated down-modulation. The smallest *invF* promoter fragment that retained hypersensitivity to rifampicin was 246 bp and contained the binding site for the activator HilA (Lostroh *et al.*, 2000).

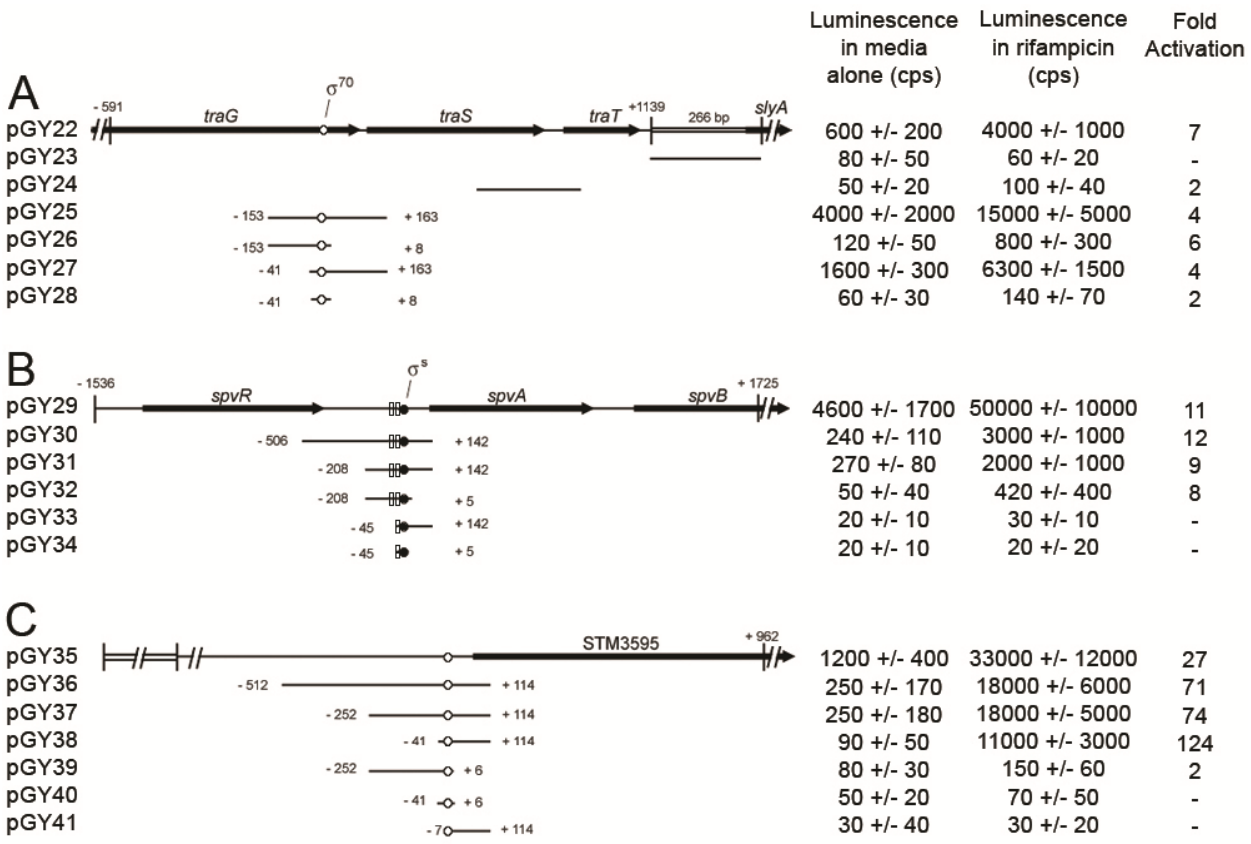

Figure 3.9. Mapping of rifampicin activated promoters. Schematics of *traS* (A), *spvA* (B) and STM3595 (C) promoter region truncations and the luminescence of the strains carrying the respective promoter-*lux* reporter fusion. Open circles indicate σ^{70} dependent +1 transcription start sites for *traS* (Ham *et al.*, 1989) and STM3595 (primer extension and 5' RACE using RNA harvested from cells), closed circles indicate σ^{S} dependent +1 transcription start sites and boxes indicate SpvR binding sites (Sheehan & Dorman, 1998).

The original library reporter construct pGY22 contained both *traGST* and *slyA*. Six sub-clones of pGY22 were constructed and assayed in *S. typhimurium* 14028 for their luminescence responses to rifampicin to identify DNA regions required for rifampicin mediated up-modulation (Fig 3.9A). A 266 bp fragment carrying the *slyA* region, a 265 bp fragment region carrying the *traT* region and a 316 bp fragment carrying the *traS* region were each cloned into pCS26 to

create plasmids pGY23, pGY24 and pGY25, respectively. It is unlikely that the promoters associated with *slyA* and *traT* are up-modulated by rifampicin since the strains carrying the respective reporters had low basal activity and were not up-modulated by rifampicin. The *traS* reporter (pGY25) had moderate basal activity and displayed rifampicin mediated up-modulation. A 3' truncation of this fragment deleting 155 bp and leaving 8 bp downstream of the transcription start site (pGY26) decreased basal activity but still displayed rifampicin mediated up-modulation. Removal of an additional 112 bp from the 5' end leaving 41 bp upstream of the start site (pGY28), decreased basal activity and abolished rifampicin mediated up-modulation. Interestingly, the reporter created by a removal of 112 bp from this fragment in pGY25, pGY27, had reduced basal activity but was able to mediate up-modulation by rifampicin. This suggested that in the case of the *traS* promoter, up-modulation can be mediated by interaction with either DNA downstream or upstream of the transcription start site.

To analyze the regions upstream of the +1 of *spvA* that were required for rifampicin mediated up-modulation (Fig 3.9B), reporter strains carrying 642 bp (pGY30) and 350 bp (pGY31) were tested. These clones that have 506 bp and 208 bp 5' to the +1 site, respectively, had similar basal activity and their level of rifampicin mediated up-modulation was similar to the original library reporter strain (pGY29). A strain carrying a construct made by truncating the 3' end of the promoter in pGY31 to the+5 nucleotide (pGY32) had lower basal activity but a similar fold increase in rifampicin mediated up-modulation when compared to reporters carrying longer DNA fragments. Deletion of 163 bp 5' truncation from the promoter in pGY31, leaving 45 bp 5' to the +1 site (pGY33) had no basal activity and did not display rifampicin mediated up-modulation. Similarly, a 50 bp fragment containing -45 to +5 of the *spvA* promoter (pGY34) had

no basal activity and did not display rifampicin mediated up-modulation. The reporter with the shortest fragment displaying rifampicin mediated up-modulation, pGY32, had sequences from -208 to +5 and encodes the binding site of a known *spvA* activator, SpvR (Sheehan & Dorman, 1998).

STM3595	cgttgTgaAagtattgcaagacgtgttcgTATcATcaaaccGTcaac		
	-35	-10	+1
E.coli σ^{70} consensus	TTGACA	TATAAT	

Figure 3.10. Transcription start site of STM3595 as determined by 5'RACE. The transcription start site is marked by GT and a +1 underneath. Sequences with similarity to the accepted *E.coli* consensus (Browning & Busby, 2004) are underlined.

Before studying the sequences required for rifampicin mediated up-modulation of the gene STM3595, the transcription start site was determined by 5' RACE (Fig 3.10). The transcription start sites for all other promoters shown in Fig 3.8 and 3.9 had been previously determined in *S. typhimurium* except *traS* which had been determined in *E. coli* (Ham *et al.*, 1989) and was assumed to be conserved in *S. typhimurium*. The transcription start site of STM3595 determined by 5'RACE using RNA isolated from *S. typhimurium* 14028 grown in the presence of rifampicin is indicated in Fig. 3.10.

Deletion analysis was used to narrow down sequences required for the rifampicin up-modulation in the STM3595 promoter (Fig 3.9C). Reporter strains carrying 626 bp (-512 to +114, pGY36)

and 366 bp (-252 to +114, pGY37) fragments had lower basal activities than the strain carrying the original library reporter (pGY35) and were up-modulated to similar absolute levels as pGY35. Thus the strains with pGY36 and pGY37 displayed higher levels of rifampicin mediated up-modulation than 14028/pGY35. Truncation of 211 bp 5' to the +1 site of pGY37 (pGY38) had even lower basal activity and higher fold levels of rifampicin mediated up-modulation when compared to reporters carrying longer DNA fragments. A 108 bp 3' truncation of the 366 bp fragment produced a reporter carrying -252 to +6 (pGY39) had low levels of basal activity and did not display rifampicin mediated up-modulation. A 52 bp fragment carrying -41 to +6 (pGY40) had no basal activity and did not display rifampicin mediated up-modulation. Since the smallest fragment of DNA mediating rifampicin mediated up-modulation spanned nucleotide sequences from -41 to +114 with respect to the +1 transcription start site, DNA downstream of +1 seemed to be necessary for rifampicin mediated up-modulation from the STM3595 promoter. Since examples of both upstream and downstream regions were seen to be required for rifampicin mediated up-modulation of different promoters, rifampicin responsive promoters may be indirectly affected by rifampicin and may work by multiple mechanisms.

3.3. *In vitro* transcription of rifampicin modulated promoters.

In vivo data from RT-PCR and *lux* reporters showed that transcription of *spvA*, *traS* and STM3595 were up-modulated by sub-MIC rifampicin and that *fliA*, *flgK* and *invF* were down-modulated by sub-MIC rifampicin. Disk diffusion assays suggested that rifampicin elicited a pattern of expression different than that of other compounds tested. Deletion studies using truncated promoters fused to the *lux* reporter suggested that down-modulation of *fliA* and *flgK* was associated with σ^{28}, while down-modulation of *invF* may require the activator HilA.

Furthermore, since RNAP *rpoB* mutants no longer displayed transcription modulation by rifampicin (section 3.1.2), it was possible that RNAP holoenzyme, DNA and rifampicin alone were enough to mediate transcription modulation by rifampicin. That is no other regulatory elements such as transcription factors or small molecules were required to mediate this effect at some promoters. To investigate this, *in vitro* transcription experiments were performed with varying amounts of rifampicin, DNA and *E. coli* RNAP. Since *E. coli* and *S. typhimurium* RNAP are 99% identical in amino acid sequence, I presumed that the *in vitro* responses were equivalent. Large quantities of *E. coli* cells from which a mixture of RNAP holoenzymes could be isolated were also readily available. Other *in vitro* experiments studying *S. typhimurium* transcription have used *E. coli* RNAP (Schaubach & Dombroski, 1999). Linear DNA templates were prepared using PCR. Two rifampicin down-modulated *S. typhimurium* genes, *invF* and *flgK*, and two rifampicin up-modulated genes, STM3595 and *traS*, were examined.

3.3.1. Rifampicin mediated down-modulation requires σ^{28} and HilA.

For transcription at *flgK* and *invF*, preliminary experiments examining transcription by RNAP-σ^{70} using different buffer conditions and incubation times failed to produce strong transcripts (not shown). One σ^{28} dependent transcription start site for *S. typhimurium flgK* transcription had been previously determined (Kutsukake & Ide, 1995). Deletion analysis (section 3.2) suggested that for *flgK*, rifampicin mediated down-modulation was associated with the previously determined RNAP- σ^{28} transcription start site. In the case of *invF*, deletion analysis suggested that RNAP-σ^{70} in conjunction with the transcription activator HilA was required to observe transcription modulation by rifampicin from *invF* because removal of the HilA binding site eliminated rifampicin mediated down-modulation. As a consequence,

recombinant His-tagged versions of σ^{28} and HilA were purified and added to *in vitro* transcription reactions for these promoters as appropriate.

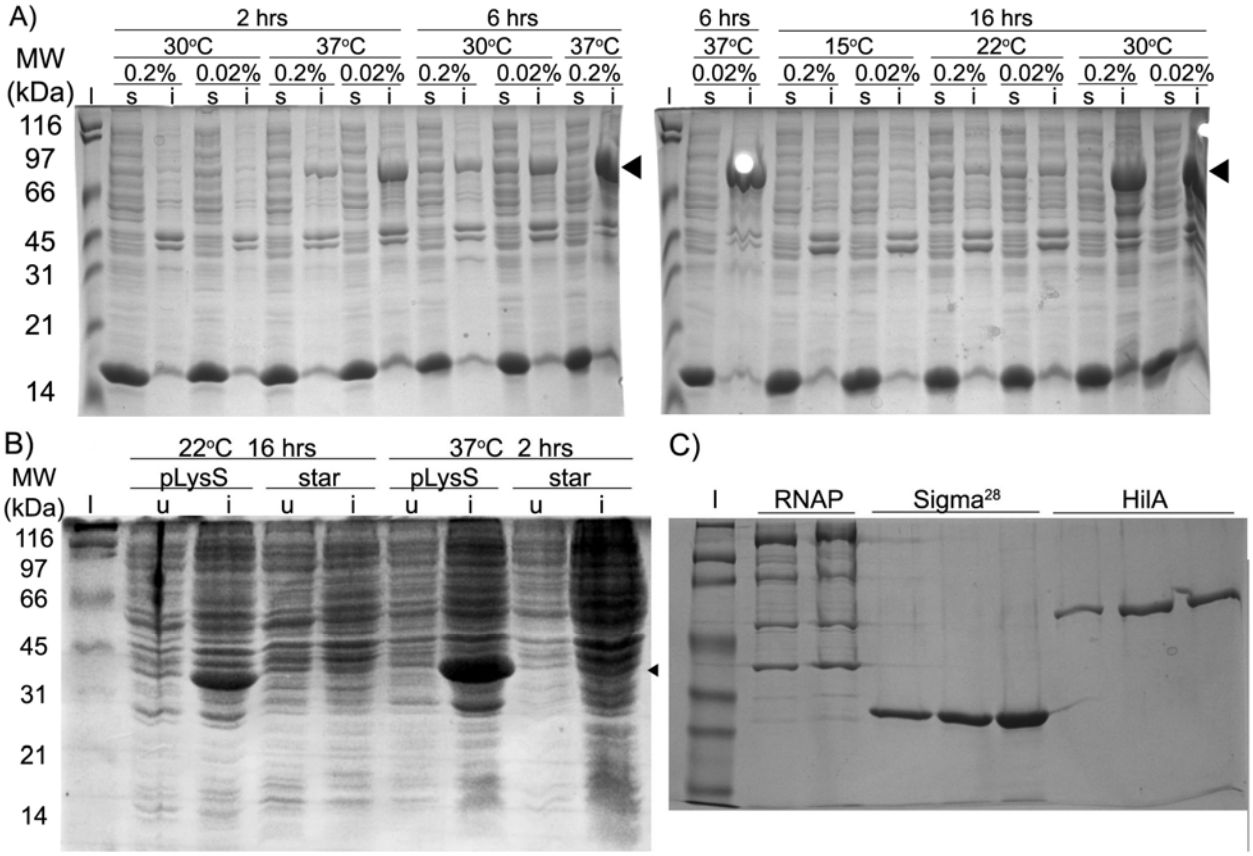

Figure 3.11. Induction and isolation of His-HilA and His-Sigma^{28}. A. SDS-PAGE of insoluble (i) and soluble (s) fractions of His-HilA from cultures grown in various induction conditions: 0.2% or 0.02% arabinose, growth at various temperatures or different induction times. B. SDS-PAGE of whole cell lysates from uninduced (u) or induced (i) cultures of BL21/pLys or BL21 star carrying pKH445 induced for 16 hrs at 22°C or 2 hrs at 37°C. C. SDS-PAGE of purified extracts of RNAP, His-Sigma^{28} or His-HilA. BioRad prestained SDS-PAGE standard protein ladder was run on each gel (l).

Since two different conditions for isolation of soluble His-HilA had been reported (Lostroh *et al.*, 2000; Rodriguez *et al.*, 2002), several parameters were optimized to obtain the best yield of soluble protein (Fig 3.11). Two different arabinose concentrations were compared for their ability to induce His-HilA from the arabinose inducible promoter P_{BAD} (Fig 3.11A). Four different post-induction incubation temperatures (15 °C, 22 °C, 30 °C and 37 °C) and three post-induction incubation times (2, 6 and 16 hrs) were also compared (Fig 3.11A). No conditions were found under which His-HilA was mostly soluble. Induction with lower arabinose, seemed to yield more protein. For isolation, expression was induced with 0.02 % arabinose and the culture incubated for 6 hrs at 30 °C or 16 hours at 22 °C (Fig. 3.11C).

As previously published, no conditions resulting in appreciable amounts of soluble His-σ^{28} could be identified. σ^{28} could not be induced in BL21(DE3), as previously published (Chadsey *et al.*, 1998), or in BL21 star (Invitrogen), but was induced in BL21(DE3) pLysS (Fig. 3.11B). If induced, more growth generally resulted in more total protein induction and more insoluble protein. His-σ^{28} induced for 2 hours at 37 °C was isolated under denaturing conditions and reconstituted as described elsewhere (Wilson & Dombroski, 1997). The resulting protein preparations appeared pure (Fig. 3.11C).

3.3.2. Optimization of *flgK* and *invF in vitro* transcription.

Several *in vitro* transcription reaction conditions were optimized: protein concentration, template concentration, activation time, extension time and order of addition. High levels of transcription from the *flgK* template required the presence of σ^{28} (Fig 3.12A). High levels of transcriptions were detected when the ratio of σ^{28}:RNAP was equal to 39:1 and stayed more or less the same

with higher ratios (Fig 3.12A). When the ratio of σ^{28}:RNAP was reduced, transcription weakened (Fig 3.12B, first 3 lanes), suggesting renaturation of σ^{28} after isolation under denaturing conditions was approximately 2.5% effective. A ratio of 39:1 was used for subsequent experiments. Varying the level of *flgK* template showed the highest transcript production at 16 nM (Fig 3.12B); 16 nM template was used for subsequent experiments. Transcription from the *invF* template was stimulated by HilA and appeared optimal when HilA was 0.3 to 0.2 nM (Fig 3.12C). When *invF* template concentration was varied, HilA dependent *invF* transcript was optimal at 16 nM of template (Fig 3.12C).

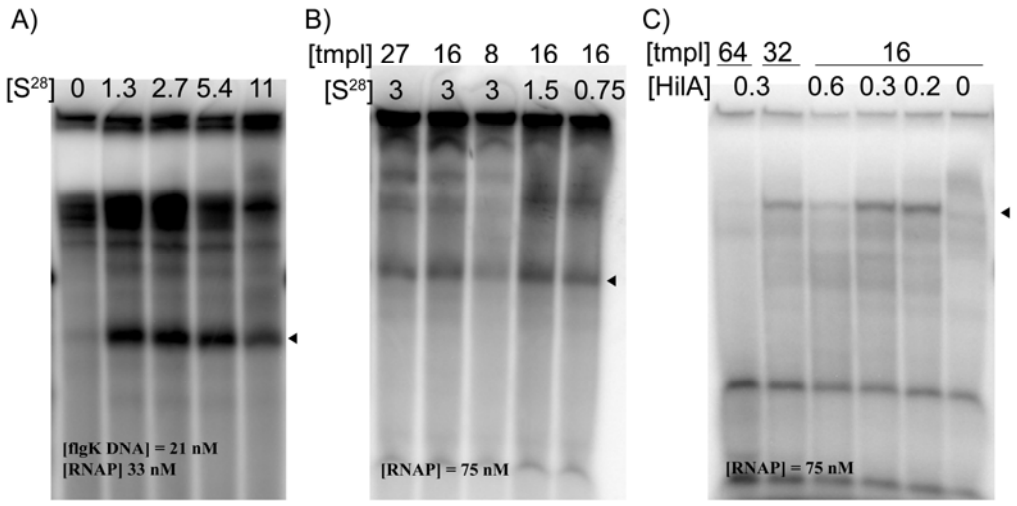

Figure 3.12. Optimization of *flgK* and *invF* transcription. A,B. Phosphorimage of denaturing polyacrylamide gels used to separate transcripts from reactions with various amounts of His-Sigma^{28} (μM) or *flgK* template (nM). C. Phosphorimage of transcripts from reactions with various amounts of His-HilA (μM) or *invF* template (nM). Arrows indicate specific transcripts.

Even after optimizing template and HilA amounts, transcript levels from *invF* were still low. Various incubation times and orders of addition were investigated to see if higher level of

transcription could be obtained. Activation of transcription by HilA was found to be stronger when HilA was added to DNA before RNAP (not shown). DNA-HilA preincubation times of 2 and 16 minute were examined; a 2 minute preincubation yielded higher levels of transcription (not shown). Extension times of 2, 5 and 22 min were examined and 5 min extension times were found to give the highest level of transcription with the least amount of non-specific transcription (not shown). Since transcription from the P_{flgK} was efficient, transcription conditions were assumed to be sufficiently optimized. Incubation times used in subsequent experiments are outlined in Figure 3.13 unless otherwise indicated.

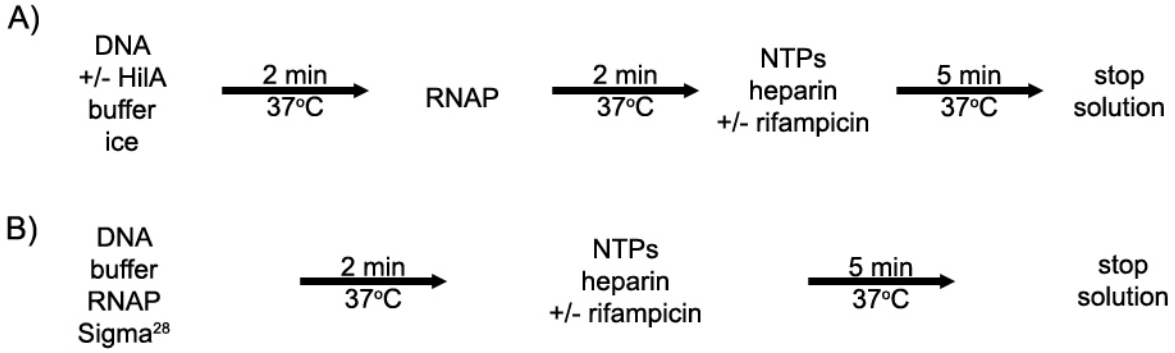

Figure 3.13. A schematic representation of incubation times used for *in vitro* transcription.

Heparin was added to limit transcription to a single round. Heparin is a polyanion which inactivates free RNAP and destabilizes some early binary complexes of RNAP and DNA. At most promoters, *E. coli* RNAP: σ^{70} which has formed an initiation complex in open complex is resistant to heparin, one exception being complexes formed at the T7 A1 promoter (Pfeffer *et al.*,

1977). In assays shown in this thesis, heparin and NTP were added simultaneously after (*E. coli*) RNAP binding, so it is unlikely that the heparin affected open complex formation or stability.

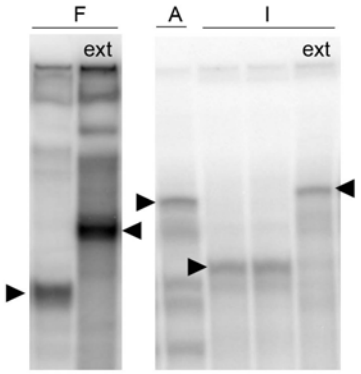

Figure 3.14. Identification of transcripts. Phosphorimage of transcripts from *flgK* (F) and *invF* (I) templates with different 3' end points separated by denaturing polyacrylamide gel electrophoresis. Templates with extended 3' ends are indicated (ext). The *abrB* transcript (A) is shown as a reference. Specific transcripts are indicated by arrows. (Transcripts from the short *invF* template are shown in duplicate.)

In gels showing multiple transcripts, transcripts of interest were identified by making DNA templates of varying lengths by PCR, since DNA templates with a truncated 3' end would lead to shorter transcripts. A shortened *invF* template was constructed using invFent for/invFent rev primers on pGY17 plasmid DNA and a shortened *flgK* template was synthesized using flgKent2 for/flgKent rev on genomic DNA. These templates were 101 bp shorter than the original templates. Transcripts from the shortened templates shifted in the expected manner and were the appropriate relative size when compared to transcripts of known length, such as the P_{abrB}

transcript from the p2only5'A/S187 on pAW44 template which gives a transcript of 206 nucleotides (Fig 3.14).

3.3.3. Rifampicin hypersensitivity of *flgK* and *invF* transcription.

Using optimized conditions, *in vitro* transcription reactions containing P_{flgK} or P_{invF} , purified His-tagged σ^{28} or HilA, respectively, were performed varying the concentration of rifampicin. Rifampicin sensitivity of transcription was compared to transcription from a control promoter. A promoter from *B. subtilis* that has -10 and -35 elements similar to the *E. coli* σ^{70} consensus sequence, P_{abrB}, was used as a σ^{70} control promoter (Fig. 3.9, 3.29). P_{fliC} was used as a control promoter transcribed by RNAP-σ^{28}. Transcription from P_{flgK} and P_{invF} was 3-fold and 10-fold more sensitive to inhibition by rifampicin than their respective control promoters (Fig 3.15, 3.16). These data suggest that the differences in the promoter sequences lead to differences in the inactivation of RNAP by rifampicin and suggested a mechanism by which promoters are down-modulated by rifampicin.

Since P_{abrB} is a *B. subtilis* promoter and its response to rifampicin in *S. typhimurium* was unknown, the effect of rifampicin on P_{abrB} was examined in *Salmonella*. An *abrB*::*luxCDABE* reporter fusion was constructed (pGY43) and the luminescence in *S. typhimurium* 14028 was determined. When luminescence of the *invF* and *abrB* reporter fusions in the presence of increasing amounts of rifampicin was compared (Fig 3.16C), the sensitivity of *invF* transcription to rifampicin was approximately three-fold greater when compared to *abrB*, comparable to results found *in vitro* (Fig 3.16B).

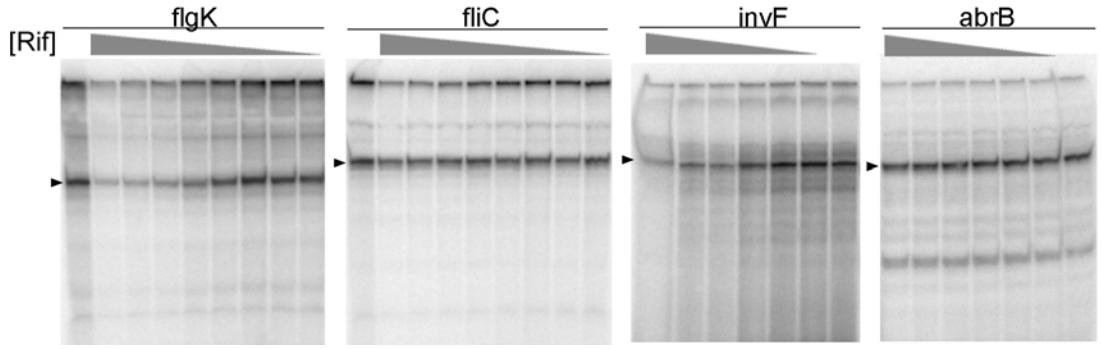

Figure 3.15. Rifampicin inhibition of *flgK, fliC, invF* and *abrB* transcripts. Phosphorimage of the products of transcription from *flgK*, *fliC*, *invF* and *abrB* promoters in the presence of varying amounts of rifampicin (50 to 0 μg ml^{-1} as shown in Fig 3.16) separated by denaturing polyacrylamide gel electrophoresis.

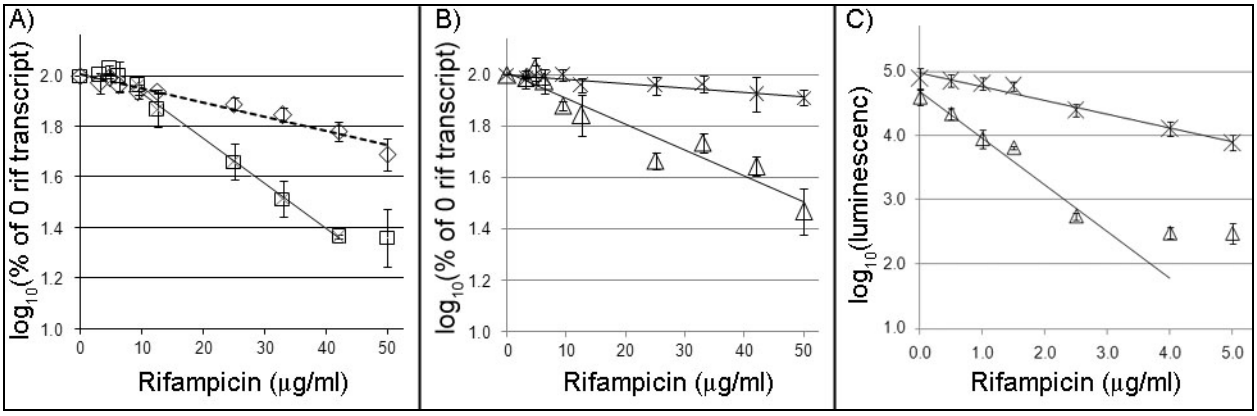

Figure 3.16. Graphs of transcription inhibition by rifampicin. A,B. *In vitro* transcript amounts in the presence or absence of rifampicin represented in Fig. 3.15 determined by spot densitometry using ImageQuant 5.2 software plotted as log10 values of the percentage of the amount of transcript in the absence of rifampicin. Templates were *fliC* (diamond), *flgK* (square), *abrB* (x) and *invF* (triangle). C. Luminescence of *abrB* reporter (x, pGY43) and *invF* (triangle, pGY17) reporter plasmids carried in *S. typhimurium* 14028 grown in the presence of various concentrations of rifampicin. Luminescence values are plotted as log10 values. Error bars represent the standard deviation of at least three experiments. The slope of the resulting trend lines were used to calculate fold differences in rifampicin sensitivity.

3.3.4. Mg^{2+} and NTP concentration effects.

In the past several years, several studies have reexamined the relationship between rifampicin, RNAP and the influence of Mg^{2+} on rifampicin sensitivity (Artsimovitch *et al.*, 2005; Feklistov *et al.*, 2008). Since some previously published data suggested that rifampicin inhibits transcription by inhibiting the catalytic activity of RNAP (Artsimovitch *et al.*, 2005), a possible mechanism of differential rifampicin sensitivity of transcription from P_{flgK} and P_{invF}, compared to the control promoters, P_{fliC} and P_{abrB}, may involve Mg^{2+}. To test this hypothesis, transcription inhibition assays were carried out at different Mg^{2+} concentrations.

In vitro transcription assays were carried out as those shown in Fig. 3.15 except final Mg^{2+} concentrations were varied. The amounts of run-off transcript produced at each concentration of Mg^{2+} in the presence or absence of rifampicin are shown in Fig. 3.17. Sensitivity of *flgK* transcripts to rifampicin showed Mg^{2+} dependence over the range of concentrations tested, 1 to 16 mM, (Fig. 3.17). The effect of Mg^{2+} observed with *flgK* transcription was similar to rifampicin susceptibility of T7 A1 transcription by *E coli* RNAP (Artsimovitch *et al.*, 2005) where higher levels of Mg^{2+} decreased the inhibition by rifampicin. In contrast, the effect of rifampicin on *fliC* transcription was not Mg^{2+} dependent over the concentration range tested. Sensitivity of transcription from *invF* and *abrB* did not differ over a similar Mg^{2+} range (not shown). Since only one of the two promoters showed Mg^{2+} dependent rifampicin sensitivity, this suggested that the level of Mg^{2+} was not a primary determinant in the polymerase promoter interactions leading to rifampicin responses.

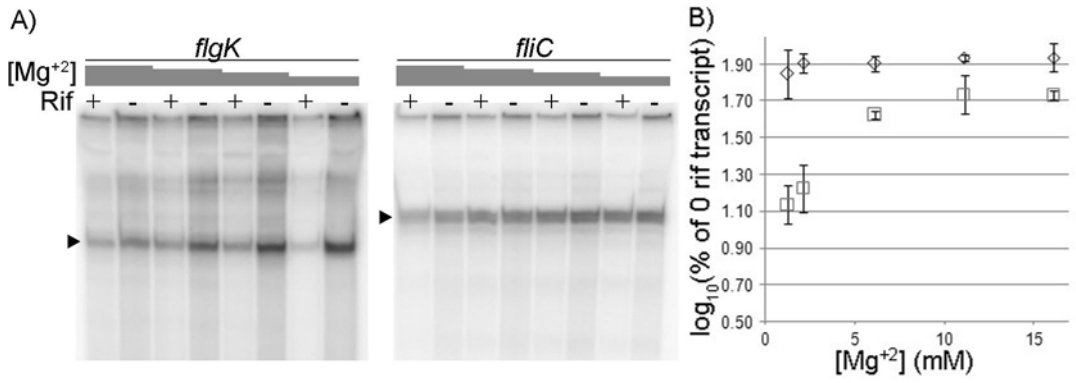

Figure 3.17. Mg^{2+} dependence of *flgK* and *fliC* transcription. A. Phosphorimages of *in vitro* transcription products from *flgK* and *fliC* promoters in the presence or absence of rifampicin (12.5 μg ml^{-1}) with decreasing amounts of Mg^{2+}. Transcripts were separated by denaturing polyacrylamide gel electrophoresis. B. *In vitro* transcription products were measured using ImageQuant 5.2 and are plotted as $\log_{10}$ of the percentage of the level seen in the no rifampicin transcript. Templates were *fliC* (diamond) and *flgK* (square). Error bars indicate standard deviation of transcript spot densities from at least three experiments.

Concentrations of initiating NTP (iNTP) have been shown to regulate transcription of rRNA in *B. subtilis* (Krasny & Gourse, 2004) and *E. coli* (Gaal *et al.*, 1997). Both NTP and rifampicin are present in the RNAP active site. The possibility that rifampicin mediated down-modulation might be affected by the concentration of NTPs was examined *in vitro*. Standard transcription assays contain 0.4 mM of all NTPs with the exception of the ^{32}P labelled NTP which is used at a lower concentration. To examine the effect of NTP, dilutions of a mixture of unlabelled nucleotide (ATP, CTP and UTP) were tested in *in vitro* transcription assays with or without rifampicin (Fig 3.18). GTP was not varied and remained at 5.1 μM (total concentration).

Decreasing NTP (ATP, CTP and UTP) concentrations in the transcription assay did not alter rifampicin sensitivity of *flgK* or *fliC* transcription. However, the overall transcription level from P_{flgK} declined sharply when the concentration of NTP was below 0.1 mM. The rifampicin

sensitivities of transcription from P_{invF} and P_{abrB} were also tested and were affected equally by lowering the NTP concentration and in both cases, overall transcription declined when the NTP concentration was below 0.025 mM (not shown). The varied minimal requirement for NTP may reflect the degree of stabilization given to the open complex by iNTPs. In *E.coli* rRNA promoters, high iNTP levels are required to stabilize open complexes. Higher iNTP levels result in greater proportions of stabilized open complexes and result in a corresponding increase in transcription (Gaal *et al.*, 1997). However, since rifampicin sensitive promoters were not preferentially affected by NTP levels, it suggested that NTP concentration was not a significant factor in rifampicin mediated down-modulation.

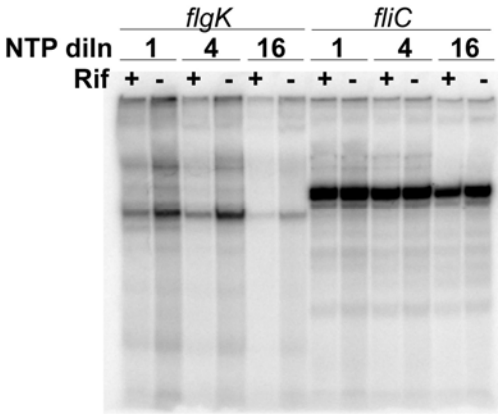

Figure 3.18. Effect of NTP concentration on *flgK* and *fliC* transcription and rifampicin inhibition. A phosphorimage of *in vitro* transcription products from *flgK* and *fliC* promoters in the presence or absence of rifampicin (12.5 μg ml^{-1}) and various NTP concentrations. The undiluted NTP concentration was 0.4 mM. Dilutions of 1:4 (4) and 1:16 (16) were tested. Transcripts were separated using denaturing polyacrylamide gel electrophoresis.

3.4 *In vitro* characterization of rifampicin up-modulated promoters.

Since rifampicin mediated down-modulation of *invF* and *flgK* could be observed *in vitro*, several rifampicin up-modulated promoters were examined *in vitro*. As was the rationale with the rifampicin down-modulated promoters, i*n vitro* transcription experiments were performed with varying amounts of rifampicin to test whether RNAP alone was sufficient to mediate rifampicin mediated up-modulation or if other factors might be required. Two promoters were examined *in vitro*, *traS* and *STM3595*.

Before *in vitro* transcription assays could be done, the transcription start sites for STM3595 and *traS* were determined. To map the transcription start site of RNA synthesized *in vitro*, primer extensions were conducted on the respective RNAs (Fig 3.19). Primer extension using the STM3595ent rev primer was also conducted on RNA harvested from cells. Analysis of $P_{STM3595}$ start transcription start sites suggested that transcription began at different nucleotides when initiated *in vitro* versus *in vivo*. As an additional method, the STM3595 *in vivo* transcription start site determined by 5' RACE. The start site was similar to the one determined by primer extension using RNA harvested from cells (Fig 3.10).

Since it appeared that the STM3595 transcription start used *in vitro* and *in vivo* differed, the DNA region containing the *in vitro* transcription start site was tested to determine whether it was functional *in vivo*. A 121 bp fragment containing the -10 and -35 elements corresponding to the *in vitro* transcription start site but not the *in vivo* start site was cloned into the *lux* reporter. The strain carrying this fusion, pGY41, showed little promoter activity and was not stimulated by

rifampicin (Fig. 3.9C). This suggested that the start site observed *in vitro* was not functional *in vivo*.

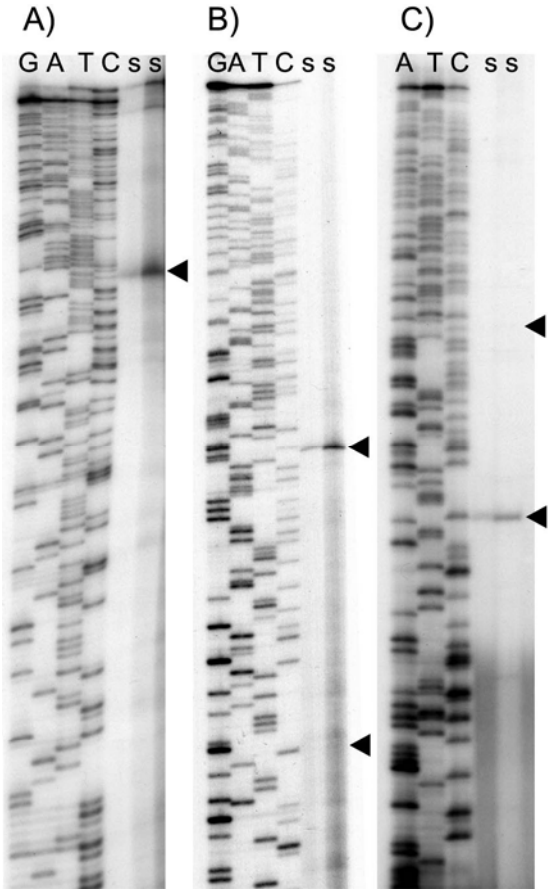

Figure 3.19. Primer extensions of *traS* and STM3595. Autoradiography of primer extension products of RNA obtained from *in vitro* transcription of *traS* (A), *in vitro* transcription of STM3595 (B) and STM3595 RNA harvested from cells (C). B,C. For reference, the upper arrows indicate the position of the start of the *in vitro* transcript and the lower the *in vivo* transcript. Sample lanes "s" were loaded with varying amounts of sample. Nucleotide sequences are shown in Fig 3.29.

Since the *tra*S transcription start had been previously determined in *E.coli*, the *S. typhimurium* +1 site of the *in vitro* P_{traS} transcript was determined (Fig 3.19). The *S. typhimurium* P_{traS} transcription start is 2 bp shifted from the transcription start found for the *E.coli* transcript (Ham *et al.*, 1989) (Fig 3.20). Although the +1 to -10 spacing is smaller than the usual 6 to 9 bp, there are other known promoters with the same spacing of 4 bp (Walker & Osuna, 2002). Since recognizable-10 and -35 elements are located appropriately with respect to the +1 found by primer extension, I assumed that this is the biologically relevant start site in *S. typhimurium*.

S. typhimurium ataaaacagaagcacTatCtcagaataataaaTATAATgaaGaaaagg

E. coli ataaaacagaagcaTTaACtcagaataataaaTATAATgaagaAaagc

-35 -10 +1

Figure 3.20. A comparison of *S. typhimurium* and *E. coli traS* transcription start sites. The *S. typhimurium* start site is labeled as determined in Fig. 3.19. An *E.coli* start site is shown for comparison (Ham *et al.*, 1989). -35 and -10 are underlined and are capitalized where nucleotides match the σ^{70} consensus. The transcription start site, +1, is capitalized.

In vitro transcriptions with templates containing P_{traS} and $P_{STM3595}$ did not show rifampicin mediated up-modulation (Fig. 3.21). In contrast to *invF* and *flgK*, in the absence of additional protein factors, *traS* transcription was quite strong (Fig 3.21). Although deletion analysis suggested that rifampicin mediated up-modulation from *traS* required DNA sequences either up-stream or downstream of the transcription start site (Fig 3.9A), a transcription factor required for activation of *traS* that binds upstream of the +1 or at any other location has not been identified.

Thus, there were no known candidates for protein factors that could be examined for rifampicin stimulation of *traS* transcription *in vitro.*

Transcription of STM3595, one of the most promoters most highly up-modulated by rifampicin in reporter assays, showed two *in vitro* products, neither of which increased in the presence of rifampicin (Fig 3.19, 3.21). Deletion studies had suggested that DNA downstream of the +1 of STM3595 RNA was required for rifampicin mediated up-modulation (Fig 3.9C), but this region of STM3595 had not been previously examined for transcription or elongation factor binding. There are no known candidates for proteins involved in rifampicin mediated up-modulation at this promoter. Since rifampicin mediated up-modulation could not be shown *in vitro*, these data suggested that RNAP alone was not sufficient for rifampicin mediated up-modulation.

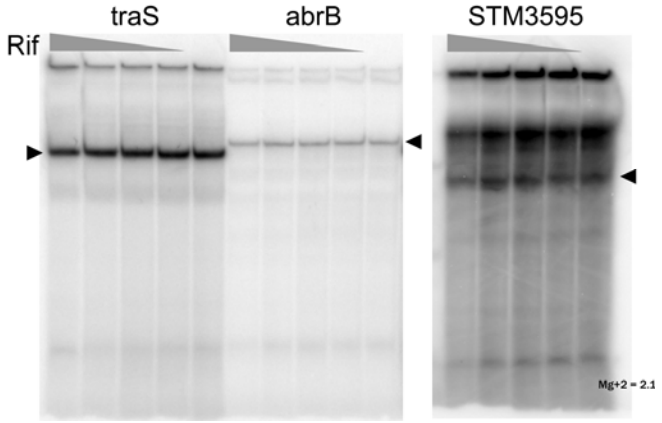

Figure 3.21. *In vitro* transcripts of *traS*, STM3595 and *abrB* in response to rifampicin. Phosphorimage of transcription products from *traS*, STM3595 and *abrB* promoters in the presence of varying amounts of rifampicin (12.5, 6.3, 3.1, 1.6 and 0 μg ml^{-1}). Transcripts are separated by denaturing polyacrylamide gel electrophoresis and arrows indicate transcript of interest.

3.5 Intracellular factors involved in rifampicin mediated up-modulation.

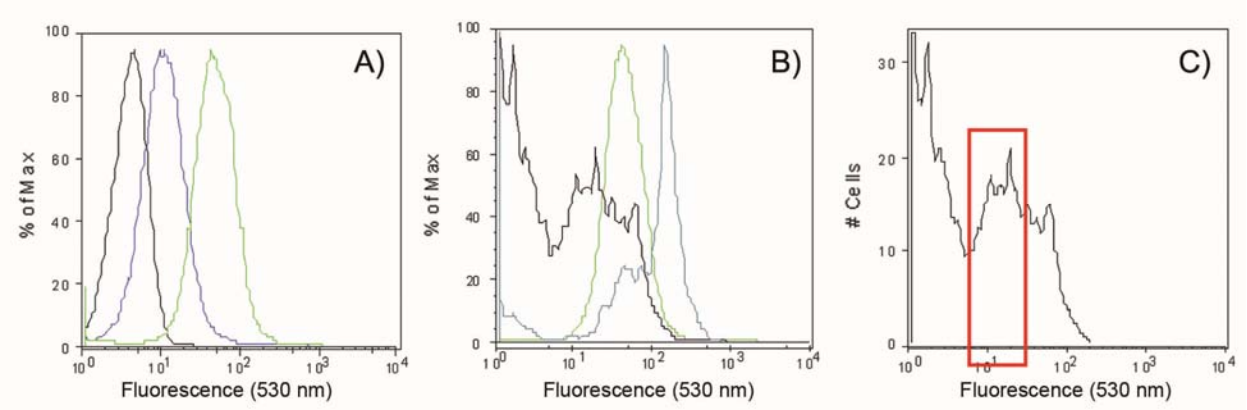

Figure 3.23. Fluorescence of 14028 *Tn*10*dTet* mutants. A. Fluorescence of *S. typhimurium* 14028 cells (black), 14028/pGY42 *Tn*10*dTet* mutants grown in the absence (blue) or presence (green) of rifampicin. B. Fluorescence of particles with fluorescence in the bottom 1% (black) or top 1% (blue) of the parent population grown in rifampicin (green). C. The trace of the bottom 1% shown in (B) with a red box representing the particles collected for a second sort.

Three lines of evidence suggested that an intracellular factor may be involved in rifampicin mediated up-modulation. First, the time course assays in section 3.1.4 showed a 2 to 3 hour lag between rifampicin addition and induction of expression. This lag may represent accumulation of a factor. Secondly, experiments with pretreated cells and spent media in section 3.1.5 showed that pretreated cells had higher promoter activity than untreated cells and spent media did not shorten the lag or increase the level of rifampicin induction. This suggested that a factor had accumulated in the cells, but not in the supernatant. Thirdly, rifampicin mediated up-modulation of transcription from STM3595 and *traS* templates could not be shown *in vitro* using only RNAP and the DNA template (section 3.4). As one approach to identify possible intracellular factor(s) involved, a screen was conducted to search for mutants that no longer displayed rifampicin mediated up-modulation of STM3595. Strain 14028 containing a STM3595::*gfp*mut3a reporter

plasmid (pGY42) was randomly mutagenized using *Tn*10*d*Tet. Cells were screened for mutants that were not up-modulated by rifampicin using fluorescence activated cell sorting (FACS) followed by screening with the Victor II plate reader.

Two different mutants were identified in the *Tn*10*d*Tet FACS screen (Fig. 3.22). Nucleotide sequencing of inverse PCR products using primer binding sites in *Tn*10*d*Tet (Fig. 2.2) showed that in the two mutants, the transposon had inserted into the *yciK* or *pcnB* genes, respectively (Fig. 3.23). The *yciK* gene encodes a putative oxidoreductase with no reported function. However, it may exist in the same transcriptional unit as *cobA* (Fig. 3.23A) and the transposon insertion could prevent transcription of *cobA*. CobA is known to be involved in de novo synthesis of cobalamin (vitamin B12). The gene for *pcnB* encodes poly(A) polymerase I. Little is known about this protein in *S. typhimurium*. In *E.coli*, poly (A) polymerase affects the rate of transcript degradation by causing 3' adenylation of transcripts. This facilitates the 3' to 5' exonucleolytic degradation of RNA with a structured 3' end (Urban & Vogel, 2008).

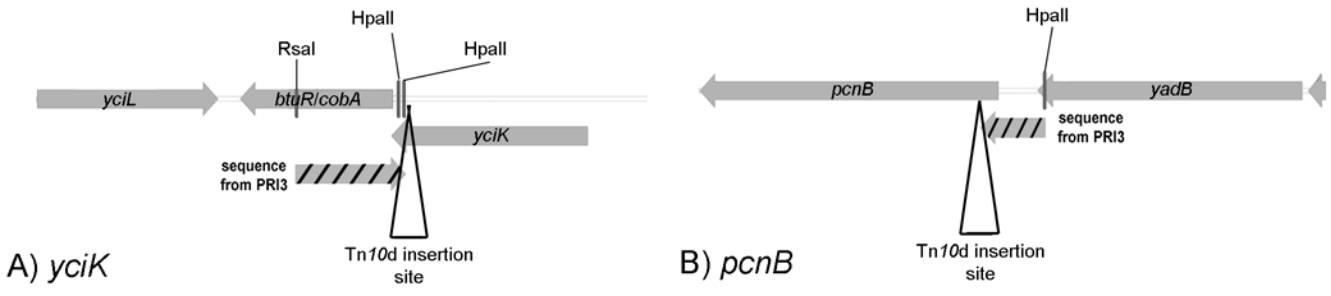

Figure 3.24. Schematic representation of *yciK* and *pcnB* insertions.

Since the *yciK* insertion reduced rifampicin mediated up-modulation of STM3595 (Fig 3.24A), the effect of this mutation was examined for the five other genes that showed either rifampicin mediated up-modulation or down-modulation. The insertion moderately affected transcription modulation by rifampicin in one other strain containing the *flgK* reporter fusion (pGY13) (Fig 3.25C). The other promoters, including the promoter for the control gene STM2901 (pGY21), had similar levels of rifampicin mediated up-modulation or down-modulation in the *yciK* mutant and the wild type backgrounds (Fig. 3.24, 3.25). For the most part, the *yciK* insertion only affected rifampicin mediated up-modulation of the STM3595.

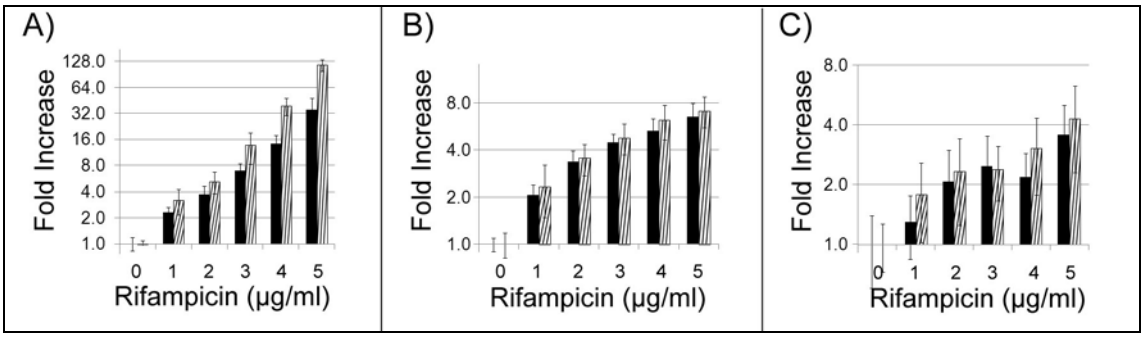

Figure 3.24. Luminescence of rifampicin up-modulated promoter reporter fusions in *yciK*::*Tn*10*d* and wildtype backgrounds. Luminescence of *yciK* mutants (black) or wildtype (grey) strains carrying STM3595 (pGY37, A), *traS* (pGY25, B) and *spvA* (pGY31, C) reporters. Cells were grown in LB supplemented with ampicillin and the indicated amount of rifampicin. Error bars indicate standard deviation of at least three experiments.

Since the *yciK* insertion may affect the levels of vitamin B12 in the cells, the role of vitamin B12 on rifampicin mediated up-modulation of STM3595 expression was examined in the *yciK*

mutant. Media for the *yciK* mutant and the wild type strains harboring the STM3595 *lux* reporter (pGY37) was supplemented with vitamin B12. Addition of vitamin B12 did not return rifampicin mediated up-modulation to levels observed in the wild type backgrounds (not shown). Therefore, it is unlikely that vitamin B12 is involved.

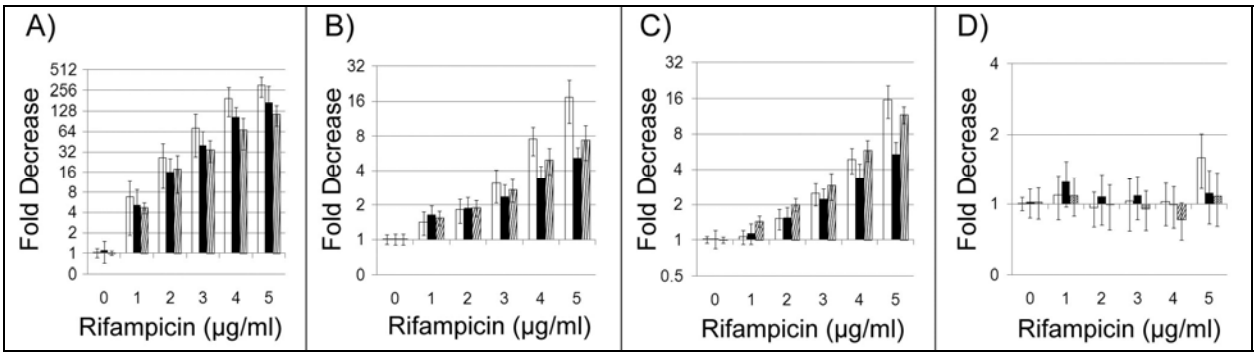

Figure 3.25. Luminescence of rifampicin down-modulated promoter reporter fusions in wildtype and mutant backgrounds. Luminescence of *pcnB* mutants (white), *yciK* mutants (black) or wildtype (grey) strains carrying *invF* (pGY17, A), *fliA* (pGY8, B), *flgK* (pGY13, C) and STM2901 (pGY21, D) reporters. Cells were grown in LB supplemented with ampicillin and the indicated amount of rifampicin. Error bars indicate standard deviation of at least three experiments.

The *pcnB* mutant showed increased sensitivity to growth inhibition by rifampicin compared to the wild type. Using a concentration of rifampicin slightly higher than the concentration used in reporter assays, growth of the mutant was noticeably more susceptible to growth inhibition by rifampicin compared to the wild type strain (Fig 3.26). When the effect of the *pcnB* mutation on the rifampicin response was examined in the other genes showing transcription modulation by rifampicin, the *pcnB* insertion mutants carrying reporter fusions to the *invF* and *fliA* promoters

showed more rifampicin mediated down-modulation than the wild type strains with the same reporter fusions (Fig. 3.25A, B).

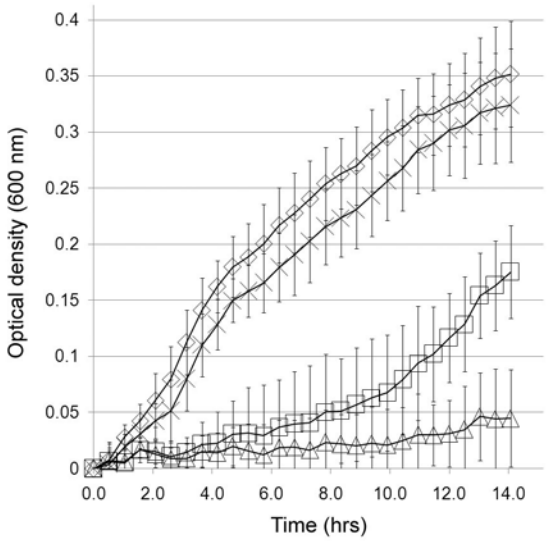

Figure 3.26. Growth curves of the wildtype and *pcnB* mutant strains in the presence or absence of rifampicin. Growth of 14028 (diamond, square) and the *pcnB* mutant (x, triangle) in the absence (diamond, x) or presence (square, triangle) of rifampicin (6 μg ml^{-1}). Error bars indicate standard deviation of at least three experiments. Cells were grown in LB supplemented with ampicillin and rifampicin as indicated.

3.6. Nucleotide sequence analysis of rifampicin modulated promoters.

3.6.1. Motif searches.

To search for common elements in the sequences of the rifampicin responsive promoters, *in silico* motif searches were conducted. Nucleotide sequences of down and up modulated promoters were examined for the presence of motifs that may be associated with transcription modulation by rifampicin. Three rifampicin down-modulated promoters, three rifampicin up-

modulated promoters and a set containing all six sequences were analyzed by the following algorithms with several settings for width, frequency, strand and palindromes: MEME (Bailey & Elkan, 1994), GLAM2 (Frith *et al.*, 2008) and Gibbs motif sampler (McCue *et al.*, 2001).

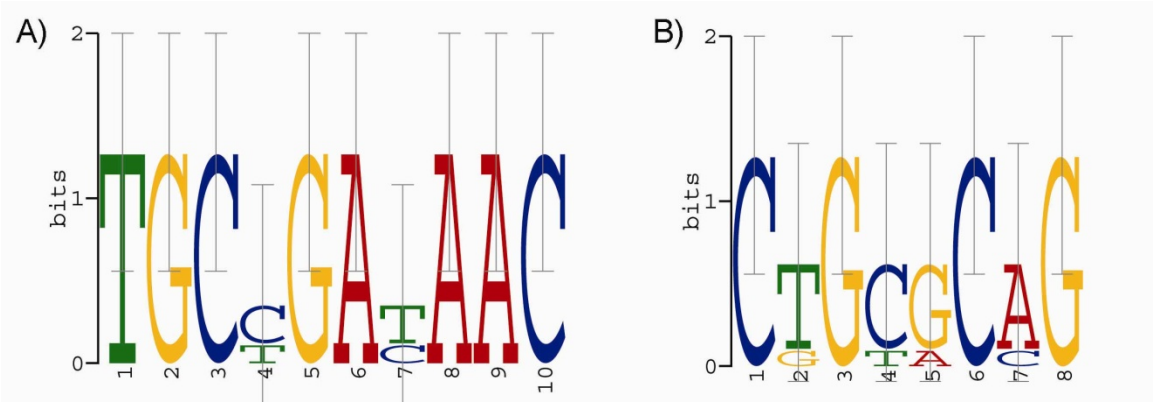

Figure 3.27. Motifs found in down- and up-modulated promoter nucleotide sequences. Motifs found in rifampicin down-modulated (A) and rifampicin up-modulated (B) promoters. See (Crooks *et al.*, 2004) for a description of sequence logos.

When examining the motifs produced by the three software programs, most motifs differed in relative position when input sequences were aligned by their transcription start site and were disregarded. However, when examining the rifampicin down-modulated promoter sequences, one motif (Fig. 3.27A) was identified by several of the analysis programs. This motif was essentially the σ^{28} -10 element with one extra nucleotide on the 5' and 3' end. The consensus sequence for *Salmonella* σ^{28} promoters is TAAA-N15-GCCGATAA (Kutsukake *et al.*, 1990). The σ^{28} -10 like element was in a similar position, centered at -32, in the *fliA* and *flgK* promoters (Fig 3.28) but 22 nucleotides downstream of the HilA-dependent +1 in *invF* (upstream of the

invF coding region). This raised the possibility that rifampicin mediated down-modulation observed was due to an effect on the σ^{28} holoenzyme. As a test of whether there was a σ^{28} dependent promoter on the *invF* template, *in vitro* transcription assays using the *invF* promoter as template were carried out adding increasing amounts of σ^{28} to the holoenzyme. The addition of several different concentrations of σ^{28} (3.0, 1.5 and 0.75 μM) to RNAP (75 nM), including amounts used for transcription of *flgK* transcription, yielded no transcripts from the *invF* template (data not shown). Therefore, it seemed unlikely that rifampicin mediated down-modulation from σ^{28} dependent promoters was a common feature of rifampicin down-modulated promoters.

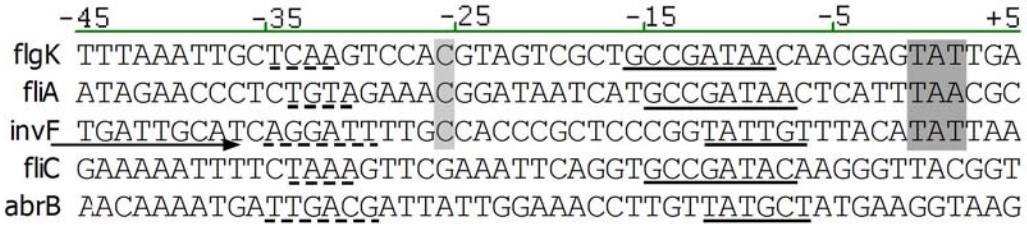

Figure 3.28. Nucleotide sequence alignment of *flgK, fliA* and *invF* promoters. Nucleotide sequences are numbered relative to the transcription start site (+1). -35 elements (broken lines), -10 elements (solid line), a partial HilA binding site (arrow) and similar sequences (shaded) are indicated.

In addition to the *in silico* analysis of rifampicin responsive promoters, a visual inspection for common nucleotide elements in sequences around the +1 was conducted. This region is relevant to rifampicin mode of action since rifampicin inhibits formation of RNA chains longer than 2 to

3 nucleotides long (Campbell *et al.*, 2001; McClure & Cech, 1978) and is proposed to inhibit formation of the phosphodiester bond between the third and fourth nucleotide (Artsimovitch *et al.*, 2005). Inspection showed four similar nucleotides -26C and TAW (where W represents T or A nucleotides) centered at +1 (Fig. 3.28, shaded); their significance is explored in section 3.6.2.

Figure 3.29. Nucleotide sequences of *STM3595, traS* and *spvA*. Alignments are numbered relative to the transcription start site (+1). Transcription start sites for *traS* (primer extension using *in vitro* RNA) , STM3595 (primer extension and 5' RACE using RNA harvested from

cells) and *spvA* (Sheehan & Dorman, 1998) are indicated. Conserved residues are shown in yellow. A palindromic motif is shown in grey.

In silico analysis of the rifampicin up-modulated promoters revealed no sequence motifs in similar positions in all three sequences. However, one palindrome (Fig. 3.27B) was found in a similar position in STM3595 and *traS* and in a different position in *spvA* (Fig. 3.29). This could be a biologically relevant motif considering deletion studies and the *E.coli traS* transcription start site. Deletion suggested that sequence downstream to the +1 was important for rifampicin mediated up-modulation of STM3595 and *traS* (Fig 3.8). Secondly, the *in vitro* transcription start site determined for *traS* may not reflect the *in vivo* transcription start site. Since high quality RNA is more readily isolated *in vitro* in comparison to RNA isolated from cells, the +1 site of *traS* transcription was determined for RNA synthesized *in vitro* (Fig 3.19). However, the *E. coli* start site identified from RNA harvested from cultured cells (Ham *et al.*, 1989) is shifted 2 bp downstream from the *in vitro S. typhimurium* start site (Fig 3.20). It seems possible that the *S. typhimurium* and *E.coli* start sites are identical when RNA is harvested from cells. As such, the palindrome in STM3595 and *traS* would be in an identical position in both sequences, four bps downstream of the +1.

3.6.2. Preliminary exploration of nucleotide sequence motifs.

To examine the biological significance of the four similar nucleotides found in the rifampicin down-modulated promoters (Fig. 3.28), they were mutated and the respective reporter fusions assayed for luminescence responses to rifampicin. The following base changes were -26C to A, -1+2TAW to GCC or both sets of mutations were made in *flgK*, *fliA* and *invF* promoter fusions

(Table 3.1). Due to cloning constraints resulting from employing a 9.4 kb plasmid, a mutant reporter with both sets of mutations in the *invF* sequence was not constructed.

Table 3.1. Nucleotide alterations of common nucleotides in rifampicin down-modulated promoters and the resulting reporter plasmids.

Promoter	flgK			fliA P2			invF	
Plasmid	pGY47	pGY48	pGY49	pGY44	pGY45	pGY46	pGY50	pGY51
Original nucleotides	C	TAT	C TAT	C	TAA	C TAA	C	TAT
Base position of alteration(s)	-26	-1,+1,+2	-26 -1, +1,+2	-26	-1,+1,+2	-26 -1,+1,+2	-26	-1,+1,+2
Replacement nucleotide(s)	A	GCC	A GCC	A	GCC	A GCC	A	GCC
Parent plasmid	pGY15	pGY15	pGY15	pGY11	pGY11	pGY11	pGY18	pGY19
Fold decrease in basal expression compared to wild type*	1.1 ±0.1	3.2± 0.2	4.4± 0.4	1.01± 0.08	7.4± 0.6	8.0± 0.8	0.30± 0.04	3.7± 0.3

*corresponds to luminescence of wild type divided by luminescence of mutant grown in 0 μg/ml of rifampicin as seen in Figure 3.30

The TAW nucleotides were important for promoter activity from all three promoters, whereas -26C was only important for one. For all three promoters, *flgK*, *fliA* P2 and *inv*F, alterations from TAW to GCC significantly reduced their basal activity (Table 3.1, Fig. 3.30, no rifampicin) suggesting that the -1,+1 and +2 nucleotides are significant for promoter strength. When examining the *flgK* and *fliA* P2 reporters, -26 C to A alterations did not affect basal activity or rifampicin hypersensitivity (Table 3.1, Fig. 3.30) indicating that -26C plays no role in rifampicin mediated down-modulation. In contrast, changing -26 C to A in the *invF* promoter increased basal activity (Table 3.1, Fig 3.30C,D). In cases where both sets of mutations were made (*fliA*

and *flgK* reporters), activity was similar to the mutants with only the TAW base changes (Fig. 3.30A, B diamond and x) having reduced basal activity when compared to the wild type. Again this suggested that positions -1 to +2 but not position -26 are important for rifampicin mediated down-modulation. Since mutations at position -1 to +2 reduced basal activity and rifampicin at 5 μg ml^{-1} reduced transcription of the reporters strains to the lower limit of detection in this assay, the sensitivity of transcription to rifampicin is reduced in the mutants when compared to the wild type reporter (Fig 3.30). The -1+1+2 positions of the *flgK*, *fliA* P2 and *invF* promoters are important for promoter activity and may be involved in rifampicin hypersensitivity in these cases.

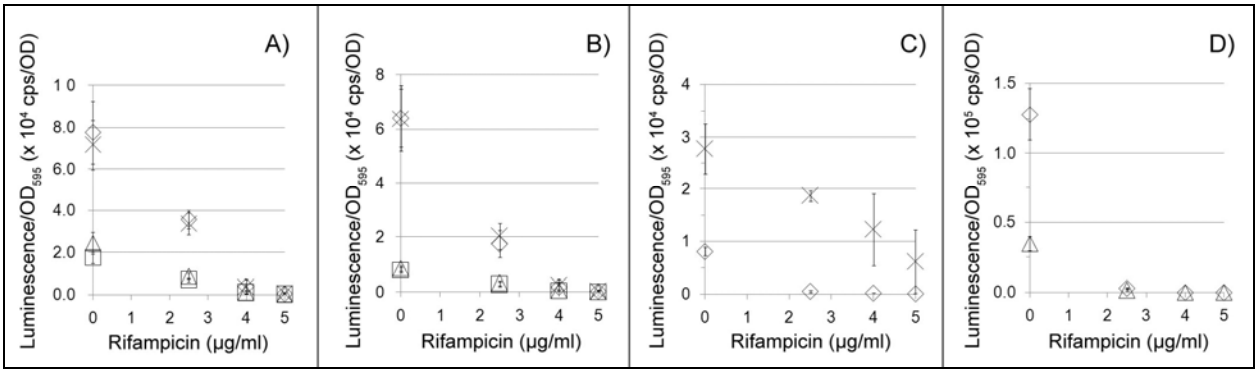

Figure 3.30. Luminescence values from mutated fusion reporters grown in the presence of varying amounts of rifampicin. Luminescence at 6 hrs of 14028 carrying A) pGY15 (diamond), B) pGY11 (diamond), or derivatives thereof with -26C to A alterations (x), with -1+2 TAW alterations to GCC (triangle) or both alterations (square). Luminescence at six hours of 14028 carrying C) pGY19 (diamond), or a derivative thereof with a -26C to A alteration (x) or D) pGY18 (diamond), or a derivative thereof with a -1+2 TAT alteration to GCC (triangle). Each symbol represents one culture condition with growth in the indicated amount of rifampicin; error bars indicate standard deviation of least three experiments.

Chapter 4. Discussion.

In *S. typhimurium* strain 14028, sub-MIC rifampicin modulated expression of at least 22 genes from 3 to 200-fold (Table1.1). Among the down-regulated genes were ones related to motility and host cell invasion. Some of the up-regulated genes encoded proteins of unknown function, while others encoded proteins related to carbon metabolism and virulence. This thesis examines the mechanisms by which sub-MIC rifampicin modulates bacterial transcription from six of these promoters (P_{fliA}, P_{flgK}, P_{invF}, P_{spvA}, $P_{STM3595}$ and P_{traS}).

Since the mode of action of rifampicin has been studied in detail (Campbell *et al.*, 2001; Floss & Yu, 2005), the simple hypothesis for the effect of sub-MIC rifampicin would be a general limitation of transcription capacity. However, the effect of sub-MIC rifampicin on transcription presented a paradox: rifampicin not only specifically down-modulated certain promoters but also up-modulated transcription for other promoters. Three promoters P_{flgK}, P_{fliA} and P_{invF} that were down-modulated by rifampicin were studied in this thesis. DNA deletion studies of P_{fliA} and P_{flgK} showed that ~50 bp fragments containing the σ^{28}-dependent promoters were sufficient to observe rifampicin mediated down-modulation in culture. For P_{invF}, a promoter specific activator binding site was required for down-modulation. In each case, transcription from the promoters showed increased rifampicin sensitivity *in vitro* relative to the controls which may reflect the down-modulation of these promoters by rifampicin when grown in culture. Since the magnitude of rifampicin mediated down-modulation observed *in vitro* was not equivalent to the effect seen in culture, an additional mechanism may contribute to down-modulation. The other three promoters examined are up-modulated by rifampicin in culture: $P_{STM3595}$, P_{spvA} and P_{traS}. Evidence presented suggested that up-modulation may be mediated by an intracellular, but not an

extracellular factor and, in two of the three promoters, nucleotide sequences downstream of the transcription start sites were required.

Below, possible mechanisms of up-modulation are discussed. Since nucleotides downstream of the transcription start site are involved with up-modulation, particular attention is paid to mechanisms relating to this region such as transcription attenuation. Lastly, the biological relevance and applications for drug discovery and therapeutics are presented.

4.1. Mechanisms of rifampicin mediated down-modulation at RNA polymerase.

As mentioned in the Introduction, in *E. coli* five DNA elements contribute to promoter recognition by RNAP:σ^{70}: the -10 hexamer, -35 hexamer, extended -10, UP element and discriminator. For the most part, detailed mechanistic and kinetic studies of transcription initiation have been only conducted in *E. coli* as a model Gram-negative organism and *B. subtilis* as a model Gram-positive organism. Since *E. coli* and *S. typhimurium* RNAP are 99% identical in amino acid sequence, promoter recognition presumably works in a similar manner. Limited studies of transcription initiation has been conducted using alternate sigma factors such as σ^{S}, the stationary phase sigma factor and σ^{32}, the heat shock sigma factor. There are a few investigations of transcription mechanism for RNAP containing σ^{28} (flagellar). -10 and -35 elements have only been identified for the *E. coli* and *S. typhimurium* σ^{28}, TAAAGTTT (-35 element) and GCCGATAA (-10 element) (Ide *et al.*, 1999).

Transcription has been functionally separated into three parts: initiation, elongation and termination. Most regulation of transcription occurs at the level of initiation. Transcription

factors will often activate or repress transcription by affecting the rate by which RNAP:DNA performs certain isomerizations during transcription initiation. These isomerizations (closed, open, initiated or elongating complex, Figure 1.3) involve rearrangements of RNAP and DNA. A closed complex is characterized by specific binding of RNAP to double stranded promoter DNA. The open complex is formed by melting of the double stranded DNA. Melting of DNA will nucleate in the -10 region of the promoter and extend from –11 to +3 while the RNAP footprint extends to +20 (Haugen *et al.*, 2008). Open complex transitions to the initiated complex as the NTPs are polymerized. Once a 7 – 12 nucleotide transcript forms, σ is released and RNAP forms a processive elongating complex. If an elongating complex is not formed, the abortive transcript is released and RNAP returns to an open complex (Record *et al.*, 1996).

Some possibilities for the mechanism of down-modulation were that sub-MIC rifampicin slows growth, causes a stress response or that minor changes in growth rate may have a differential effect on some promoters. Alternatively, rifampicin may have specific effects at certain promoter-RNAP complexes. Using a bacterial *lux*-reporter system, RT-PCR and *in vitro* techniques, the work described in this thesis attempted to test various aspects of transcription and to characterize the mechanism by which sub-MIC rifampicin modulates transcription of certain *S. typhimurium* promoters.

Sub-cloning, testing of promoter fragments and *in vitro* transcription experiments described in this thesis suggested that rifampicin mediated down-modulation occurs directly at the level of RNAP-promoter interaction. Sub-cloning studies showed that ~50 bp fragments containing only the minimum promoter elements (-35, -10 and +1) of σ^{28}-dependent P_{flgK} and P_{fliA} were

sufficient for down-modulation by rifampicin, suggesting that no upstream trans-acting factors were necessary for down-modulation at these promoters (Figure 3.7). Furthermore, HilA was required for P_{invF} down-modulation by rifampicin, but this may just reflect the technical problem of having sufficient transcripts to see inhibition. Thus, it seemed unlikely that HilA was a general element of rifampicin mediated down-modulation at P_{invF}, P_{flgK} and P_{fliA}.

It is possible that different holoenzymes might be differentially sensitive to rifampicin. It has previously been reported for one pair of promoters that RNAP-σ^{70} (P_L promoter) is less sensitive to rifampicin than RNAP-σ^{32} (P_{groE} promoter) (Wegrzyn *et al.*, 1998). However, *in vivo* and *in vitro* transcription experiments in this thesis showed that differential rifampicin sensitivity occurred for both RNAP-σ^{70} transcription from P_{invF} and RNAP-σ^{28} transcription from P_{flgK} (section 3.3.3). Transcription by the two forms of polymerase holoenzymes from the control promoters (P_{abrB} and P_{fliC}) were similarly sensitive to rifampicin, further indicating that, under the conditions used, rifampicin hypersensitivity was not due to the sigma factor associated with the holoenzyme. Thus, it seems that hypersensitivity to rifampicin inhibition results from differences in specific interactions between RNAP and rifampicin down-modulated promoters.

Based on recent literature on mechanisms of transcription initiation, I explored two reaction conditions for *in vitro* assays, varied nucleotide and Mg^{2+} concentrations. These two factors are linked as there is an active site Mg^{2+} in RNAP and NTPs are the substrates of RNAP. Furthermore, during the course of this work a study appeared suggesting that rifampicin inhibition was affected by Mg^{2+} (Artsimovitch *et al.*, 2005). The rifampicin sensitivity of transcription from the flagellar promoter, P_{flgK}, displayed Mg^{2+} dependence (Fig. 3.17) but

transcription from P_{invF} did not. While this is counter to the notion of a single mechanism for rifampicin hypersensitivity, it leaves open the possibility that the effect of Mg^{2+} on rifampicin action might be promoter dependent. Altering NTP concentrations had no differential effect on rifampicin inhibition *in vitro* when compared to control promoters. While no role for Mg^{2+} in rifampicin mediated down-modulation was uncovered, it was clear that rifampicin hypersensitivity of transcription *in vitro* was similar to the rifampicin mediated down-modulation observed in culture. This implies that DNA:RNAP interactions modulate rifampicin binding and in turn that small molecules such as antibiotics can directly modulate transcription.

Transcription from P_{flgK} followed the previously suggested hypothesis that rifampicin inhibition of transcription acts by affecting binding of Mg^{2+} to the RNAP active site (Artsimovitch *et al.*, 2005). A more recent study by Feklistov *et al.* (2008) challenged the relationship between rifampicin and Mg^{2+} found by Artsimovitch *et al*., tested several assertions made in 2005 and found them false. However, Feklistov *et al.* tested only one promoter. In the work presented here, it appeared that one of the four promoters tested, the *flgK* promoter but not the *abrB*, *invF* or *fliC* promoters, showed Mg^{2+} dependent rifampicin sensitivity (Fig 3.17). Since rifampicin binds to the RNAP and the same enzyme was used in all tests, one might expect that all of the promoters would display the same rifampicin sensitivity, or at least responses correlating to the sigma factor used. These findings suggest there may be unknown effects involved that could explain rifampicin mediated down-modulation. As with all *in vitro* experiments, buffer conditions or order of additions may contribute to the magnitude of Mg^{2+} dependent rifampicin sensitivity and may partially explain differences observed here and in other studies. Studies using alternative techniques to measure transcription initiation (Mekler *et al.*, 2011) may need to

be employed to elucidate the role of Mg^{2+} in rifampicin inhibition of transcription initiation and certainly a wider range of promoters needs to be investigated.

4.1.1. The role of +1 region in transcription initiation.

In some promoters, such as rRNA promoters, the +1 region and the iNTP, has been shown to be important for regulating transcription initiation. The iNTP is the first nucleotide of the transcript and base pairs at the +1 base of the template strand at the promoter. At the *rrn* promoters, the open complex is highly unstable, decreasing the possibility of transcription initiation. Therefore, efficient transcription will only occur when there are high concentrations of iNTP. When NTP pools are high, such as when the cell actively growing, the iNTP will base pair to the +1 nucleotide on the template strand of DNA, stabilize the open complex and increase the likelihood of transcription initiation (Gaal *et al.*, 1997; Krasny & Gourse, 2004).

Data for consensus nucleotides in the +1 region of *E. coli* promoters shows a very weak nucleotide bias. Recently, a modified high throughput 5' RACE protocol was used to determine approximately 1700 transcription start sites in *E.coli* (Mendoza-Vargas *et al.*, 2009). The bases AAT seem to be weakly preferred at positions +1 to +3 (Fig 4.1). The information content of the motif is less than 0.2 bits at any position (Fig 4.1), 2 being the highest bits per position possible. Higher binding site conservation corresponds to higher information content (measured in bits). Typical information content of highly conserved positions in transcription factor binding sites, such as the CAP binding site, is usually between 1 and 2 bits (Crooks *et al.*, 2004; Schneider *et al.*, 1986). When the +1 to +3 motif of *E.coli* promoters compared to the TAW (Fig 3.28) consensus sequence at -1 to +2 of the rifampicin down-modulated promoters studied here, there

is one difference. The -1 of the rifampicin down-modulated promoters has a T bias where no bias exists in *E. coli* promoters. This base difference may reflect some characteristic of rifampicin down-modulated promoters that renders them hypersensitive to rifampicin inhibition.

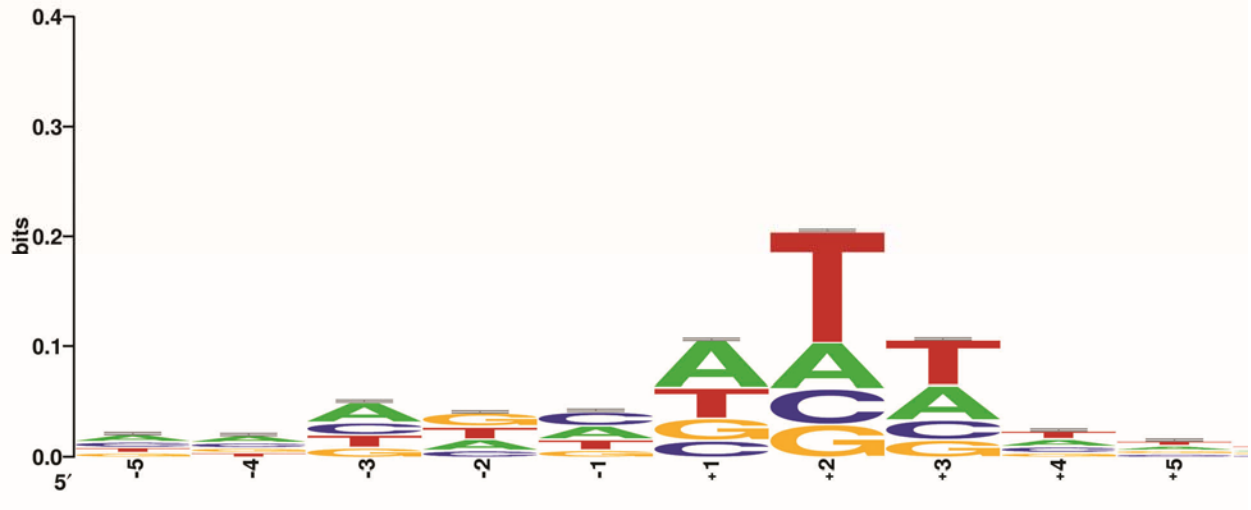

Figure 4.1. Sequence logo of the +1 region of *E. coli* promoters. Data was obtained by a high throughput pyrosequencing strategy and compiled by Mendoza-Vargas *et al.*(2009). Their data was used to create a sequence logo using WebLogo software (Crooks *et al.*, 2004).

4.1.2. A working model of rifampicin mediated down-modulation.

A working model for rifampicin mediated down-modulation might involve the +1 region of the rifampicin down-modulated promoters. The accepted model for inhibition of transcription by rifampicin is that its binding to RNAP sterically inhibits extension of the RNA chain when it is 2-3 nucleotides long (Campbell *et al.*, 2001; Feklistov *et al.*, 2008; McClure & Cech, 1978). Elongating RNAP:DNA complexes are resistant to rifampicin (Carpousis & Gralla, 1985). When the elongating complex has formed, the nascent RNA chain presumably blocks binding of

rifampicin and subsequent inhibition. Certain DNA sequences of the rifampicin down-modulated promoters such as the TAW consensus may render rifampicin down-modulated promoter:RNAP complexes more sensitive to inhibition by rifampicin. This may involve rifampicin down-modulated promoter:RNAP complexes which form initiated complexes more slowly or have altered structures compared to typical promoters. This would allow more time for rifampicin to affect transcription initiation causing a higher proportion of the DNA:RNAP complexes at those particular promoters to be inhibited by rifampicin, would decrease the rate of transcription initiation and cause down-modulation of transcription.

4.2. Other possible mechanisms of rifampicin mediated down-modulation.

The levels of rifampicin mediated down-modulation observed *in vitro* were lower than the responses seen in culture. It seems possible that a secondary mechanism exists which potentiates rifampicin mediated down-modulation in culture in addition to the rifampicin hypersensitivity of rifampicin down-modulated promoters. Several mechanisms which may act in addition to rifampicin hypersensitivity (discussed in section 4.1) are presented below. This includes the role of poly(A) polymerase (*pcnB* insertion mutant identified by FACS) and several *Salmonella* regulators (known regulators of invasion and motility) in rifampicin mediated down-modulation, the activator HilA being the most promising candidate.

FACS screening identified a mutant with an insertion in *pcnB* that had increased rifampicin mediated down-modulation relative to the wildtype strain (Fig. 3.25). In *E.coli*, *pcnB* encodes the enzyme poly(A) polymerase which adenylates specific transcripts at their 3' end, destabilizing them; consequently, *pcnB* mutants usually have increased levels of the respective

transcript when compared to wildtype (Urban & Vogel, 2008). A direct effect of poly(A) polymerase seems unlikely to explain rifampicin mediated down-modulation since expression of the reporter construct was decreased in *pcnB* mutants. However, transcripts that are decreased in *pcnB* mutants are indirectly regulated by poly(A) polymerase. In these cases, poly(A) polymerase destabilizes the transcript of a repressor for these transcripts which increases the amount of gene of interest transcript (Urban & Vogel, 2008). A similar phenomenon may be occurring at rifampicin down-modulated promoters.

The *Salmonella pcnB* mutant defective in rifampicin mediated up-modulation of STM3595 that was isolated by FACS displayed increased susceptibility to growth inhibition by rifampicin (Fig 3.26). The increased susceptibility to rifampicin may reflect a general role of poly(A) polymerase in *Salmonella* physiology. Since rifampicin resistant mutants no longer display down-modulation, perhaps the opposite, mutants which are more susceptible to rifampicin mediated growth inhibition displaying stronger transcription down-modulation, is not surprising.

Other *Salmonella* regulators such as PhoP and SirA were tested for their involvement in rifampicin mediated down-modulation by rifampicin. The BarA/SirA two component system has been shown to negatively influence motility and invasion in *Salmonella* (Teplitski *et al.*, 2003). Rifampicin also down-modulates expression of invasion and motility genes. Reporter fusions genes that showed rifampicin mediated down-modulation (*invF* and *sopB*, Table 1.1) known to be positively regulated by SirA, had a lower basal level expression in the *sirA* mutant but were still down-modulated by rifampicin (not shown). The PhoPQ system negatively regulates invasion genes (Teplitski *et al.*, 2003). In *Salmonella*, PhoPQ is a two component

system regulated by magnesium ion concentrations, acidic pH and cationic peptides. PhoPQ is important for *Salmonella* survival in host cells, resistance to antimicrobial peptides and acid pH (Song *et al.*, 2008). Promoter-reporter fusions carried in a *phoP*::*cam* mutant showed similar patterns of down-modulation as the same reporter fusions carried by the isogenic parent (not shown). SirA and PhoP are regulators of individual rifampicin down-modulated promoters, but are unlikely to be primary determinants of rifampicin mediated down-modulation.

The effect of rifampicin on some rifampicin down-modulated genes may be indirect, for example, involving HilA. Of the promoters examined in this thesis, this would only include P_{invF}. Deletion studies using *invF* promoter-reporter fusions suggested that rifampicin mediated down-modulation required the HilA binding site (Figure 3.8). HilA protein was required to observe rifampicin mediated down-modulation from *in vitro* transcripts (section 3.3) but this could have just reflected the technical problem of having sufficient transcripts to detect inhibition. Several genes (not examined in this thesis) such as *sopA*, *sopB*, and *siiA*, that are down-modulated by rifampicin (Table 1.1) are known to be HilA regulated (Thijs *et al.*, 2007). Repression of the HilA regulon is mediated by Hha (Teplitski *et al.*, 2003). Lux expression in B9(2), a *S. typhimurium* reporter strain containing the *lux* genes fused to the *hha* promoter region, was up-modulated by rifampicin (Figure 4.2). Thus, it is possible that rifampicin mediated up-modulation of *hha* may lead to down-modulation of HilA and consequent repression of *invF*, *sopA*, *sopB* and *siiA*. Strain B9(2) was initially isolated in a preliminary screen of a partial *S. typhimurium lux* reporter library that was discarded (Goh, unpublished). The original 6528-clone library used in the second large screen for rifampicin responsive reporters, from which the fusions used in this thesis were obtained, had approximately 2.7 fold

coverage (Bjarnason *et al.*, 2003). This is likely not saturating. This may explain why a *hha* reporter was not re-isolated and thus not included in Table 1.1.

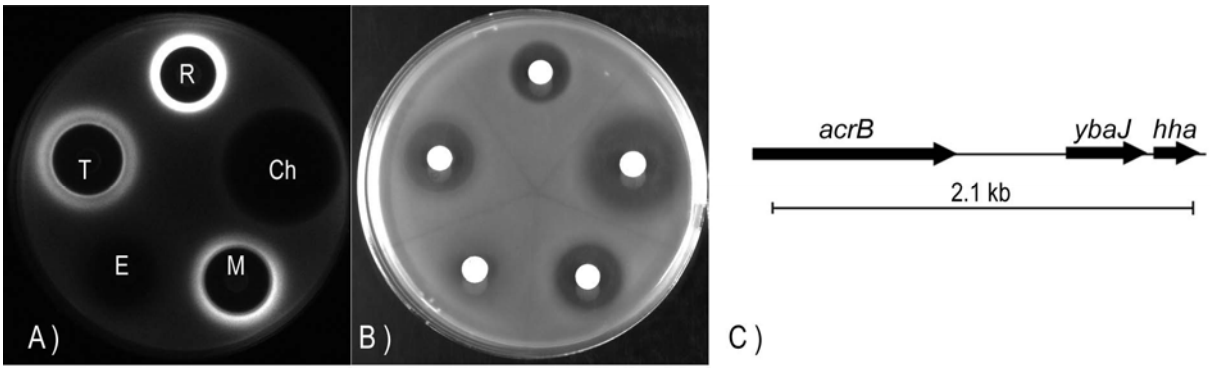

Figure 4.2. A disk diffusion assay of the putative *S. typhimurium* 14028 *hha lux* reporter strain B9(2). Luminescence (A) and growth inhibition (B) in the presence of various antibiotics are shown. Paper discs containing rifampicin (R, 30 μg), chloramphenicol (Ch, 30 μg), minocycline (M, 10 μg), erythromycin (E, 15 μg) or tetracycline (T, 10 μg) are placed on top on the overlay. C) The line labeled 2.1 kb indicates the *Sau*3A fragment cloned into the *lux* reporter plasmid carried by strain B9(2).

4.3. Possible mechanisms of rifampicin mediated up-modulation.

Transcription of the rifampicin up-modulated promoters was not stimulated by rifampicin *in vitro* (section 3.4). Growth studies, testing of truncated promoter fragments, and *in vitro* transcription experiments suggested that rifampicin mediated up-modulation may involve factors other than RNAP. Time course (section 3.1.4) and spent media (section 3.1.5) experiments suggested that these factors, if they exist, accumulated intracellularly because a 2-3 hour lag existed between rifampicin addition and up-modulation. Since spent media from rifampicin treated cells did not increase the magnitude or speed of rifampicin mediated up-modulation, this unidentified factor must not be a stable secreted factor. DNA mapping studies (section 3.2)

suggested that rifampicin mediated up-modulation of the three promoters studied may involve at least two discrete mechanisms since DNA upstream and downstream of the core promoter elements (-35, -10, +1) were required for rifampicin mediated up-modulation in different cases. In the case of STM3595 and *traS*, DNA downstream of the +1 was needed for up-modulation. Nucleotide sequences upstream of the -35 element at the *spvA* and *traS* promoters affected rifampicin mediated up-modulation. These sequences are unlikely to interact directly with RNAP at initiation, suggesting that other intracellular factors interact with these upstream or downstream DNA sequences. Motif searches using DNA sequences from the three up-modulated promoters yielded only one consensus sequence, CTGCGCAG (Fig 3.27B). This sequence is a palindrome and an exact match is present in the downstream region of STM3595, with one mismatch in the downstream region of *traS* (sites are shifted two bp) and with one mismatch in the upstream region of *spvA* (Fig. 3.29). However, it difficult to hypothesize why this motif would be present in two different locations. *In vitro* transcription studies were consistent with the need for additional factors inasmuch as RNAP and DNA alone were not sufficient to demonstrate rifampicin mediated up-modulation *in vitro* (section 3.4).

A *Tn*10*d* mutant that was defective in rifampicin mediated up-modulation for all promoters tested was not found, this may be because that such a mutant(s) is lethal, a protein factor is not involved or a more extensive search is required. Transcription factors that are essential in *E. coli* and are presumably essential in *Salmonella* include elongation factors such as NusA, NusG or ρ (Cardinale *et al.*, 2008). GreA and GreB are not essential in *E. coli* but are essential for organisms such as *Mycoplasma pneumonia* (Stepanova *et al.*, 2007).

4.3.1. Known regulatory factors which bind RNA polymerase.

A strong consensus DNA binding site may not exist in the sequence of the rifampicin up-modulated promoters if the protein or small molecule factor which mediates this effect binds RNAP and not DNA. Two known regulatory factors which bind RNAP are DksA and guanosine tetraphosphate (ppGpp). DskA is a transcription factor, like GreA and GreB, which binds the RNAP secondary channel (Paul *et al.*, 2004). ppGpp is a small molecule important for stringent response, a bacterial program responsible for decreasing rRNA synthesis and consequently ribosome production when the cell undergoes stress conditions including amino acid starvation (Srivatsan & Wang, 2008). Recent studies have elucidated the mechanism of action of ppGpp at promoters involved in cell physiology as well as promoters related to virulence. In conjunction with DskA, ppGpp directly inhibits rRNA promoters by reducing the half life of open complexes. ppGpp also potentiates the expression of genes involved in amino acid biosynthesis by enhancing an isomerization step leading to open complex formation (Paul *et al.*, 2005). Upon nutrient starvation or entry into stationary phase, ppGpp promotes expression of two regulators of enterohaemorrhagic *E. coli* virulence, Ler and Pch (Nakanishi *et al.*, 2006). In addition, DksA and Hfq (a RNA chaperone and post-transcriptional regulator) play a role in intercellular spread of *Shigella flexneri* in epithelial monolayers (Sharma & Payne, 2006).

In *E.coli*, two enzymes synthesize ppGpp, RelA and SpoT. A microarray analysis of a Δ*relA*Δ*spoT* mutant of *Salmonella* and the isogenic parent strain found ppGpp regulates both the invasion and intracellular virulence programs (Thompson *et al.*, 2006). Rifampicin appears to down-modulate genes involved in invasion and up-modulate other virulence genes, including the *spvRAB* operon (Table 1.1). Transcription modulation in response to rifampicin differs from the

response to ppGpp, which up-regulates both *spvRAB* and *invF* (Pizarro-Cerda & Tedin, 2004). Although rifampicin and ppGpp seem to regulate the same groups of *Salmonella* genes, the gene expression patterns are different, suggesting that ppGpp is not involved in transcription modulation by rifampicin.

4.3.2. Elongation and termination factors.

The fact that regions downstream of the +1 were required for rifampicin mediated up-modulation of *traS* and STM3595 suggested that, at these promoters, rifampicin may act after transcription initiation. Rifampicin mediated up-modulation may involve regulation of transcription elongation, attenuation, termination or anti-termination. Elongation factors in *E. coli* include GreA, GreB, NusA, NusG; termination factors include Mfd and ρ, while RfaH is an anti-termination factor related to the regulation of virulence genes (Roberts *et al.*, 2008). Some studies have examined expression patterns in elongation factor deficient or over-expressing mutants. In many cases, these factors bind directly to a subunit of RNAP and have no known preference for DNA sequence. The evidence for each of these factors as mediators of rifampicin mediated up-modulation is discussed below.

An *E. coli* microarray study employing Δ*greA*, Δ*greB*, Δ*greA*Δ*greB* and *greA* overexpressing strains analyzed the genes affected by these mutations (Stepanova *et al.*, 2007). I compared the *S. typhimurium* genes up-modulated by rifampicin to the transcription patterns in elongation factor mutants. The only GreA regulated gene found by Stepanova *et al.* (2007) that was also affected by rifampicin was *talA*. Transcription of *talA* is positively regulated by rifampicin and negatively regulated by GreA (Stepanova *et al.*, 2007). Overall, the lack of correspondence

between genes subject to transcription modulation by rifampicin and loss of GreA and GreB is inconclusive. Complicating the analysis is the observation that many genes modulated by rifampicin in *Salmonella* are related to virulence and are not found in *E. coli*.

The functions of *E. coli* NusA, NusB, NusE and NusG proteins are quite complex and are seemingly contradictory as they have been shown to be involved in either or both anti-termination and termination. Antitermination complexes are multi-component RNAP elongating complexes, which are resistant to pausing and termination (Mooney *et al.*, 2009) and contain NusA, NusB, NusE and NusG proteins (Prasch *et al.*, 2009). Antitermination complexes at ribosomal (*rrn*) operons are comprised of NusA, NusB, NusE (ribosomal protein S10) and NusG, while those formed at λ bacteriophage genes also include the λ N protein (Prasch *et al.*, 2009). When at λ anti-termination complexes, NusA binds to *nut* sites (Prasch *et al.*, 2009). These *nut* sites are comprised of highly conserved *boxA*, *boxB* and *boxC* sites (Prasch *et al.*, 2009). The λ *boxA* consensus is thought to be 5' CGCUCUUA 3' (Prasch *et al.*, 2009). However, when NusA forms an anti-termination complex at *rrn* promoters, NusA specificity is determined by *boxA*, and sometimes a *boxC* sequence; the *rrn boxA* consensus sequence is 5' UGCUCUUUA 3' and as such differs from the λ *boxA* (Prasch *et al.*, 2009). λ anti-termination complexes are resistant to both ρ–dependent and independent termination while *rrn* complexes are only resistant to ρ–dependent termination (Prasch *et al.*, 2009).

GreA and GreB bind to RNAP in the secondary channel of RNAP (Vassylyeva *et al.*, 2007) and NusG has been proposed to bind the β' subunit of RNAP (Mooney *et al.*, 2009). The specificity of DNA binding sites for the loading of these factors onto RNAP is not known. NusA binds to

the β subunit of RNAP, its known binding sites are discussed below (Yang *et al.*, 2009). Mfd will terminate arrested elongating complexes (Roberts *et al.*, 2008). Mfd may also bind to the β subunit of RNAP (Roberts *et al.*, 2008), while ρ does not bind to a specific consensus sequence but binds to naked, untranslated RNA (without ribosomes) with a preference for nucleotide sequences that are C-rich and lacking secondary structure (Cardinale *et al.*, 2008). *E.coli* and *Salmonella* RfaH bind to *ops* sites, 5' GGCGGTAG 3'; this sequence often occurs with a direct repeat and a conserved thymine residue two nucleotides down from the *ops* (operon polarity suppressor) site (Fig 4.3).

Sequences of rifampicin up-modulated promoters were examined for *rrn boxA* and *ops* sites, as these were the only known consensus sequences for possible elongation and termination factors. Moderate matches to the *rrn boxA* consensus sequence were found in *traS* (matching in 6/9 positions) and *spvA* (one matching in 7/9, two matching in 6/9 positions) transcripts. A moderate match to the *ops* sequence was present in the sequence of STM3595 which starts at +35 relative to the transcription start site (Fig 4.3). RfaH, the protein which binds to *ops* sites, is involved in production of the bacterial cell envelope, hemolysin toxin, F pilus, as well as, *siiA* expression (SPI-4, a rifampicin modulated gene, Table 1.1) (Bailey *et al.*, 1997; Main-Hester *et al.*, 2008). Previous studies have shown that a 6 bp alteration in the consensus from 5' GGCGGTAGCGC 3' to 5' CCGGGATGC 3' only had a 2-fold affect on proximal gene expression but a 55-fold change in more distal gene expression as measured by RT-PCR (Main-Hester *et al.*, 2008). Given that such a large nucleotide alteration had such a small effect on proximal gene expression, it is reasonable to hypothesize that a RfaH homologue may be involved in rifampicin mediated up-modulation of STM3595 and also *traS*. However, this cannot be the only

mechanism since *siiA* is down-modulated by rifampicin (Table 1.1), while STM3595 is up-modulated by RfaH but has a weak match to consensus.

EC *rfaQ* CAGTATTCAGGTAGCTGTTGAGCCTGG**GGCGGTAGC**GTG
EC *rfbB* CAGTGCTCTGGTAGCTGTTAAGCCAGG**GGCGGTAGC**GTG
EC *wza* CAGTGTATTGGTAGCTAAAAAGCCAGG**GGCGGTAGC**GTG
EC *kpsM* CAGTGTATTGGTAGCTGTTAAGCCAAG**GGCGGTAGC**GTA
EC pHly152 *hlyC* TCCCGGTTTACGGGTAGTTTCCGGAAG**GGCGGTAGC**ATG
EC LE2001 *hlyC* CCGGTTGATACGGGTAATTTCCGGAAG**GGCGGTAGC**ATG
EC F *traR* CGAGTGCCCTGTGCGTGAAAAGGGATG**GGCGGTAGC**GTG
EC J96 *hlyC* GCTGGTTGATGACTGTTAATTCCAGAA**GGCGGTAG**TCTG
ST SL1344 *siiA* AAGCGTATTGGTAGCAGGAAGCCAAG**GGCGGTAGC**GTT
ST LT2 *rfbB* CAGTGCACTGGTAGCTGATGAGCCAGG**GGCGGTAGC**GTG
ST 14028 STM3595 CTGTGGGCAAGGTAGTAGTTTTGACCT**GGCGTTA**TTTTT

Figure 4.3. The *ops* sites of various *E.coli* and *S. typhimurium* genes. The *ops* site is in bold and the direct repeat is underlined. This figure is modified from other sources (Bailey *et al.*, 1997; Main-Hester *et al.*, 2008; Wang *et al.*, 1998).

4.3.3. Transcription attenuation and intrinsic terminators.

Rather than protein elongation or termination factors being involved, an intrinsic terminator or transcription attenuation may be mediating up-modulation of $P_{STM3595}$ and P_{traS} by rifampicin. Intrinsic terminator sequences are regions are characterized by a hairpin followed by a run of U residues (Roberts *et al.*, 2008). A brief description of attenuation is described in section 1.4.3. To investigate this possibility, secondary structures of these two rifampicin up-modulated transcripts were analyzed using RNA folding software. Several RNA secondary structures were predicted when the first 163 nucleotides were analyzed. Secondary structures with similar features in both *traS* and STM3595 transcripts are shown (Fig. 4.4), but no candidate intrinsic terminators were identified. Interestingly, the consensus sequence (Fig 3.27B) observed in

rifampicin up-modulated promoters appeared to be base paired in a similar location in both STM3595 and *traS* secondary structures (Fig. 4.4B and C, respectively). Finer deletions than those shown in Figure 3.9 or nucleotide alterations would need to be constructed to determine the importance of the consensus nucleotides in forming secondary structure involved with rifampicin mediated up-modulation.

Since intrinsic terminators could not be identified from RNA secondary structure predictions using the first 163 nucleotides of the *traS* and STM3595 transcripts (Fig 4.4), mRNA sequences were specifically examined for transcription attenuator features such as inverted repeats. The REPuter program (Kurtz *et al.*, 2001) was used to search for the inverted repeats within the transcript sequences that would be present in the stem loop of a intrinsic terminator. Sequences downstream of the inverted repeats were examined for runs of U nucleotides. Since only one inverted repeat with a run of U's was found, this allowed for a shorter input sequence into the RNA folding software. The software predicted one possible intrinsic terminator which encoded a peptide from the shorter STM3595 transcript sequence. The small 13 codon ORF in the leader sequence of STM3595 is displayed (Fig 4.5). Although leader peptides are usually found upstream of intrinsic terminators, a ribosome binding site within the terminator sequence would work by a functionally similar mechanism. In the presence of rifampicin, RNAP would pause allowing the ribosome to catch up to RNAP, preventing secondary structure formation and thereby preventing termination. If rifampicin is not present, RNAP would not pause, secondary structure would form and terminate transcription. This occurs in attenutation of the *pyrC* gene, in which the stem of a leader region hairpin includes part of the *pyrC* ribosome binding site

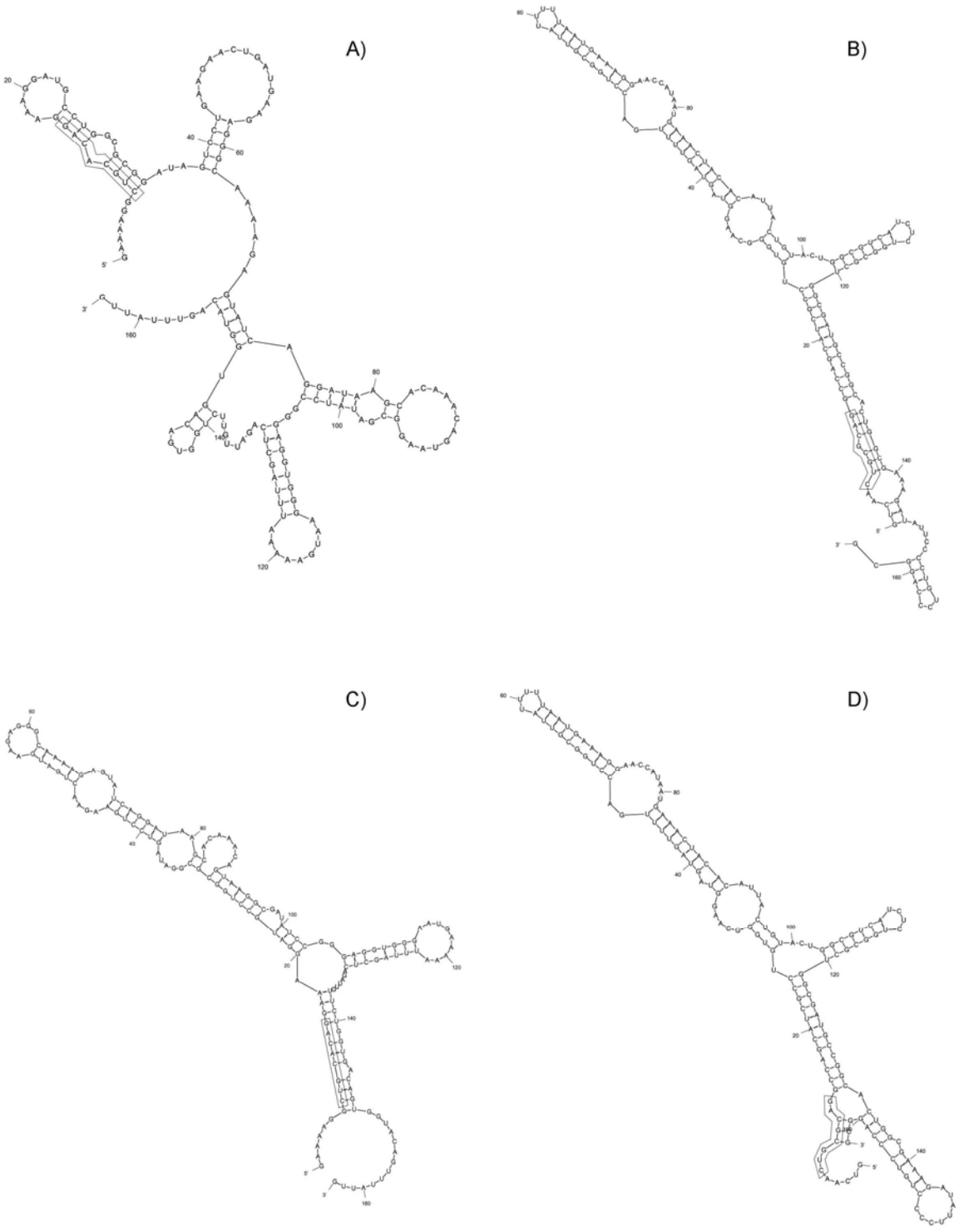

Figure 4.4. Predicted RNA secondary structures of *STM3595* and *traS* transcripts.
Selected secondary structures of *traS* (A,C) and STM3595 (B,D) transcripts from +1 to +163 as predicted by Mfold (Zuker, 2003). The consensus sequence shown in Fig. 3.27 is boxed.

(Turnbough & Switzer, 2008). Nucleotide sequences could be substituted within the putative stem loop structure or within the UTP tract to test this hypothesis.

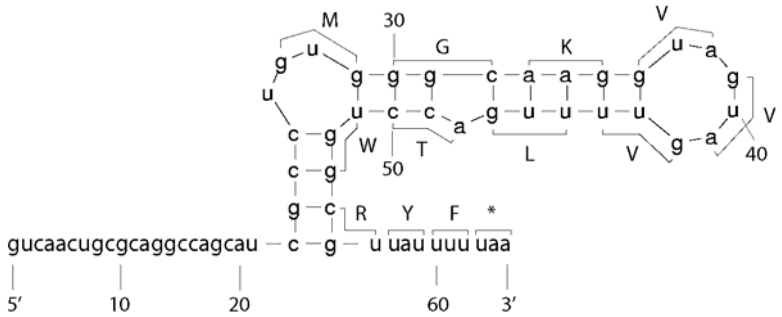

Figure 4.5. Predicted secondary structure of the nucleotides from +1 to +65 nucleotides. Numbering is relative to the +1 transcription start. Capital letters follow the IUPAC single letter code for amino acids and correspond to the amino acid predicted by accepted codon usage tables. The structure is as predicted by Mfold (Zuker, 2003).

4.4 Rifampicin resistance.

Four rifampicin responsive reporter fusions tested in a *S. typhimurium* rifampicin resistant background (mutant in the β subunit of RNAP) no longer displayed transcription modulation by rifampicin (Fig 3.2). This suggested that transcription modulation by rifampicin was mediated at the level of RNAP and not by rifampicin binding to another target. This is consistent with a previous study showing that rifampicin resistance abolished rifampicin mediated up-modulation of quorum sensing promoters from various bacterial genera (Goh *et al.*, 2002). Although

different rifampicin resistant mutants have varying phenotypes related to transcription termination, temperature sensitivity, *etc*. (Jin & Gross, 1989), only one rifampicin resistant mutant was employed in this study, *S. typhimurium* R306. R306, according to *E. coli* numbering, has a H526Y mutation in the RNAP beta subunit which corresponds to the *E. coli* allele *rpoB2*. Interestingly, in *E. coli*, *rpoB2* confers a 20-fold decrease in F' plasmid stability, temperature sensitivity, increased 5-fluorouridine sensitivity (5-fluorouridine resistant mutants have altered K_m's for ATP and UTP) and altered termination at the *trp* transcriptional terminator (Jin & Gross, 1989; Yanofsky & Horn, 1981). Jin and Gross (1989) proposed that rifampicin resistant phenotypes may reflect structure and function relationships within different areas of the β subunit. Similarly, different rifampicin resistant mutants may have different degrees of transcription modulation by rifampicin reflecting the involvement in that region in transcription modulation by rifampicin and the associated phenotypes, such as defects in transcription termination, may give hints to the mechanism of transcription modulation by rifampicin at that promoter.

4.5. Differential responses of reporter strains in liquid versus sold media.

Differences in transcription modulation by rifampicin from strains grown on solid or in liquid media were observed. The control strain (STM2901 fusion, pGY21) was unresponsive to rifampicin in liquid culture (Fig. 3.5) but showed increased luminescence in response to rifampicin on solid media (Fig. 3.3). Differential expression in response to sub-MIC antibiotics between strains grown on solid or liquid media was shown for rifampicin mediated up-modulation of quorum sensing promoters (Goh *et al.*, 2002). Transcriptome analysis comparing expression between cultures grown on solid or liquid cultures showed that up to 32 % of the *S. typhimurium* functional genome can be expressed differentially under the two conditions

(Wang *et al.*, 2004). Differences in STM2901 expression in liquid and solid media are an example of culture methods having an impact on expression.

Disk diffusion plate assays have many advantages over liquid media assays. Most studies examine the effects of antibiotics in broth culture. Sub-MIC transcription effects can occur over a narrow concentration range. In disk diffusion assays, this is illustrated by a narrow light induction zone (Fig 3.4ii). The use of sub-optimal antibiotic concentrations in liquid media may explain why these seemingly ubiquitous effects on bacterial transcription have not been described previously in literature. Compared to assays in liquid medium that can only employ one concentration at a time, disk diffusion assays test a large concentration range.

4.6. The response of rifampicin modulated promoters to various antibiotics.

A wide response range of promoters to antibiotics has been previously been demonstrated with five other reporter fusions and four different classes of antibiotics (Goh *et al.*, 2002). The responsiveness of promoter reporter fusions to a diverse range of antibiotics seen in disk diffusion assays (section 3.1.3) may hint at the sensitivity of these reporters to perturbations in bacterial metabolism. The ability of antibiotics to elicit so many effects may also emphasize that antibiotics are not randomly chosen compounds but compounds preselected for bioactivity. Antibiotics are not like off the shelf chemicals or compound libraries; they have undergone extensive selection for bioactivity: by evolution in bacteria and in the laboratory when screened for growth inhibitory properties at high concentrations.

The observation that many antibiotics induce transcription responses in many promoters led to the suggestion that detection and classification of pharmaceutically active compounds may be possible by monitoring transcription of selected promoter clones (Goh *et al.*, 2002). As mentioned in section 1.3, it has proposed that mode of action identification or even preliminary compound identification is possible by comparing the expression profiles induced by unknown compound to profiles induced by known compounds. The pattern of luminescence produced by the rifampicin responsive reporter fusions to various compounds shown in section 3.1.3 could be considered a less comprehensive version of the mode of action identification panels. These seven promoter-*lux* reporter fusions may be limited in their ability to identify different modes of action, as their response pattern may only be able to determine whether compounds have a similar mode of action to rifampicin. Any compound that elicits a pattern of expression similar to rifampicin (inducing up-modulation of *traS, spvA*, STM3595 and STM2901 in the wildtype background that are abolished in the R306 background and shows negative responses with *fliA*, *flgK* and *invF* reporters) could be predicted to have a similar mode of action as rifampicin. These reporters may not be as useful for identifying other kinds of antibiotics since promoter reporter fusions were not specifically chosen for their unique and specific responses to other classes of antibiotics. Mode of action panels often include at least one-promoter fusion that has been chosen for its characteristic response to each class of antibiotic. For example, some macrolide responders, some aminoglycoside responders, some beta-lactam responders, *etc*.

The response of rifampicin modulated promoter-*lux* reporters to other compounds was examined (section 3.1.3). This was carried out by testing the rifampicin responsive reporter fusions against various antimicrobials. Many promoters responded to other antimicrobials but did not elicit a

pattern similar to rifampicin; as such, the promoter-*lux* reporters showed a rifampicin specific response pattern. Three of the seven reporter fusions tested were weakly responsive to hydrogen peroxide and erythromycin. Six of the seven tested reporter fusions tested in section 3.1.3, including promoters for invasion and flagellar genes, were strongly induced by tetracycline. Studies with *S. typhimurium* DT104 also showed tetracycline induced transcription of invasion and flagellar genes and in addition showed increased invasion of HeLa cells (Weir *et al.*, 2008). Therefore, if the appropriate reporter strains are used (*i.e.* reporters for promoters related to virulence), antibiotic induced responses on disk diffusion assays could be useful predictors for screening compounds which elicit phenotypic changes. Since all the antimicrobials elicited different patterns of light induction, it is likely that these compounds induce transcription modulation by distinct mechanisms.

4.7. Biological relevance of transcription modulation by rifampicin.

Screening of a *S. typhimurium* promoter-reporter library revealed that rifampicin differentially affected the transcription of many *S. typhimurium* virulence genes depending on their involvement in two virulence programs (Yim *et al.*, 2006). Virulence genes associated with intracellular growth in macrophages *spvAB* (Table 1.1) and SPI-2 genes (not shown) were up-modulated by rifampicin. Other virulence genes, not known to be associated with SPIs, such as *traS* and *yijP* were also up-modulated by rifampicin (Table 1.1). Genes involved in intestinal invasion associated with the type III secretion system encoded on SPI-1 and its secreted effectors (Teplitski *et al.*, 2003) showed rifampicin mediated down-modulation. These genes included *invF*, *sopA* and *sopB*. Such differential regulation in distinct environments and between types/routes of infections is often observed and discussed relative to SPI-2 (Knodler *et al.*, 2002;

Watson *et al.*, 1999). Rifampicin may be mimicking natural cues that cause switching of virulence modes from penetration of epithelial cells to growth and survival in macrophages occurring when the salmonellae move from the intestine into macrophages during systemic infection.

The genes found to show rifampicin mediated down-modulation are similar to genes repressed by cationic microbial peptides (Bader *et al.*, 2003) and bile (Prouty & Gunn, 2000; Prouty *et al.*, 2004b). Rifampicin, bile and cationic microbial peptides cause transcriptional repression of the SPI-1 genes, its effectors and motility genes in *S. typhimurium* (Bader *et al.*, 2003; Prouty & Gunn, 2000; Prouty *et al.*, 2004b; Yim *et al.*, 2006). Both rifampicin and cationic antimicrobial peptides (*e.g.* polymyxin B) are broadly used antibiotics while bile has been shown to have antibacterial activity (Gunn, 2000). The active component of bile for the induction of bile and antimicrobial resistance genes has been identified as deoxycholate (Prouty *et al.*, 2004a). Other than their antibacterial properties, it is unclear what relationship there is between these three molecules that could account for their similar transcription profiles. Structurally diverse natural products cause potassium leakage and biofilm formation (Lopez *et al.*, 2009). Analogously, but highly unlikely, is the hypothesis that polymyxin B and deoxycholate may be RNAP inhibitors. Alternatively, host produced antimicrobial peptides and bile would be naturally present in the intestine, but it is unlikely that rifampicin, which is produced by a soil microorganism, would be present in the intestine except during therapy. Rifampicin may be mimicking a signal in the gut or macrophage that causes *Salmonella* to switch from invasion to intracellular survival.

The function of the gene most highly up-modulated by rifampicin identified in my studies, STM3595, is unknown but several observations suggest it is important for *S. typhimurium* survival in macrophages. In the PubMed Conserved Domain Database the protein encoded by STM3595 is annotated as a putative acid phosphatase with a PAP2 domain (Marchler-Bauer *et al.*, 2009). Proteins with PAP2 domains have been classified as non-specific acid phosphatases, enzymes which catalyze phosphomonoester hydrolysis with optimal activity in low pH conditions (Ishikawa *et al.*, 2000). Other genes encoding *S. typhimurium* proteins with PAP2 domains include *phoN, mig-13* (*ybjG*) and *pgpB*. Like STM3595, *phoN* and *ybjG* may be regulated by PhoP (Eguchi *et al.*, 2004; Kasahara *et al.*, 1991; Song *et al.*, 2008). The PhoP/Q system is activated by low pH (and low Mg^{2+}) and PhoP activated genes are expressed maximally in acidified phagosomes (Alpuche Aranda *et al.*, 1992). P_{traS} and P_{spvA}-reporter fusions carried in a *phoP*::*cam* mutant showed similar up-modulation as the isogenic parent strain while the strain carrying the STM3595 reporter fusion (pGY35) was no longer activated by rifampicin suggested that STM3595 is positively regulated by PhoP (not shown). Others have also found that STM3595 is positively regulated by PhoP (Song *et al.*, 2008). Interestingly, several PAP2-containing proteins have been identified in a FACS screen for *Salmonella* genes expressed within macrophages; one such gene was *mig-13* (Valdivia & Falkow, 1997). A gene located beside the rifampicin up-modulated gene *traS*, *traT*, was identified in the same screen (Valdivia & Falkow, 1997). In *E. coli*, TraS and TraT are important for surface exclusion during conjugation and TraT has been shown to be important for complement resistance (Sukupolvi & O'Connor, 1990). Although STM3595 is known to be positively regulated by the transcription factor PhoP, little is known about the direct regulation and function of this gene.

4.8. Sub-MIC and its implications for virulence and motility.

In laboratory studies, MIC values are often used to determine whether a given bacterial isolate is resistant or sensitive to a given drug. The MIC value is dependent on many factors; it can vary greatly from organism to organism, as well as strain to strain. It is also highly dependent on media, aeration, inocula, incubation time, temperature or even the type of agar (Lorian, 1991). This is why defined growth conditions are always used in medical laboratories. The accepted variability in an MIC when compared between multiple medical laboratories is four-fold (average MIC ± two-fold) (Espinel-Ingroff *et al.*, 2005; Huys *et al.*, 2010). This becomes a problem when the breakpoint that determines whether an organism is to be classified as sensitive or resistant to a given antibiotic straddles these values.

When referring to antibiotic concentrations in the body, antibiotic concentration will greatly vary depending on many factors. Given the plethora of organisms and their respective diverse MICs, non-uniform distribution of drug and patient noncompliance to proper dosing regimens, a given antibiotic will certainly exist at sub-MICs for most antibiotic treatments. Although the MIC value determined in culture does not reflect the amount of drug required in a given tissue to inhibit bacterial growth, the term Peak/MIC is often seen in clinical literature. The peak value is the concentration of given drug reached in a given body compartment (Mouton et al., 2005). The peak for a given drug will vary from adult to adult depending on age, health and other genetic and environmental factors (this is one of the aspects that personalized medicine wishes to address). The Peak/MIC value has no specific meaning since MICs are determined in culture and cannot be determined in the body but may be useful for estimations in the body such as following. On average, for a 600 mg oral dose of rifampicin, the serum peak is 7-10 μg/ml and

the serum half life is approximately three hours (Sanofi-Aventis Canada Inc., 2010). The rifampicin MIC for 20 *M. tuberculosis* strains in 7H-9 broth with Tween 80 varied between 0.005 to 0.02 μg/ml (Lorian, 1991). The concentration of rifampicin in the serum is many times over the *M. tuberculosis* MIC with Peak/MIC ratios ranging from 350 to 2000. Relative to the rifampicin MIC of *S. typhimurium* 14028 in LB, ~12 μg/ml, this is in the sub-MIC range.

Since promoters for virulence and motility are selectively repressed by sub-MIC rifampicin, this may prove to be an advantage in treating salmonellosis. Although flagellae are not absolutely required for the virulence of *S. typhimurium*, their presence increases the ability to invade mammalian cells (Jones *et al.*, 1992; Schmitt *et al.*, 2001). In addition to the genes *invF*, *fliA* and *flgK* which were studied in this thesis, screening of the *S. typhimurium* DNA-*lux* fusion library suggested that rifampicin down-modulated other genes related to host cell invasion and motility such as *sopA*, *sopB*, genes of SPI-4 and the class II flagellar operon *flhBA* (Table 1.1). Multiple mechanisms exist for co-regulation of motility and invasion (Ellermeier & Slauch, 2003; Iyoda *et al.*, 2001; Teplitski *et al.*, 2003) and down-modulation of P_{fliA} and P_{invF} by rifampicin would be expected to interfere with some of these pathways (Fig. 4.6). For example, reduced transcription of *fliAZY* would reduce both motility and virulence. As *fliA* encodes σ^{28}, reduced *fliA* transcription would decrease expression of the class III flagellar genes and *fliA* (it is auto-regulated) (Kutsukake, 1997). In addition, FliZ positively regulates *hilA* and positively affects secretion of invasion proteins (Iyoda *et al.*, 2001). HilA and InvF are activators of transcription of virulence effectors secreted by SPI-1 (Darwin & Miller, 2001) and HilA directly down regulates *sopA*, *sopB*, and *siiA* (first gene of SPI-4, STM4257) (Thijs *et al.*, 2007). All of these genes belong to the group of promoters selectively down-regulated by rifampicin (Table 1.1).

Thus we predict that *S. typhimurium* invasion and motility would be repressed by sub-MIC rifampicin and may contribute to the clinical functionality of rifampicin.

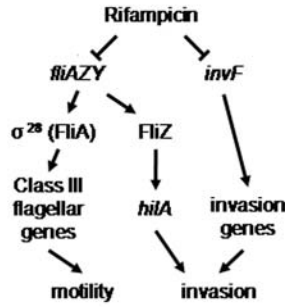

Figure 4.6. A schematic of putative virulence and motility pathways affected by rifampicin.

A well-studied precedent for the use of antibiotics to repress virulence function rather than growth inhibition is the use of macrolide antibiotics (such as erythromycin and azithromycin) in diffuse panbronchiolitis and cystic fibrosis infections. Macrolides do not reduce the bacterial load but inhibit the expression of virulence determinants and also have immunomodulatory effects (for a comprehensive review, see (Tateda *et al.*, 2007)). Briefly, in *P. aeruginosa*, production of toxins, pigments, alginate, pili and flagella are inhibited by macrolides (Tateda *et al.*, 2007). As a consequence, cell adherence and biofilm production is also inhibited (Tateda *et al.*, 2007). These effects are proposed to be mediated, at least in part, by inhibition of the quorum sensing system (Tateda *et al.*, 2007). Antibiotics may be used to kill or inhibit bacterial growth and to decrease bacterial pathogenicity. In addition, such studies may provide guidance on antibiotics-pathogen combinations not to be used if pathogenicity is induced. An example of

a poor combination would be using tetracycline to treat *Salmonella* since tetracycline induces invasion and motility in *S. typhimurium* strain DT104 (Weir *et al.*, 2008). Clearly, this analysis would contribute to a more rational and effective use of antibiotics.

4.9. Concluding remarks.

Sub-MIC rifampicin down and up-modulates transcription of many genes in *S. typhimurium*. Genes involved in host cell intestinal invasion and motility are down-modulated, while those involved in carbon metabolism and other virulence functions appeared up-modulated. The mode of action of rifampicin at P_{fliA}, P_{flgK}, P_{invF}, P_{spvA}, $P_{STM3595}$ and P_{traS} is much more complicated than initially anticipated. The transcription of some of the down-modulated promoters is hypersensitive to rifampicin, causing transcription to be down-modulated by rifampicin. Other mechanisms may cause further down-modulation by rifampicin. Rifampicin mediated up-modulation may involve intracellular factors and transcription attenuation. It is unclear whether common factors mediated both up-modulation and down-modulation by rifampicin but it is clear several mechanisms are involved.

When any given promoter or set of promoters is induced or repressed by a given stimulus, one mechanism, such as a specific transcription factor, is often studied. From this, one may have the impression that gene regulation is simple. However, it is clear that multiple levels of regulation exist for expression of a given gene. A gene often has several promoters; a second promoter may be internal to an operon and correspond to a shorter transcript. Each promoter can be transcribed by a different set of sigma factors and transcription factors. This allows the integration of many different environmental stimuli and flexibility in the sets of genes transcribed in response to a

given stimulus. Regulation often exists at the level of transcription initiation, but can also occur during transcription elongation, termination or post-transcriptionally. Like any other group of promoters, modulation of this group of rifampicin responsive promoters has multiple levels.

Most antimicrobials are microbially produced, biologically active small molecules. They represent a fraction of the small molecules produced by microbes. Decades of work and billions of dollars worth of discovery, chemistry and testing were employed to find useful compounds to inhibit growth of other microbes. Given that antibiotics are not randomly selected molecules but molecules selected by evolution and selected again by pharmaceutical companies, perhaps it is not surprising that so many antibiotics have a wide variety of effects on transcription and consequently phenotype. They are used clinically at concentrations much higher than those likely to occur in the environment. It has long been thought that antibiotics are agents of inter-microbial warfare. One microbe will use these molecules to kill or inhibit the growth of competing microbes living in the same environmental niche. Since both functionalities of antibiotics have been shown in the laboratory, surely both are possible in nature. Studying the sub-MIC properties of antibiotics and other small molecules will help to understand how they affect gene expression and will allow a more detailed exploration of their potential as therapeutic agents, antimicrobial or otherwise. These studies may also elucidate the "natural" function of antibiotics: modulatory signaling molecules, weapons of inter-microbial competition or both!

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