RIFAMPICIN INDUCED TRANSCRIPTION MODULATION IN *SALMONELLA ENTERICA*
SEROVAR TYPHIMURIUM

By

GRACE YIM

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Abstract.

Sub-inhibitory concentrations of antibiotics modulate global transcription patterns in bacteria. Genes involved in stress responses, metabolism, virulence, motility and other functions have been identified as being activated or repressed in response to rifampicin by screening a promoter-*lux* library of 6528 clones in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) 14028. Rifampicin differentially regulates the transcription of *S. typhimurium* virulence genes. Transcription of genes involved in intracellular growth, survival and replication in macrophages was up-regulated. Motility genes and genes associated with intestinal invasion, SPI-1 and its secreted effectors, were transcriptionally down-regulated by rifampicin. Rifampicin induced transcription modulation in a concentration dependent manner with differential responses as great as 200 fold in some cases. Testing of eight rifampicin responsive promoters in one of six *S. typhimurium* 14028 regulatory protein and sigma factor mutants: *crp, fnr, ihfB, fis, hns* and *rpoS* showed that for most of the promoter-mutant combinations rifampicin induced transcription modulation (RITM) was independent of the regulators. In a few cases, RITM did appear to depend on the factor tested and in those cases the altered protein may work co-operatively with other unidentified regulatory mechanisms to cause RITM of the tested promoter. However, no single regulator tested affected RITM for all promoters suggesting that different mechanisms may elicit RITM and RITM may be promoter dependent. Possible mechanisms will be discussed. Rifampicin induced changes in regulatory proteins or RNA polymerase may mimic intestinal cues that promote switching of virulence modes from penetration of epithelial cells to intracellular survival and proliferation.

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2DGE	2-dimensional gel electrophoresis
2D-TLC	2-dimensional thin layer chromatography
CAMP	cationic microbial peptide
CTD	carboxy terminal domain
CYP	cytochrome P450
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N'N'-tetra-acetic acid
EMSA	electrophoretic mobility shift assay
iPCR	inverse polymerase chain reaction
LB	Luria-Bertani
LBA	Luria-Bertani agar
MDR	multidrug resistance
MIC	minimal inhibitory concentration
mRNA	messenger ribonucleic acid
NTD	amino terminal domain
OD	optical density
PCR	polymerase chain reaction
PXR	pregnane X receptor
qPCR	quantitative polymerase chain reaction
RDR	rifampicin down-regulated
RIDR	rifampicin induced down-regulation
rif	rifampicin
RITM	rifampicin induced transcription modulation
RIUR	rifampicin induced up-regulation
RNA	ribonucleic acid
RNAP	RNA polymerase
rRNA	ribosomal ribonucleic acid
RRP	rifampicin responsive promoter

RT-PCR	reverse transcriptase polymerase chain reaction
RUR	rifampicin up-regulated
SCV	*Salmonella* containing vacuole
SM	small molecule
SPI	*Salmonella* pathogenicity island
TB	tuberculosis
TBE	tris-borate EDTA
TE	tris-EDTA
TF	transcription factor
TTSS	type III secretion system
Tris	tris(hydroxymethyl)aminomethane
wt	wild type

Acknowledgements.

<u>Chapter 1. Introduction.</u>

1.1 Antibiotics.

Antibiotics are molecules that kill or inhibit the growth of bacteria or fungi. The word itself means "against life." In the past 50 years, antibiotics have been critical to the control of many infectious diseases. Many antimicrobials are natural compounds that have been isolated by screening growth medium of bacterial and fungal species for antimicrobial activity. Generally, antibiotics work by blocking crucial processes in the microbial cells. Hence, antibiotics have been classified according to structure and mode of action. Several examples of such classes are the rifamycin, quinolone, macrolide and aminoglycoside antibiotics. Rifamycins bind and inhibit DNA-dependent RNA polymerase (RNAP) (21). Quinolones block DNA synthesis by targeting DNA gyrase and topoisomerase, enzymes responsible for DNA supercoiling and deconcatenation of interlinked daughter chromosomes following DNA replication (35, 80), respectively. Macrolide and aminoglycoside antibiotics both inhibit protein synthesis. Macrolides block the elongation of the nascent peptide chain by binding to the 50S subunit of the ribosome (159, 176) while aminoglycosides bind to the A site16S rRNA in the 30S subunit (120, 150, 188).

What is the role of antibiotics in nature? Antibiotics have been found to have two distinct functions, one at high concentrations and the other at concentrations lower than the drug's minimal inhibitory concentration (MIC). The common human use of antibiotics has been to employ these small molecules (SMs) in a concentrated and purified form to treat fungal and bacterial infections of humans. In the past several years, a second functionality of antibiotics has emerged, the modulation of bacterial gene transcription at sub-lethal dosages (26, 38, 50, 183). In light of antibiotic modulation of bacterial transcription, it is questionable whether the natural purpose of antibiotic production by microbes is to inhibit growth of neighboring microbes for inter-microbial competition. The concentrations of antibiotics found in the soil or in the environment are likely to be significantly lower than those encountered in clinical settings and would not aid in intermicrobial competition (64). While antibiotics have been isolated for inhibition of microbial growth, other SMs have been shown to have a diverse array of biological activities other than growth inhibition such as antiviral, antifungal, antitumor and immunosuppressive (24, 33).

This thesis research was done to address the question of whether antibiotics are able to differentially regulate bacterial gene transcription and perhaps act as signaling molecules and not as chemicals for intermicrobial competition. To do this, a promoter-*lux* library of approximately 6500 clones in the Gram-negative bacterium, *S. enterica* serovar Typhimurium 14028 (*S. typhimurium*) was obtained (11). This library was screened for promoters whose activity was altered by rifampicin (50), aminoglycoside (unpublished), trimethoprim (unpublished) and macrolide-lincosamide-streptogramin (179) antibiotics, each at sub-inhibitory concentrations. Each antibiotic was shown to modulate global transcription patterns (~ 5% of genome) and both induction and repression was observed. For rifampicin, this was particularly unexpected as rifampicin slows bacterial growth by inhibiting transcription (see Section 1.2).

This thesis is an extension of the library screening mentioned above and the resulting publication is reference 50 (see appendix 7.1). Specifically, this thesis attempts to characterize RITM using a subset of the 192 rifampicin responsive clones collected in the original screening of the library (50). The 192 rifampicin responsive clones were reassayed to obtain a smaller number of robustly responsive clones (33 were chosen) for detailed study. Nucleotide sequences from rifampicin responsive clones showed that promoters were associated with genes involved in stress responses, metabolism, virulence and other functions. The 33 clones were also tested against a concentration gradient of rifampicin. Since the promoters represented genes for a wide range of cellular functions, global regulatory mutants were transformed with eight rifampicin responsive promoter (RRP) reporters to test whether RITM was mediated by these global regulatory proteins. Possible regulatory mechanisms and directions for further studies of RITM are proposed.

1.2 Rifampicin.

Rifampicin is an antibiotic of particular of interest and is the focus of this study. It belongs to the ansamycin family of antibiotics, which are named for their basket-like structure (ansa=handle in Latin). It is a semisynthetic compound produced from rifamycin SV, a biosynthetic precursor of rifamycin B. The producer organism of rifamycin B is *Amycolaptosis mediterranei*, an Actinomycete originally classified as *Streptomyces mediterranei* and then renamed *Nocardia mediterranea*. Rifampicin inhibits growth of many Gram-positive bacteria,

particularly *Mycobacteria,* and possesses excellent bioavailability. It is used in the treatment of leprosy and other AIDS-associated mycobacterial infections. (44) Rifampicin is also one of the primary antimicrobials used to treat tuberculosis (TB) as it has a strong sterilizing activity against populations of *Mycobacterium tuberculosis*, the causative agent of TB. In 1993, the World Health Organization (WHO) declared TB to be a global health emergency. In 1997, it was estimated that 1/3 of the human population, ~ 2 billion people, were infected with TB and the numbers were continuing to grow. It is primarily a disease of poverty, with 90% of those infected being in developing countries. (96)

The biochemical target for rifampicin in the bacterial cell is the β subunit of RNAP (60). The crystal structure of rifampicin complexed with *Thermus aquaticus* RNAP has been solved (21) and has confirmed and refined models for rifampicin mode of action (Figure 1.1). Rifampicin binds RNAP deep within the main DNA/RNA channel and blocks the path of the elongating RNA transcript (21). This locks RNAP into an abortive initiation complex that can only synthesize transcripts 2-3 nucleotides long (118). Consequently, elongating RNAP is resistant to rifampicin as the transcript is already inhabiting the DNA/RNA channel (165). Further evidence supporting RNAP as the bacterial target of rifampicin is the almost exclusive mapping of rifampicin resistance to the *rpoB* gene, that encodes the β subunit of RNAP, in many organisms (77, 79, 110).

In addition to its inhibitory properties on bacterial cells, rifampicin has been shown to have activity on viral and mammalian cells. The reverse transcriptase of some viruses is inhibited by rifampicin (7, 93) but rifampicin was found not potent or selective enough for use as a clinical antiviral. Multidrug resistance (MDR) in hepatocyte cells is also inhibited by rifampicin through the repression of drug efflux pumps (30, 142), enhancing drug accumulation at target cells (42). Rifampicin has hepatic toxicity (mostly in patients with underlying liver disease) as well as immunosuppressive properties (55). It has been suggested that the immunosuppressive properties of rifampicin are due to activation of the human glucocorticoid receptor (20). However, there is evidence showing that rifampicin does not bind to human glucocorticoid receptor (66). Rifamides have also been reported to inhibit NF-$\kappa\beta$ activation, providing an additional mechanism for immunosuppression (139). Another reported human target for

A

B
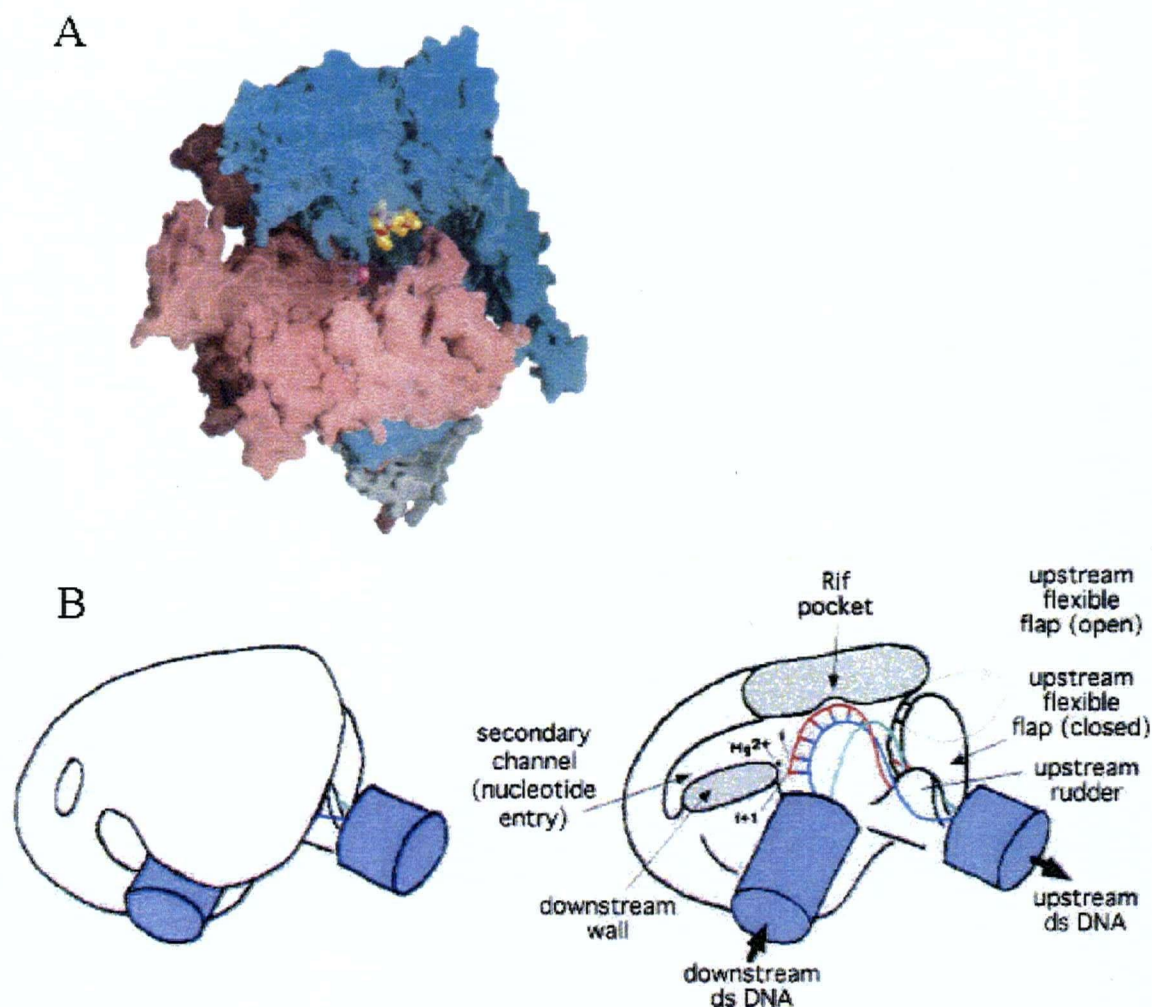
Figure 1.1.A. Three-dimensional structure of *T. aquaticus* core RNAP in complex with rifampicin generated using GRASP. GRASP is a molecular visualization and analysis program that is particularly useful for the display and manipulation of the surfaces of molecules and their electrostatic properties (125). The molecular surfaces of the RNAP subunits are color coded (β, cyan; β', pink; ω, gray; the α subunits are behind the RNAP and are not visible). The Mg^{+2} ion chelated at the active site is shown as a magenta sphere. Rifampicin is shown as CPK atoms (carbon, orange; oxygen, red; nitrogen, blue). (Figure obtained from (21))

Figure 1.1.B. Schematic model of the structure of a ternary transcription complex. Double-stranded DNA is represented as blue cylinders. The DNA template strand is shown as a blue line; the non-template strand, a cyan line; the RNA transcript, a red line. Very little information is available to position the non-template DNA strand within the model; it is shown here for illustrative purposes only. (Left) View with intact RNAP molecule. (Bottom) Same view but with parts of the RNAP cut away (shown in gray) to reveal the inner workings of the complex, which are labeled. (Figure obtained from (192))

rifampicin is the pregnane X receptor (PXR), a xenobiotic and steroid receptor. Through PXR, rifampicin has been reported to activate human cytochrome P4503A4 (CYP3A4), a major CYP isozyme in the human liver for metabolizing endobiotics and xenobiotics *e.g.* the contraceptive, estrogen ethinylestradiol (15). PXR activation also inhibits CYP7A1, another CYP isozyme involved in bile acid synthesis, and has led to rifampicin treatment of bile acid induced cholestasis (98). Rifampicin thus affects bacterial, viral and mammalian targets.

1.3   Bacterial transcription modulation by antibiotics.

Many antibiotics have been observed to change bacterial transcription and protein expression patterns. As mentioned above, the Davies laboratory has demonstrated transcription modulation in response to several antibiotic classes in *S. typhimurium*. Other groups have carried out similar studies using proteomic and DNA microarray approaches. Using 2D gel electrophoresis (2DGE) of *Escherichia coli* proteins, various protein synthesis inhibitors were found to induce protein expression in a manner similar to a mild or strong heat shock (puromycin, kanamycin and streptomycin) or cold shock (chloramphenicol, erythromycin, fusidic acid, spiramycin, tetracycline) (183). Cold shock related antibiotics were also found to slightly up-regulate transcription of ribosomal proteins and down-regulate heat shock proteins (183). 2DGE studies in *Haemophilus influenzae* treated with six translation inhibitors from (183) showed similar results (38). Similarly, DNA microarray analysis of *Streptococcus pneumoniae* treated with puromycin, tetracycline, chloramphenicol or erythromycin showed differential mRNA levels for genes involved in the synthesis of ribosomal proteins, amino acids and purine as well as genes required for the heat shock response (124). In an *E. coli* MG1655 microarray study, rifampicin and ampicillin were shown to up-regulate genes involved with nucleotide salvage and purine biosynthesis, while kanamycin up-regulated heat shock genes and norfloxacin up-regulated genes involved in the SOS response (161). Microarray analysis of *E. coli* and *H. influenzae* treated with antibiotic DNA synthesis inhibitors has shown activation of osmotic stress response genes (26) and induction of the DNA (SOS) repair system (49), respectively. Studies with enterohaemorrhagic *E. coli* have shown that prophage genes and prophage encoded toxin genes are up-regulated by quinolones and trimethoprim (65, 86, 116, 193). Many groups have reported modulation of bacterial gene expression by antibiotics showing that antibiotics have multiple effects other than inhibition through the traditional antibiotic target.

1.4 Virulence modulation.

In addition to genes involved in metabolism and stress, antibiotics have been found to regulate the expression of virulence genes. By RT-PCR and reversed passive latex agglutination, rifampicin has been shown to down-regulate transcription of toxin (*stx1*, *stx2*) and attachment genes (*eaeA*) and decrease toxin production, respectively, in *E. coli* 0157:H7 (113). Studies using both 2DGE and DNA microanalyses showed that *S. typhimurium* subjected to sublethal concentrations of cationic antimicrobial peptides (CAMPs) have elevated levels of PhoP/PhoQ and RpoS virulence regulon transcription, while repressing the transcription of genes required for flagella synthesis and the invasion-associated type III secretion system (4). In both *Pseudomonas aeruginosa* and *Proteus mirabilis*, subinhibitory concentrations of mupirocin and macrolide antibiotics inhibited flagella formation and motility (69, 81, 122). Subinhibitory concentrations of macrolides, clindamycin and piperacillin/tazobactam have been observed to decrease alginic acid production, biofilm formation and virulence factor production in *P. aeruginosa* (45, 72, 73, 121, 174). Production of *Pseudomonas* virulence proteins is likely down-regulated by macrolides through down-regulation of the quorum sensing systems, *lasR* and *rhlR* (175). In the Gram-positive bacteria *Staphylococcus aureus*, sublethal concentrations of clindamycin suppressed virulence factor syntheses at the transcription level (64). *Streptococcus pyogenes* and *S. aureus* exoprotein protein secretion was reduced in response to subinhibitory concentrations of linezolid when measured by protein production, enzymatic activity (48) and 2DGE (9). In many pathogenic bacteria, antibiotics induce modulation of virulence factor expression showing both the potentially harmful and positive side effects that need to be taken into account when using antimicrobials for human treatment.

1.5 Pathogenicity and virulence of *S. typhimurium*.

The majority of virulence factors present in the *Salmonella* species are encoded within *Salmonella* Pathogenicity Islands (SPIs) or on the virulence plasmid (109). The SPIs are *Salmonella* specific and present in most *Salmonella spp.* (54). Currently, there are five known SPIs (Figure 1.2) (109), each is expressed during a specific time during the infection (54). The SPIs were likely acquired through horizontal gene transfer and can turn benign bacteria into pathogens (54). The *Salmonella* virulence plasmids vary in size depending on the serovar and can restore virulence to plasmid cured *Salmonella* (57). The known *Salmonella* virulence

6
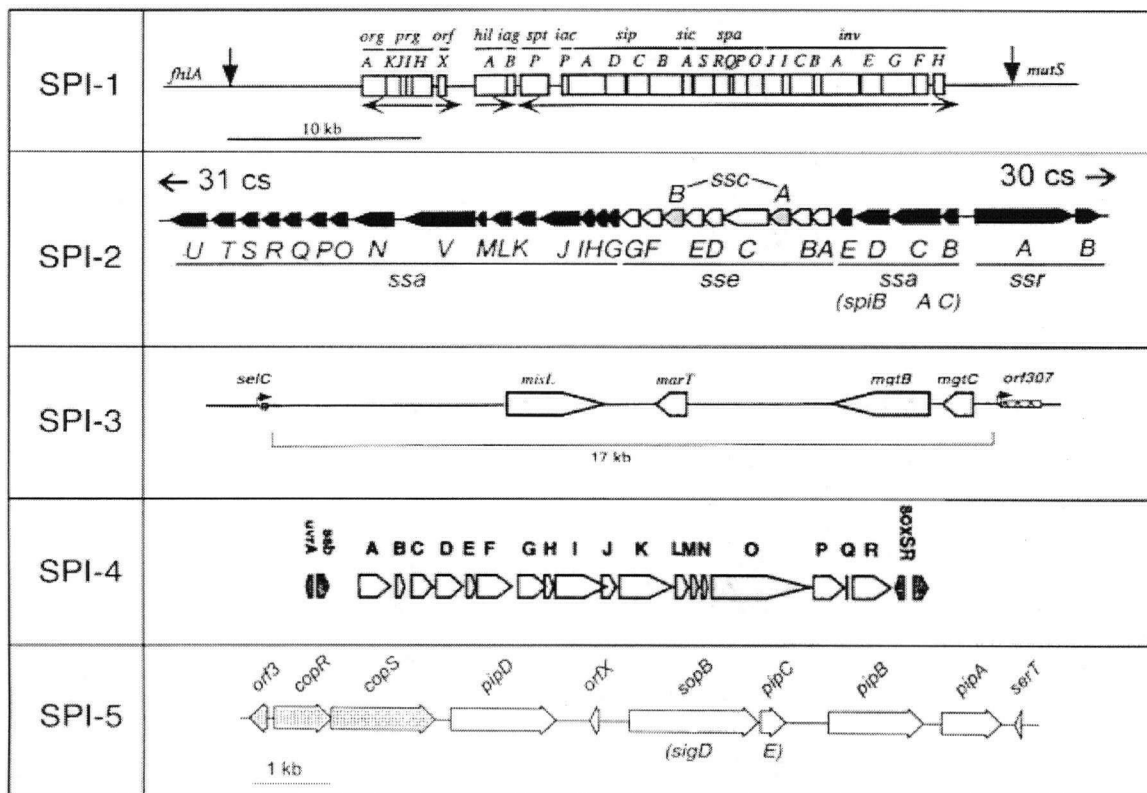
Figure 1.2. Schematic drawings of *Salmonella* pathogenicity islands. See reference (109) for detailed information regarding genes located within the SPIs. (Figure obtained from (109)).

plasmids contain the Salmonella Plasmid Virulence, *spv*, genes (27).

Salmonella strains have acquired many determinants that have enabled them to live a variety of pathogenic lifestyles (54). *S. enterica* is a facultative intracellular pathogen that causes different types of disease depending on the host and serotype. *S. typhimurium* infection is limited to gastroenteritis in humans but causes a systemic (typhoid like) infection in mice. In humans, *S. enterica* serovar Typhi infection leads to typhoid fever, a systemic infection, but does not cause typhoid fever in non-primates (68). Thus, mice infected with *S. typhimurium* are often used as an animal model for human typhoid fever (109). The cause of salmonellae infection is usually from ingestion of contaminated food or water. During systemic infection, bacteria travel from the stomach and colonize the intestinal epithelial cells. At the intestinal wall, injection of proteins using the type III secretion system (TTSS) encoded on SPI-1 induces epithelial cells to take up *Salmonella* (46). *Salmonella* disseminates to local mesenteric lymph nodes where it invades phagocytic cells and often survives. Proteins encoded on SPI-2, *slyA* and the *spvRABCD* allow *S. typhimurium* to survive and replicate in epithelial and macrophage cells inside a unique membrane-bound vacuole (SCV) (68). *Salmonella* travels to extraintestinal sites, *i.e.* spleen and liver, via CD18 positive phagocytes (184), resulting in a systemic infection.

1.6   Bacterial RNA polymerase and transcription regulation.

The cellular machinery responsible for transcription in the cell is RNA polymerase (RNAP). The RNAP core enzyme is competent for transcription elongation and is composed of five subunits, $\beta\beta'\alpha_2\omega$ (17) (Figure 1.1). The 3.3 Å crystal structure of *T. aquaticus* RNAP revealed its crab claw-like structure (192). The active site which binds the DNA:RNA complex is formed by the β and β' subunits (89). There is a Mg^{+2} bound at the beginning of the secondary channel through which NTP diffuses into the active site (192). The 5' end of the RNA transcript exits the active site via the main DNA/RNA channel (89). Each α subunit has two independently folded domains, the αCTD (carboxy terminal domain) and the αNTD (amino terminal domain) (12). Dimerized αNTD directs $\beta\beta'$ assembly while αCTD is a DNA binding module important for interactions with promoters and transcription factors (TFs) (52).

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To begin transcription at a specific promoter sequence, the core enzyme must bind a σ factor and form the holoenzyme, $\sigma\beta\beta'\alpha_2\omega$. There are two families of σ factors, σ^{54} and σ^{70}. The members of the σ^{70} family have four domains joined by linkers (22). σ^{70} is the main σ factor and allows RNAP to recognize the majority of promoters in the *E.coli* genome (75). *E. coli* has 6 other σ factors: σ^{28}, σ^{32}, σ^{S}, σ^{E}, σ^{Fec1} and σ^{54}. There are also anti-σ factors that bind and sequester σ factors away from RNAP (62). The region where the σ factor binds DNA relative to the transcription start site, +1, differs depending on the σ factor family. The σ^{70} family recognizes −10 and −35 hexamers while the σ^{54} family recognizes −12 and −22 elements. The σ^{54} family has no sequence similarity to the σ^{70} family (19). Unlike the σ^{70} family, σ^{54} absolutely requires an activator for transcription (19).

TFs down or up-regulate transcription by influencing RNAP. Most TFs bind DNA specifically at or upstream of a promoter (17). In the *E. coli* genome, there are >300 predicted TFs (145); 49% percent of genes are regulated by multiple TFs. There are a few global regulatory proteins that influence a large number of genes while in other cases TFs only regulate 1-2 genes (112). In *E. coli*, seven global regulatory proteins (CRP, FNR, FIS, IHF, ArcA, NarL, Lrp) directly modulate 51% of the genome (112). Global regulators are TFs that are defined by their pleiotropic phenotypes, their ability to regulate diverse metabolic pathways and are not proteins which are part of cellular machinery *i.e.* ribosome or RNAP (51). Global regulators often work with specific "local regulators", *i.e. melAB* is regulated by both CRP, a global regulator, and MelR, a local regulator (185). The mechanism of this kind of co-regulation varies. Most TFs work independently of other TFs to up or down regulate transcription from a specific promoter, but there are some TFs with a more complex mechanism where repressors and activators interact directly with each other, *i.e.,* the CytR-CRP regulation of CytR dependent promoters (17, 25).

1.6.1 The 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 (168). 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. The flagellar regulon consists of >50 genes and >17 operons

with FlhD$_2$C$_2$ as the master regulator. FlhD$_2$C$_2$ activates the σ^{70} dependent class II genes (102). Class II genes encode proteins that compose the flagellar export system and the basal body proteins as well as genes encoding σ^{28} (91) and FlgM, the anti-σ^{28} factor (92, 134). 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 (71). 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.

1.6.2 The virulence regulons.

There are two groups of virulence genes in *S. typhimurium*: those involved with the enteropathogenic phase of infection (intestinal invasion) and those involved in the systemic phase of infection. The SPI-1 genes and their associated effector proteins are key to the enteropathogenic phase of infection. However, the SPI-1 genes are not required for systemic infection. The primary regulator for SPI-1 expression is HilA, a SPI-1 encoded protein in the OmpR/ToxR family (6). HilA expression is positively influenced by a multitude of proteins: CsrAB, Fis, Fad, FliZ, OmpR/EnvZ, HilC and HilD (177). HilA expression has also been shown to be negatively influenced by many factors: PhoPQ, PhoBR, Lon protease, Ams, HupB and Hha (177). Currently, HilA is thought to activate two operons encoded on SPI-1: *prgHIJK-orgABC* and *invFGEABC-spaMNOPQRS-sicA-sipBCDA-iacP-sicP-sptP* (103). As seen from this operon structure, *invF* is transcriptionally activated by HilA. InvF positively regulates SPI-1 encoded effectors which are secreted by the SPI-1 encoded TTSS (37). SPI-1 encoded effectors are transcribed from a promoter just upstream of *sicA* creating a truncated version of the *invF* transcript (31). In addition, there are at least seven virulence factors encoded by genes located outside SPI-1: SopA, SopD, SopE, SopE2, SlrP, SspH1 and SopB (SigD) (152). The transcription of these seven proteins is thought to be activated by InvF but not HilA (37).

Known regulators and genes that are involved in the systemic phase of infection include: *slyA*, *spvR*, *spvABCD* and 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 (23, 28, 63, 133, 162). The regulator for SPI-2 is likely *ssrAB* which encodes a two-component regulatory

system (43). SlyA is regulator required for systemic infection and survival in macrophages (18, 23) but not for enteric infection (186). SlyA has also been shown to stimulate *ssrA* transcription (43). The *spv* genes are also involved in systemic infection and survival in macrophages (23, 61, 101, 114). Although it is unclear how *slyA*, *spvRABCDE* and SPI-2 could be co-regulated expression of all three loci has been implicated in systemic infection and intracellular survival.

1.7 Thesis Objective.

The objective of this thesis research was to characterize RITM and to formulate a hypothesis for possible mechanisms of RITM in *S. typhimurium* 14028. This objective was addressed in the following four ways: i) 33 promoters for further study from 192 previously isolated (50) rifampicin responsive library clones was selected. ii) The nucleotide sequence of the smaller set of RRPs was determined and previously reported pathways involving RRPs were examined to find candidate regulators of RITM. iii) Eight RRPs were tested in global regulatory mutants to determine if these regulators were involved in RITM. iv) Possible regulatory mechanisms and directions for further studies of RITM were proposed.

Chapter 2. Methods and materials

2.1 Bacterial strains and culture methods.

Strains used in this study are listed in Table 2.1. Luria-Bertani (LB) broth or LB agar (LBA) plates were used for routine growth of strains; these were prepared as described previously (157). M9 agar plates for crp⁻ strain analysis and *lacZ* assays and SOC broth for electroporation were prepared as described previously (157). All media components were obtained from BD BioSciences (Oakville, Ontario). Cultures were grown at 37°C. Overnight liquid cultures were grown in shaking test tubes or flasks while cultures in 384 or 96-well plates were grown without shaking. Media were supplemented with tetracycline (15 μg/ml), chloramphenicol (30 μg/ml), ampicillin (100 μg/ml), kanamycin (25 μg/ml) and rifampicin at various concentrations as appropriate. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO) or from the laboratory collection.

2.2 Lux reporter screening.

2.2.1 Library construction and screening.

A random promoter-*luxCDABE* library consisting of 6528 clones (17 x 384-well plates) in *S. typhimurium* 14028 was obtained from Dr. M.G. Surette (University of Calgary). Briefly, this library was constructed by partially digesting *S. typhimurium* 14028 genomic DNA with *Sau*3AI and by ligating the resulting fragments into the *Bam*HI site of the *lux*-reporter plasmid pCS26-*Pac* (Figure 2.1). Genomic DNA-reporter constructs were transformed into *E. coli*, *E. coli* plasmid DNA was harvested and plasmid DNA was used to transform *S. typhimurium* 14028. Further details on library construction can be obtained from Bjarnasson *et al.*(11). Methods for screening the library with rifampicin and erythromycin can be obtained from Goh *et al.* (50) which is provided in Appendix 7.1.

2.2.2 Screening of 192 rifampicin positives.

Luminescence measurements were taken in 96-well sterile, clear bottom microtitre plates with white opaque walls (Costar #CS3610, Fisher Scientific, Canada) and seed cultures were grown in clear, sterile polystyrene plates (Fisher Scientific, Canada). Seed culture plates typically contained 150 μl of LB with kanamycin per well. The 96-well seed culture plates containing the 192 rifampicin positives (2 x 96-well plates) described in Section 3.2 were inoculated from
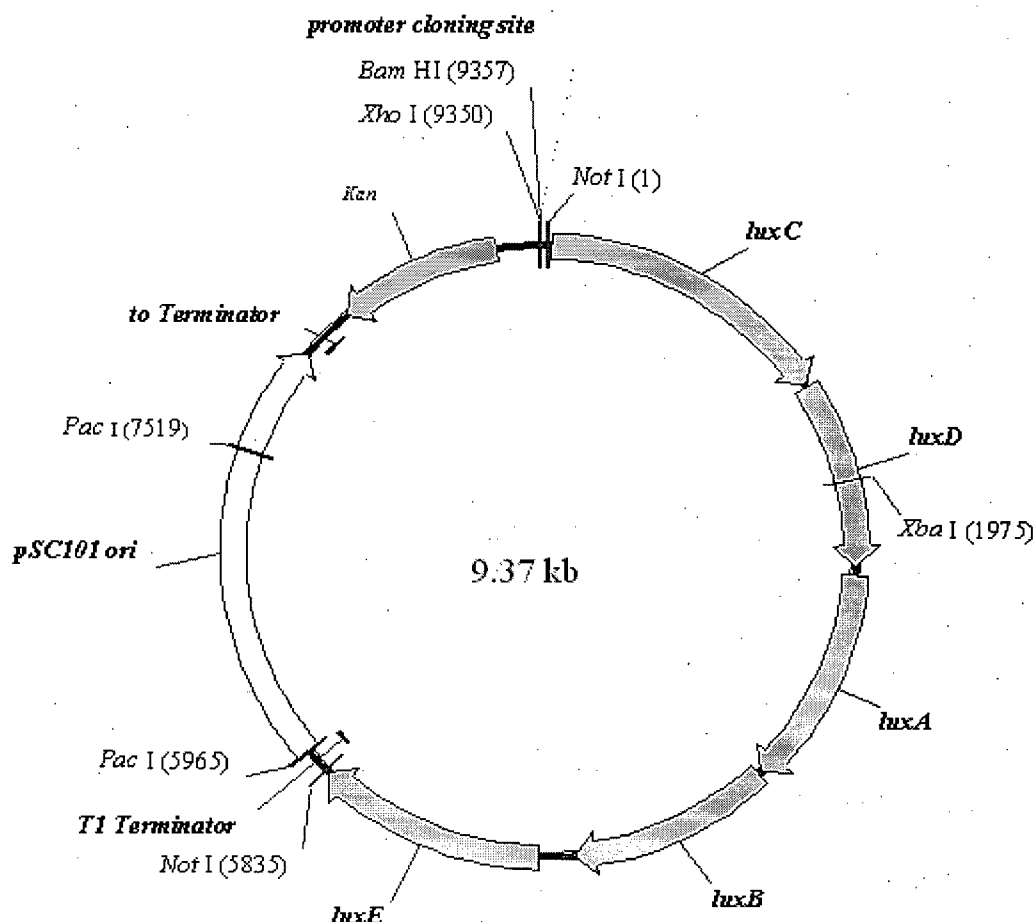
Figure 2.1. Promoter-*lux* reporter plasmid pCS26-*Pac*. Random genomic fragments were cloned into the *Bam*HI site (see (11) for details of library construction). Promoters drive expression from the *luxCDABE* operon producing luminescence (light at 490 nm) and light production is used as a measure of promoter activity. 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 LuxCDABE catalyzed reaction is as follows where R represents a long carbon chain:

$$RCHO + FMNH_2 + O_2 \rightarrow FMN + RCOOH + H_2O + light\ (490\ nm)\ (119).$$

Due to the pSC101 origin of replication, the plasmid is maintained at 3-5 copies in the cell. No exogenous substrate is needed to produce light as the *luxCDE* genes are present.

Table 2.1. List of strains.

	Genotype	Source and/or reference
E. coli strains		
M182	Δ(*codB-lacI*), *galK16*, *galE15*(gal^S), *LAM*$^-$, *e14*$^-$, *relA1*, *rpsL150*, *spoT1*	J.Green (190)
JRG4830	M182 Δ*crp*	J.Green (190)
JRG4864	M182 Δ*hns*; cam^R	J.Green (190)
JRG4747	M182 Δ*fnr*; cam^R	J.Green (190)
MC4100	*araD139*, Δ(*argF-lac*)*169*, *LAM*$^-$, *e14*$^-$, *flhD5301*, *fruA25*, *relA1*, *rpsL150*, *rbsR22*, *deoC1*	R. Hengge-Aronis (94)
RH100	MC4100 *rpoS360 zfi3251*::Tn*10*	R. Hengge-Aronis (94)
None given	MC4100 *lrp*::Tn*10*	C.J.Dorman (111)
RM313	MC4100 *arcA1 zjj*::Tn*10*	G. Sawers (153)
RM611	MC4100 *narP253*::Tn*10*d *narL215*::Tn*10*	G. Sawers (153)
CLG149	MC4100 *fis*::*cat*	C.Gutierrez (16)
S. typhimurium strains		
14028 pCS26-*Pac*	Wild type with *lux*-reporter plasmid	M.G.Surette (11)
PP1037	LT2 *crp-773*::Tn*10 trpB223*	Salmonella Genetic Stock Centre (SGSC) (158)
	14028 *crp-773*::Tn*10*	This study
TH1764	LT2 *fis-1*::*tet leuA414 hsdSB*(r$^-$m$^+$) *fels*$^-$	R. Osuna (137)
	14028 *fis-1*::*tet*	This study
DL3157	14028 *hns-6*::Tn*10*	D.L. Low (127)
JE3999	LT2 *metE205 ara-9 ihfB*::*cat*	J.C. Escalante-Semerena (140)
	14028 *ihfB*::*cat*	This study
TN2336	LT2 *leuBCD485 pepT81*::MudJ (mini-mud *lac*+ kan^R) *fnr-2*::Tn*10* (*fnr = oxrA*)	C.G. Miller
	14028 *fnr-2*::Tn*10*	This study
SF1005	14028 *rpoS*, amp^R	SGSC (158)
	SL1344 *sseA*::*lacZ*, amp^R	B. Coombes, UBC
	SL1344 *sseD*::*lacZ*, amp^R	B. Coombes, UBC
	SL1344 *sseE*::*lacZ*, amp^R	B. Coombes, UBC

frozen stocks (or previous seed culture plates) using a 96-well pin replicator and were incubated overnight at 37°C. The 96-pin replicator was also used to inoculate the screening plates containing 150 μl of LB kanamycin with or without rifampicin (2.5 μg/ml) per well from the seed culture plate. One freshly inoculated screening plate was placed in a Victor II Multi-label Counter (PerkinElmer, Boston, MA), incubated at 37°C and luminescence measured every hour for 16-21 hrs. Microtitre plates read with the Victor II were sealed with a Breathable Sealing

Membrane (#163340, Nalge Nunc, Naperville, IL) without shaking. The 192 positives were measured twice in each condition, LB kanamycin with or without rifampicin, for a total of eight overnight readings. Peak luminescence readings for replicates were averaged. Thirty-three clones with three-fold or greater differential luminescence in response to rifampicin and readings greater than 1000 cps were selected for further analysis.

2.2.3 Screening for rifampicin effects.

Overnight liquid cultures (5 ml) were grown from single colonies for each of the 33 promoter clones and the strain containing the empty vector control. For each clone, 150 µl of overnight culture/well were placed in one column (eight wells) of a 96-well plate, to create a seed culture plate. As each 96-well plate contained twelve strains, three seed plates were created. Solutions of LB supplemented with kanamycin and 0, 0.16, 0.31, 0.63, 1.3, 2.5, 5.0 and 10 µg/ml of rifampicin were made and 150 µl of each solution were placed in the columns of the screening plate using a Multidrop 384 (Thermo Labsystems, Waltham, MA). Column A contained 10 µg/ml of rifampicin, column B contained 5.0 µg/ml of rifampicin, *etc*. Screening plates were inoculated from the seed plate using a 96-pin replicator and luminescence read as described in Section 2.2.2.

2.3 Plasmid DNA isolation.

Plasmid DNA was isolated from 2 ml overnight cultures by alkaline lysis and phenol:chloroform (1:1) extraction as described previously (157) except the final resuspension of the plasmid DNA was in Tris-HCl buffer (10 mM, pH 8.5).

2.4 Colony PCR and PCR methods.

PCR primers are listed in Table 2.2. Standard PCR was conducted as previously described (34). For colony PCR, instead of a DNA solution a portion of an agar colony was transferred to a standard PCR mix using a sharp, sterile, wooden toothpick.

2.5 Nucleotide sequencing.

PCR products used as template in a sequencing reaction were cleaned with QIAquick PCR purification kit (Qiagen, Mississauga, Ontario). Nucleotide sequencing reactions (20 µl) were

performed with BigDye Terminator v3.1 Cycle Sequencing Kit (ABI, Foster City, CA) according to manufacturer's instructions using PCR product or plasmid DNA as template. Excess dye terminators were removed using CentriSep columns (Princeton Separations, Adelphia, NJ). Nucleotide sequencing was performed at the NAPS Unit (Vancouver, BC) or at the CBR Nucleotide Sequencing Facility (Victoria, BC).

Table 2.2 List of primers.

Primer	Nucleotide Sequence (5' to 3')	Reference/Source
pCS26for	TGGCAATTCCGACGTCTAAG	This study
pCS26rev	CACTAAATCATCACTTTCGG	This study
pri1	ACA TGA AGG TCA TCG ATA GCA GGA	(126)
pri2	GGC TGT TGA GTT GAG GTT GAC GAA	(126)
pri3	AAC AGT AAT GGG CCA ATA ACA CCG	(126)
pri4	CGA GTT CGC ACA TCT TGT TGT CTG	(126)
ihfb_for	GCT AAA GGC GAG TAA TCC	This study
ihfb_rev	GGT GGC AGT AAT GAC GAC	This study
fis_for	AGC ATT ATC TGG ACA CTG GG	This study
fis_rev	GAG GTT CAC ATT CCG CTT TC	This study
catintF	AAT GTA CCT ATA ACC AGA CCG	Lab stock
catintR	ATA TTG GCC ACG TTT AAA TC	Lab stock

2.6 Analysis of insert sequences.

Plasmid inserts of the 33 clones identified in Section 3.2 were sequenced using forward (pCS26for) and reverse (pCS26rev) primers (see Table 2.2) that hybridize to either side of the pCS26-*Pac Bam*HI site. Insert sequences were compared to the annotated *S. typhimurium* LT2 chromosome and virulence plasmid nucleotide sequence (117) available in the TIGR database (www.tigr.org) using the batch nBLAST command available on the UBC Bioinformatics (UbiC) local BLAST server. Annotation in TIGR, information from EcoCyc (83), RegulonDB (156) and literature were used to identify putative ORFs, promoters, operons, regulators and pathways for genes encoded on the plasmid inserts (see Appendix 7.3 for compiled information). Gene orientation, computational operons and experimental operons reported in the literature or in the databases listed above were used to name and identify putative promoters. Figure 2.2 shows an example of the process by which promoters were identified. Analyses were done in a similar fashion for the other 32 promoter-*lux* constructs.

2.7   Construction of *S. typhimurium* 14028 regulatory mutants.

Generalized transduction using P22HT*int* was performed as described elsewhere (172) with the following modifications: transductants were selected on LBA plates containing 10 mM EGTA and the appropriate antibiotic. Transductants were single colony purified a minimum of two times and then cross-streaked on LBA plates with P22H5 lysate to confirm sensitivity to P22. P22HT*int* lysates were also streaked on LBA to confirm complete lysis of the donor strain.

2.8   Verification of transductants.

2.8.1   PCR and sequencing of transductants.

14028 *fis-1::tet* and 14028 *ihfB::cat* were verified by PCR followed by nucleotide sequencing. The PCR product from 14028 *fis-1::tet* was obtained using the primers fis_for and fis_rev and the PCR product from 14028 *ihfB::cat* was obtained using the primers catintF and ihfB_for. The PCR products were cleaned and sequenced as described in Section 2.5. Sequencing for the *fis⁻* and *ihfB⁻* strains was performed using one PCR primer and the PCR product as template.

2.8.2   Genomic DNA isolation.

Genomic DNA was isolated from 2 ml overnight cultures as follows (59). Cells were resuspended in 400 µl TES (50 mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, pH7.5). Sarkosyl (17 µl of a 30% w/v stock solution), Proteinase K (5 µl of a 10 mg/ml stock solution) and RNase A (2 µl of a 20 mg/ml stock solution) were added to the cell suspension and incubated at 37°C for 30-60 minutes, or until the solution cleared. Ammonium acetate (200 µl of a 8M stock solution) was added and mixed well. The solution was extracted twice with an equal volume of phenol:chloroform (1:1) and then with an equal volume of chloroform. DNA was precipitated with an equal volume of isopropanol. DNA was reprecipitated in ethanol by the slow addition of 1/10 vol. sodium acetate (3.0M, pH 5.2) and incubation at room temperature for 15 minutes. DNA pellets were obtained by centrifugation at 11,000 g for 15 minutes, washed in 1 ml of cold 70% ethanol, dried, resuspended in 50 µl TE (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at 4°C.

2.8.3   iPCR methods.

iPCR products were obtained from the constructed 14028 *fnr-2::*Tn*10* strain as described
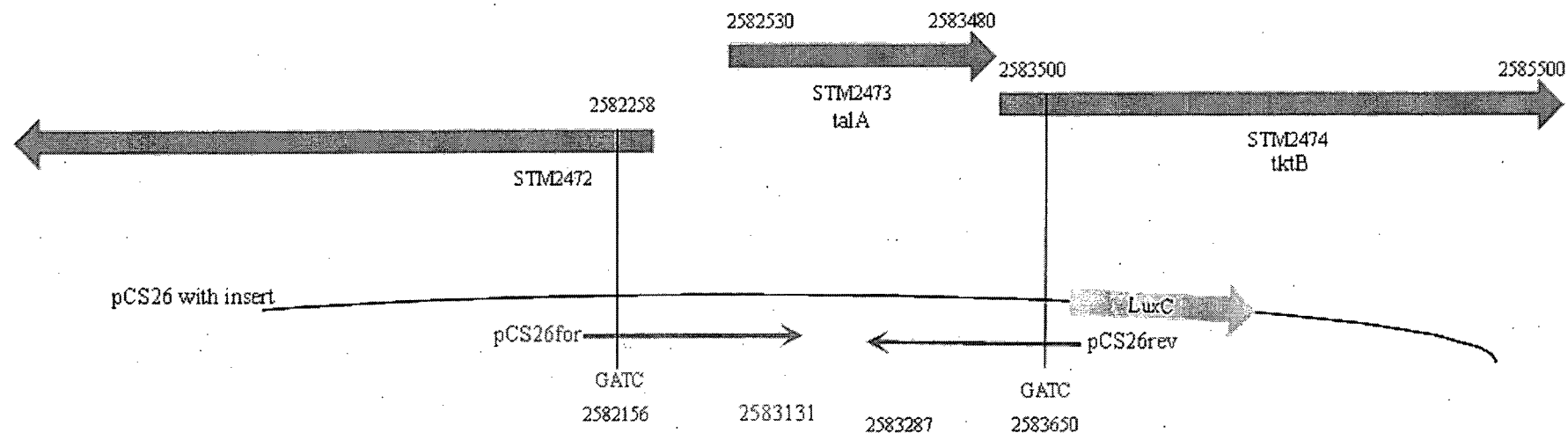
Figure 2.2. Analysis of *S. typhimurium* clone B-D08. The dark grey filled arrows show annotation for the insert sequence and its flanking sequences. Numbers above the filled arrows indicate chromosomal coordinates for the beginning and end of the indicated ORF. The line arrows indicate the sequence obtained from the indicated primer: pCS26for or pCS26rev. Numbers below line arrows indicate the chromosomal coordinates of the insert sequence from the respective primer. GATCs represent the *Sau*3AI sites that are the ends of the *Salmonella* insert. The corresponding promoter for this construct has been identified as *talA*. *talA* and *tktB* are known to constitute an operon, *talA* being the first gene in the operon (information from RegulonDB).

previously (126, 132) with a few modifications: ~ 5 µg of genomic DNA was digested overnight at 37°C with 25 U of *Hpa*II (Roche, Laval, Quebec) according to manufacturer's instructions and inactivated 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, Canada) in a total volume of 25 µl as per manufacturer's instruction for 16 hrs at 12°C. Ligation mix (2.5 µl) was used as template in a 25 µl PCR reaction (34) with the primers pri1 and pri2 (see Table 2.2). PCR product from the pri1/pri2 PCR reaction (2.5 µl) was used as template in a 25 µl PCR reaction (34) with primers pri3 and pri4 (see Table 2.2). The final PCR product was treated and sequenced as described in Section 2.5 using pri3.

2.8.4 Phenotypic verification of *crp⁻* strain.

To assay for the presence of the *crp⁻* mutation, strains were tested for growth on M9 agar with 0.4% glycerol as described elsewhere (158). Stable transductants were incubated on M9 agar plates with the respective carbon sources for 24 hrs at 37°C. Putative *crp⁻* strains showed no growth on 0.4% glycerol and growth of large colonies on 0.4% glucose. The *S. typhimurium* 14028 wild type strain grew well on both carbon sources.

2.9 Transformation of *E. coli* and *S. typhimurium*.

All transformations were done by electroporation as described in the Gene Pulser™ manual (BioRad, Mississauga, Ontario). In short, electrocompetent cells were prepared by inoculating 100 ml of LB with 1 ml of overnight culture and grown to an OD_{600} of 0.4-0.6. For the following washings, cells were pelleted by centrifugation at 4000g for 15 minutes. Cells were washed twice with ice cold, sterile ddH_2O, once with 100 ml then once with 50 ml. Cells were then washed with 2.0 ml of cold, sterile 10% glycerol and resuspended in 200 µl of cold, sterile 10% glycerol. Aliquots of cell suspension (40 µl) were stored in 0.5 ml microcentrifuge tubes and kept at -80°C for later use. When required aliquots were thawed on ice, mixed with 0.5 to 1.0 µl of plasmid DNA and placed in 0.1 cm cuvettes. Cells were electroporated using a BioRad Gene Pulser™ (Mississauga, Ontario) at 200 Ω, 25 µF and 1.8 kV, resuspended in 1.0 ml of SOC and incubated, shaking at 37°C for 1 hr. SOC cell suspension (typically 50 µl) was plated on LB agar with kanamycin (pCS26 antibiotic marker). Single colonies were streaked on LBA supplemented with kanamycin and the appropriate antibiotic marker for the strain.

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2.10 Testing of mutant arrays.

To prepare the *E. coli* and *S. typhimurium* seed culture plates all wells of a 96-well plate were filled with 150 μl of LB kanamycin. Each well was inoculated with a single agar colony of a different mutant-reporter clone using a sterile toothpick. Solutions of LB kanamycin with 0, 2.5 and 5.0 μg/ml of rifampicin were made for *S. typhimurium*. Solutions of LB kanamycin with 0, 1.0 and 2.5 μg/ml of rifampicin were made for *E. coli*. One solution was used to fill an entire 96-well luminescence plate (96 x 150 μl) using a Multidrop384 (Thermo Labsystems, Waltham, MA). At least four plates of each concentration of rifampicin were read for luminescence, each plate being read for 16-21 hrs. Screening plates were inoculated from the seed plate using a 96-pin replicator and luminescence read as described in Section 2.2.2. The average and standard deviation were calculated at each time point using the four replicate measurements.

2.11 β-galactosidase assays.

Overnight cultures of *lacZ* reporter strains were diluted 1:200 into 5 ml of melted 0.7% (soft) agar and poured over room temperature M9 agar plates supplemented with glucose (0.4%), L-histidine (40 μg/ml) and X-gal (40 μg/ml). Histidine was added to plates as SL1344 is a histidine auxotroph. X-gal was added as the substrate for *lacZ*. Transcription was scored by the intensity of blue which was indicative of cleaved X-gal: 0 = no response, + = weak response, ++ = intermediate response and +++ = strong response. Sensi-discs (BD BioSciences, Oakville, Ontario) or premade sterile filter discs holding antibiotic solution were placed on top of the soft agar overlays. M9 plates were incubated at 37°C for one night (*sseA* reporter strain) or three nights (*sseD* and *sseE* reporter strains) depending on the density of growth.

Chapter 3. Results.

3.1 Screening of *S. typhimurium* promoter-*lux* library with rifampicin and erythromycin.

A random promoter-*luxCDABE* library consisting of 6528 (17 x 384-well plates) clones in *S. typhimurium* was screened for changes in transcription caused by sub-MIC concentrations of rifampicin and erythromycin (50). This library was screened several times with rifampicin and erythromycin, measuring luminescence production at two to four time points depending on whether it was one of the initial screens or a rescreen. After each screen/rescreen responsive clones were selected and assayed in the next screen. Responsive clones were defined as clones displaying at least three times differential luminescence between LB supplemented with kanamycin at 25 μg/ml (for plasmid maintenance) and LB supplemented with kanamycin at 25 μg/ml and the antibiotic being tested. The initial screens suggested that each antibiotic affected transcription of ~ 5% of *S. typhimurium* genes. Of the 5%, 192 of the most reproducibly responsive rifampicin clones were chosen (2 x 96-well plates) and comprised the selection of rifampicin responsive promoters (RRPs) for later study. Some of these 192 clones had their plasmid inserts partially sequenced. Sequences encoded genes for a variety of cellular functions. For details please refer to Appendix 7.1 which is the publication resulting from this data (50) and Appendix 7.2 for the corresponding sequences.

3.2 Selection of rifampicin responsive clones for further study.

A set of consistently responding clones was selected from the 192 clones in Section 3.1 for detailed study. To obtain this subset of clones, the 192 clones were rescreened a minimum of two times in the presence of 2.5 μg/ml rifampicin reading every hour for at least 16 hours. More frequent measurement of the 192 clones was possible since only two 96-well plates were being measured as opposed to the entire library as was done in Section 3.1. Measuring luminescence more frequently allowed different times of peak expression to be distinguished. Luminescence often peaked sharply late in exponential phase and the time of peak expression differed slightly due to different growth rates between the control (LB) and test (LB + rifampicin) conditions and also between clones (see Figure 3.1). In the case shown in Figure 3.1, *STM1252* peaked at the same time in both conditions; however, *STM1252* and *cirA* peaked at a different time. If the luminescence reading was taken at four hours, this would correctly identify the peak expression of *STM1252* but the peak value of *cirA* would be identified as
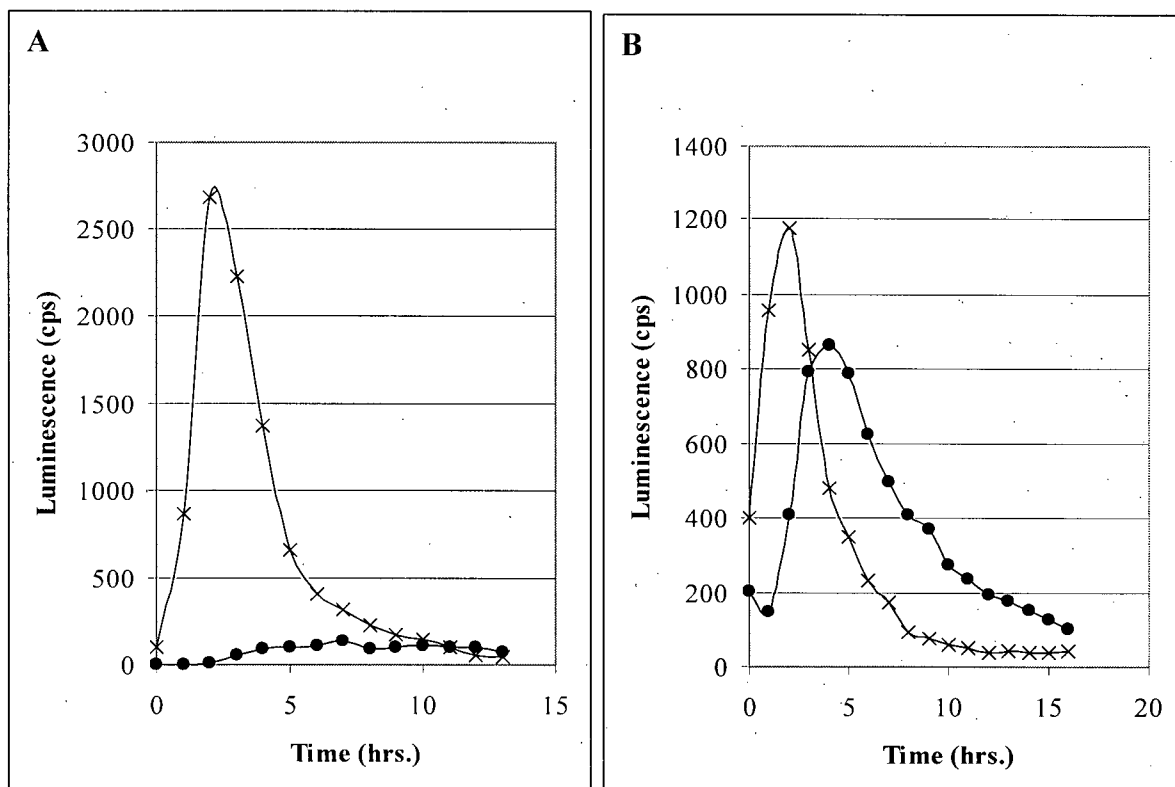
Figure 3.1. Differential peak luminescence of promoter-*lux* reporters. Luminescence of
S. typhimurium 14028 reporters *STM1252::luxCDABE* (•) and *cirA::luxCDABE* (X) in LB
supplemented with kanamycin at 25 μg/ml (A) and LB supplemented with kanamycin at 25
μg/ml and rifampicin at 2.5 μg/ml (B).

almost three times lower than its maximum value. The peak luminescence values were used to compare expression with and without rifampicin. Forty-eight clones had three-fold or greater differential response in response to rifampicin. From these 48 clones, 15 clones were discarded as they had readings below 1000 cps in both LB kanamycin with or without rifampicin. Thus, 33 highly differentially transcribed and strong promoters were chosen so that RITM could be easily detected in later analyses.

3.3 Rifampicin concentration dependence of responsive clones.

To determine the dynamics of the rifampicin response and the optimal concentration for subsequent assays, the luminescence of the 33 clones identified in Section 3.2 was measured at different concentrations of rifampicin. Rifampicin concentrations ranging from 10 – 0.1 μg/ml at two-fold dilutions and 0 μg/ml were measured. The response to rifampicin was concentration dependent for all *S. typhimurium* clones tested (Figure 3.2). The reduced luminescence at 10 μg/ml of rifampicin for clones may have been the result of a general growth limitation. The minimal inhibitory concentration (MIC) of *S. typhimurium* in LB kanamycin was ~ 12 μg/ml.

3.4 Sequencing of rifampicin responsive clones.

To identify the genes that were most strongly affected by rifampicin, the plasmid inserts of the 33 clones identified in Section 3.2 were sequenced from the 3' and 5' ends and analyzed as described in Materials and methods. Insert size estimated from annotation and confirmed by PCR ranged from 0.3 to 4.3 kb. Infrequently, inserts contained two noncontiguous fragments of genomic DNA. Where two fragments were present, usually only one promoter was in the appropriate direction to drive *lux* expression. In the cases where there were two or more genes appropriately oriented, the genes had been computationally or experimentally reported to be in the same operon. In such cases constructs were named for the first gene in the operon (which was always present in the insert fragment). In the cases where there were two possible promoters from different operons, the construct was named for the promoter closest to the *lux* operon. Genes encoding a variety of functions were identified. Most notably, genes involved in virulence, motility and metabolism were found. A summary of insert characteristics is provided in Table 3.1. Supplemental data on the 33 RRPs found in various databases is listed in Appendix 7.3.
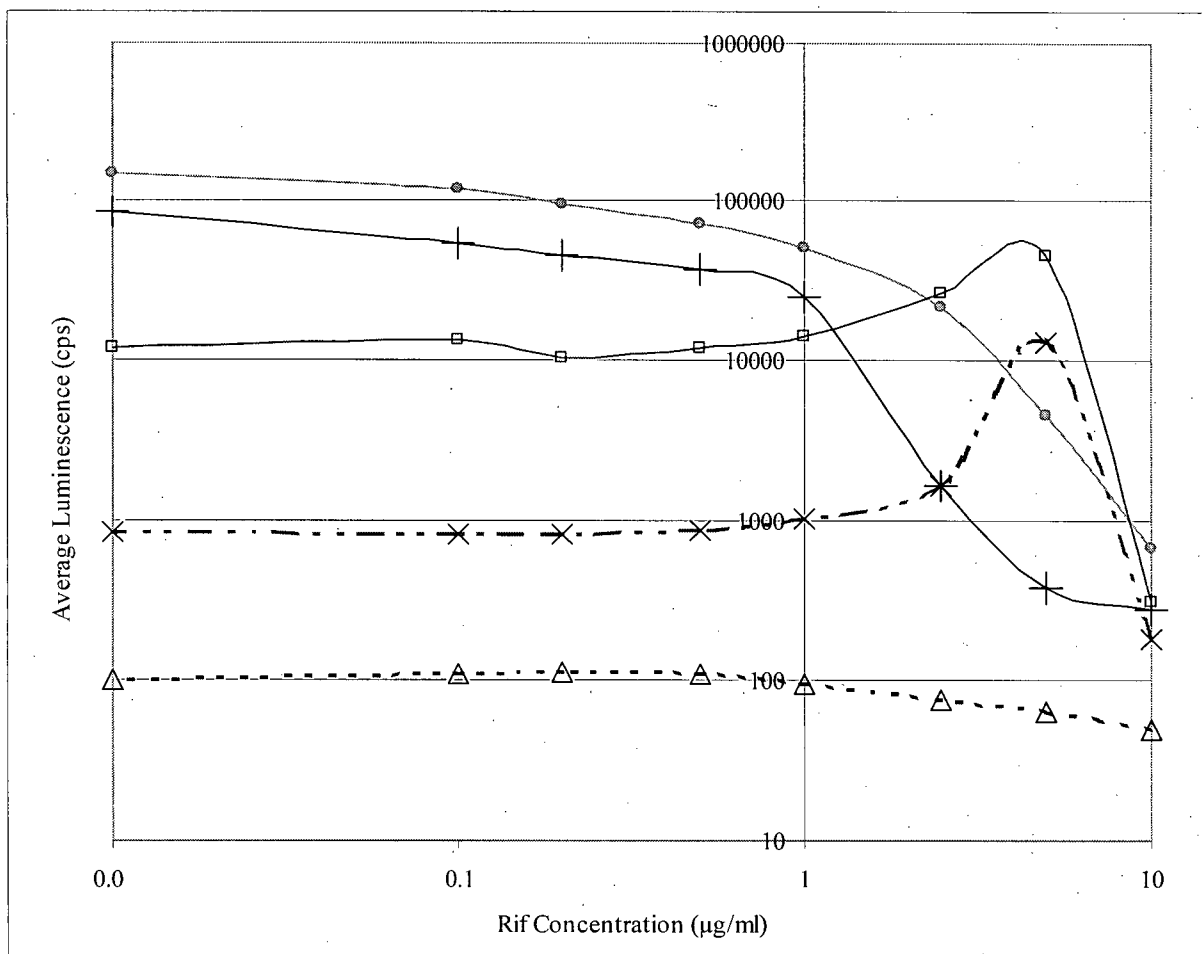
Figure 3.2. Concentration dependence of rifampicin induced transcription modulation.
S. typhimurium 14028 with *fliA* (●), *invF* (+), *STM3595* (X) and *ucpA* (□) *luxCDABE*
reporters and pCS26 without insert (Δ) grown in LB supplemented with kanamycin at 25 μg/ml
and the indicated concentration of rifampicin were grown for 16-24 hrs. Peak luminescence
values from each time course are plotted here.

Six pairs of duplicate clones (*STM3595, yijP, osmE, invF, ucpA* and *talA*), were found during sequencing. This was not surprising as the library was predicted to have a three-fold redundancy (11). Pairs of similar clones did not necessarily encode identical fragments, having different 5' and 3' ends and/or an additional fragment from elsewhere in the chromosome; such clones responded similarly to rifampicin with respect to the direction and magnitude of response but often differed in basal expression levels. Different basal expression levels could be due to background expression driven by the other promoters in the fragment. Results from duplicate clones were removed from Table 3.1 for the sake of clarity.

3.5 Transcription Profiling of *S. typhimurium* 14028 global regulatory mutants.

3.5.1 Construction of a 56-strain *S. typhimurium* 14028 mutant array.

Analysis of promoters in Section 3.3 revealed that rifampicin affected a broad range of promoters. It seemed possible that rifampicin was modulating a global transcription regulator or activating one or more stress responses. To examine these possibilities, a selection of RRP containing fusions were transformed into *S. typhimurium* 14028 strains carrying mutations in one of six major transcription and sigma factors: CRP, FNR, FIS, H-NS, IHF and σ^S. Where necessary, mutant alleles were introduced into the 14028 background using P22HT*int* mediated generalized transduction. Strains were confirmed by PCR or iPCR followed by nucleotide sequencing and phenotypic analysis. The RRPs were chosen to include ones that were either up or down-regulated and to reflect the diversity of cellular functions influenced by rifampicin. The four constructs containing up-regulated genes were *STM3595, spvAB, ucpA* and *talA*. The four down-regulated genes were *fliA, flhBA, cirA* and *STM1328*. The six regulator mutants and the wild type strain containing the eight RRP fusions resulted in a 56-strain array. Each strain except the *ihfB⁻* strain with the *cirA::luxCDABE* reporter was validated for the appropriate insert size by colony PCR using the primers pCS26for and pCS26rev. In general, the strains were assayed using rifampicin at 0, 2.5 µg/ml and 5.0 µg/ml.

3.5.2 Regulators of tested promoters under control conditions.

Levels of expression in the regulator mutants grown in LB supplemented with kanamycin but without rifampicin were used to determine which regulators positively or negatively influenced the tested RRPs (Table 3.2). Significant differences, greater than 2.5-fold, in luminescence

Table 3.1. Characteristics of rifampicin responsive promoters in *S. typhimurium* 14028.

Promoter identification	Light units (cps)			Fold induction /repression by Rif			
	LB	LB+Rif (2.5 µg/ml)	LB+Rif (5.0 µg/ml)	Rif (2.5 µg/ml)	Rif (5.0 µg/ml)	Insert Size (kb)	Putative Function
STM3595	800	1,600	13,000	↑2.0	↑16	3.3	unknown
STM4454/treB	4,200	18,000	54,000	↑4.3	↑13	2.1	carbon metabolism
STM0425/thiI	1,900	5,200	16,000	↑2.7	↑8.4	~3.6	RNA modification
STM1444/slyA	1,000	2,600	5,200	↑2.6	↑5.2	1.8	virulence
STM1154/yceE, STM1155/htrB	10,000	26,000	38,000	↑2.6	↑3.8	2.9	membrane biosynthesis
STM0389/yaiA	2,600	4,700	10,000	↑1.8	↑3.8	0.4	unknown
STM2445/ucpA	12,000	26,000	45,000	↑2.2	↑3.8	0.8	carbon metabolism
STM4118/yijP	2,600	3,900	8,700	↑1.5	↑3.3	1.2	virulence
STM2473/talA	3,100	4,500	9,200	↑1.5	↑3.0	~4.3	carbon metabolism
pSLT040-041/spvAB	4,700	7,000	13,000	↑1.5	↑2.8	3.3	virulence
STM2287 (intragenic)	2,900	3,700	7,100	↑1.3	↑2.4	0.2	unknown
STM1311/osmE	9,300	13,000	18,000	↑1.4	↑1.9	1.5	osmotic stress
STM1597/ydcW	3,500	2,900	6,400	↑0.8	↑1.8	1.4	carbon metabolism
intragenic of STM2946	150	230	240	↑1.5	↑1.6	0.2	unknown
STM0940/ybjX	10,000	7,400	14,000	↑0.7	↑1.4	0.3	membrane biosynthesis
STM1833	100	130	130	↑1.3	↑1.3	0.8	unknown
STM3248/garR/yhaE	380	830	420	↑2.2	↑1.1	3.1	carbon metabolism
STM2899/invF	84,000	1,600	370	↓53	↓230	1.1	virulence
STM1091/sopB	45,000	850	280	↓53	↓160	1.6	virulence
STM2066/sopA	16,000	220	140	↓73	↓114	1.7	virulence
STM1956/fliA (σ^F)	150,000	22,000	4,600	↓6.8	↓33	0.7	motility
STM1914-1913/flhBA	6,600	1,400	250	↓4.7	↓26	3.2	motility
STM1328	2,500	110	100	↓23	↓25	1.5	unknown
STM1248	2,800	150	150	↓19	↓19	0.4	unknown
STM1183/flgK	94,000	26,000	5,200	↓3.6	↓18	1.4	motility
STM4257-STM4258	3,200	200	240	↓16	↓13	3.7	virulence
STM2199/cirA	2,200	680	520	↓3.2	↓4.2	2.7	inorganic ion metabolism
ilvC	3,100	3,200	1,800				amino acid synthesis
ilvLG	390	170	110				amino acid synthesis
pCS26	100	80	60				promoterless vector

between mutant and wild type strains occurred in quite a few cases indicating that the tested regulator positively or negatively affected transcription of the respective promoter. Differences between mutant and parent luminescence levels that were greater than or equal to 2.5-fold are described below for each RRP fusion:

i. *flhBA::luxCDABE*

Basal expression levels in the *crp⁻* and *fis⁻* strains were reduced by 17-fold and 15-fold, respectively, when compared to the wild type. This suggested that CRP and Fis positively regulated *flhBA* transcription under the conditions tested.

ii. *fliA::luxCDABE*

fliA is another class II flagellar gene (91) and as with *flhBA*, it was positively regulated by CRP and Fis. Basal level expression in *crp⁻* and *fis⁻* mutants was 21 times and 23 times lower, respectively, when compared to the parent strain.

iii. *cirA::luxCDABE*

Expression levels in the *fis⁻* and *ihfB⁻* strains were six-fold and 34-fold lower, respectively, than the wild type strain suggesting that Fis positively influenced *cirA* transcription. However, lower luminescence in the *ihfB⁻* strain likely did not reflect lower transcription levels of *cirA* but a low level of reporter plasmid. PCR product could not be obtained from the insert of this strain and kanamycin resistance, assayed by a filter disc method, was lower than in other *ihfB⁻* strains (results not shown here).

iv. *STM1328::luxCDABE*

The *fnr⁻* and *rpoS⁻* mutations caused an 11-fold and a 6-fold increase, respectively, in the basal expression when compared to the parent. The data implies that FNR and σ^S negatively influence expression of *STM1328* under the conditions examined.

v. *spvAB::luxCDABE*

The *crp⁻*, *fis⁻* and *hns⁻* strains showed a 20-fold, 18-fold and five-fold increase in luminescence compared to the parent, respectively. In contrast, the *rpoS⁻* mutation caused a 70-fold decrease in the control expression level when compared to the parent. CRP, Fis and H-NS have a negative influence on *spvAB* expression and σ^S is likely required for *spvAB* transcription under the conditions tested.

vi. *STM3595::luxCDABE*

The *fis⁻* and *hns⁻* mutations caused a five-fold and four-fold increase, respectively, in control expression when compared to the parent strain, suggesting that both Fis and H-NS have a negative influence on *STM3595* transcription. The *ihfB⁻* mutation caused a three-fold decrease in control expression compared to the parent strain. IHF may have a positive influence on *STM3595* transcription.

vii. *ucpA::luxCDABE*

The fusion in the *crp⁻* strain produced 11 times less luminescence under control conditions when compared to the parent suggesting that CRP is a transcriptional activator of *ucpA*.

viii. *talA::luxCDABE*

A three-fold and seven-fold increase in control expression was observed in the *crp* and *fis* mutants, respectively, when compared to the parent strain suggesting that both CRP and Fis negatively influence *talA* transcription. Compared to the parent, the *ihfB⁻* strain had a 3-fold decrease in expression implying that IHF is an activator of *talA*.

All the regulator mutants tested influenced transcription of at least one RRP confirming that these regulators are indeed global regulators with widespread influence on overall expression of the *S. typhimurium* genome.

3.5.3   The majority of rifampicin induced transcription modulation is independent of the tested regulatory proteins.

The overall response of the 56-strain array to rifampicin is shown in Figures 3.3 and 3.4. As in Section 3.3, most strains displayed the strongest rifampicin induced down-regulation (RIDR) or rifampicin induced up-regulation (RIUR) at 5 µg/ml. In the majority of promoter – mutant combinations, RITM was similar for both the wild type and mutant strain. Although the magnitude of RITM changed from as much as six to 120-fold (as in the case of RIDR of the *STM1328::luxCDABE* reporter in the 14028 *rpoS::amp* mutant), as described in Table 3.3, there were no obvious patterns in these differences. Thus, for the purposes of this analysis, the differences were not considered relevant. This suggests that in most cases studied rifampicin was not modulating transcription of the eight RRP reporters through any of the regulatory proteins: CRP, FNR, FIS, H-NS, IHF and σ^S.

Table 3.2. Basal expression of promoter-*lux* reporter constructs in wild type *S. typhimurium* 14028 and isogenic mutant strains.

	Basal Expression (cps)	Basal Expression of mutant/wt	Basal Expression of wt/mutant		Basal Expression (cps)	Basal Expression of mutant/wt	Basal Expression of wt/mutant
14028 fliA	47111		1.0	14028 STM3595	1313	1.0	
14028 rpoS⁻ fliA	25066		1.9	14028 rpoS⁻ STM3595	1101	0.8	
14028 crp::Tn10 fliA	2296		20.5	14028 crp::Tn10 STM3595	1578	1.2	
14028 fis::tet fliA	2041		23.1	14028 fis::tet STM3595	7058	5.4	
14028 ihfB::cat fliA	57355		0.8	14028 ihfB::cat STM3595	460		2.9
14028 fnr::Tn10 fliA	86569		0.5	14028 fnr::Tn10 STM3595	2756	2.1	
14028 hns::Tn10	53906		0.9	14028 hns::Tn10 STM3595	4703	3.6	
14028 flhBA	5807		1.0	14028 ucpA	14214		1.0
14028 rpoS⁻ flhBA	3005		1.9	14028 rpoS⁻ ucpA	30808		0.5
14028 crp::Tn10 flhBA	338		17.2	14028 crp::Tn10 ucpA	1266		11.2
14028 fis::tet flhBA	400		14.5	14028 fis::tet ucpA	6271		2.3
14028 ihfB::cat flhBA	3129		1.9	14028 ihfB::cat ucpA	10401		1.4
14028 fnr::Tn10 flhBA	9690		0.6	14028 fnr::Tn10 ucpA	8661		1.6
14028 hns::Tn10 flhBA	6836		0.8	14028 hns::Tn10 ucpA	8081		1.8
14028 cirA	1004		1.0	14028 talA	2730	1.0	
14028 rpoS⁻ cirA	737		1.4	14028 rpoS⁻ talA	1306	0.5	
14028 crp::Tn10 cirA	2061		0.5	14028 crp::Tn10 talA	7671	2.8	
14028 fis::tet cirA	168		6.0	14028 fis::tet talA	17800	6.5	
14028 ihfB::cat cirA	30		33.5	14028 ihfB::cat talA	966		2.8
14028 fnr::Tn10 cirA	1563		0.6	14028 fnr::Tn10 talA	1724	0.6	
14028 hns::Tn10 cirA	1591		0.6	14028 hns::Tn10 talA	3795	1.4	
14028 STM1328	1551	1.0		14028 spvAB	15786	1.0	
14028 rpoS⁻ STM1328	9387	6.1		14028 rpoS⁻ spvAB	227		69.5
14028 crp::Tn10 STM1328	3530	2.3		14028 crp::Tn10 spvAB	310256	19.7	
14028 fis::tet STM1328	2179	1.4		14028 fis::tet spvAB	276633	17.5	
14028 ihfB::cat STM1328	1076	0.7		14028 ihfB::cat spvAB	14900	0.9	
14028 fnr::Tn10 STM1328	17273	11.1		14028 fnr::Tn10 spvAB	13490	0.9	
14028 hns::Tn10 STM1328	2555	1.6		14028 hns::Tn10 spvAB	72316	4.6	

Fold changes in basal expression greater than 2.5 have been highlighted

3.5.4   Several promoters display rifampicin induced regulation dependent on the tested regulatory protein.

For several fusion-mutant combinations in which RIUR occurred in the parent strain, in the mutant strain RIUR did not occur or switched from RIUR to RIDR. This suggested that the respective regulator was involved in RIUR for that promoter. In the *crp*⁻ strain containing the *spvAB::luxCDABE* reporter, RITM changed from 2-fold RIUR in 14028 to no effect in the *crp*⁻ strain. For the same construct, the *fis* mutation changed the effect from 2-fold RIUR to 7-fold RIDR. In the *fis*⁻ strain containing the *STM3595::luxCDABE* reporter, the effect changed from 5-fold RIUR in the wild type to 3-fold RIDR in the mutant. For the *talA::luxCDABE* construct, the *fis* mutation abolished the 5-fold RIUR. However, there was no single regulator that abolished RITM for all eight RRPs. The influence of the tested regulators seemed to be promoter specific, utilizing different mechanisms depending on the promoter. Rifampicin may be causing RITM through novel regulatory mechanisms or activating/repressing an intermediate regulator(s) that was not examined in this study. In the cases in which RITM was dependent on the regulator tested, the tested regulator may be working co-operatively with a rifampicin responsive factor to regulate the RRPs. Table 3.3 shows the result of the 56-strain array summarized in terms of fold change.

Several mutants reported in Section 3.5.2 showed very low basal levels of expression of the *lux* reporters. In the case of the *flhB* reporter in the *crp* and *fis* mutants, the fold change of luminescence caused by rifampicin changed from strong down-regulation to no effect, these results could not be used to determine whether the respective regulator was involved in RIDR as transcription may have already reached its lower limit. Luminescence from the *spvAB* reporter in the *rpoS* mutant and from the *talA* reporter in the *ihfB*⁻ was low and rifampicin did not induce up-regulation. This suggests that σ^S and IHF are required for transcription from the respective promoters. Hence, it was difficult to determine if these respective regulators were involved in RIUR. These cases are indicated by an asterisk (*) in Table 3.3.

Figure 3.3. Luminescence of *S. typhimurium* 14028 and isogenic mutants in response to rifampicin. Strains were grown in LB with kanamycin and 0 µg/ml (open bars), 5.0 µg/ml (black bars) or 2.5 µg/ml (grey bars) of rifampicin.

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Figure 3.4. Luminescence of *S. typhimurium* 14028 and isogenic mutants in response to rifampicin. Strains were grown in LB with kanamycin and 0 μg/ml (open bars), 5.0 μg/ml (black bars) or 2.5 μg/ml (grey bars) of rifampicin.

Table 3.3. Summary of rifampicin induced transcription modulation.

	fliA	flhB	cirA	STM1328	STM3595	spvAB	ucpA	talA
14028	↓↓	↓↓	↓↓	↓	↑	↑	↑	↑
14028 crp::Tn10	↓	*	↓↓	↓	↑↑		↑	↑
14028 fis::tet	↓	*	↓	↓	↓	↓↓	↑↑	
14028 hns::Tn10	↓↓↓	↓↓	↓	↓	↑	↑	↑↑	↑
14028 ihfB::cat	↓↓↓	↓↓	↓	↓	↑	↑	↑	*
14028 fnr::Tn10	↓↓	↓↓	↓↓	↓↓↓	↑	↑	↑↑	↑↑
14028 rpoS::amp	↓↓	↓	↓↓	↓↓↓	↑↑	*	↑	↑

Fold change was calculated by dividing luminescence in LB supplemented with rifampicin and kanamycin by luminescence in LB with kanamycin. Two to five-fold is depicted as one arrow, six to 15-fold by two arrows and 16-fold and greater as three arrows. No arrows indicate there was less than two-fold difference between induced and non-induced conditions. * indicates promoters in which regulator mutations resulted in very low luminescence.

3.6 Transcription profiles of eight E. coli global regulatory mutants.

3.6.1 Construction of a 80-strain E. coli mutant array.

Prior to examining the eight promoter constructs in S. typhimurium, a larger number of E. coli transcription and sigma factor mutants were inspected: lrp, fis, arcA, narLP, crp, hns, fnr and rpoS. The E. coli parent strains, MC4100 and M182, and the isogenic mutants were transformed with the same RRP reporters tested in S. typhimurium. Each strain was confirmed for the appropriate reporter by PCR amplification of the insert fragment using the primers pCS26for and pCS26rev. Since a larger number of E. coli mutants were readily available in isogenic parent strains and previous studies of heterologous expression of S. typhimurium genes in E. coli had successfully shown similar responses in both organisms, E. coli was initially tested for this work. (For example, both E. coli (128, 136) and S. typhimurium (106) SlyA proteins activated transcription of hlyE (aka clyA and sheA), a novel pore-forming toxin, and displayed hemolytic activity in an E. coli K-12 strain. The regulation of spvRAB, Salmonella plasmid virulence genes, by σ^S and H-NS was shown to be similar when expressed in E. coli (154) and S. typhimurium (41, 131).) However, in our study three of the four chosen promoters no longer displayed RIUR in the E. coli hosts. There were also low transcription levels for many of the strains examined. Mutants were constructed in Salmonella to address these problems. Some interesting results did arise from the limited E. coli studies. As the rifampicin MIC in LB when used against E. coli MC4100 and M182 (the parent strains used in this study)

was ~9 µg/ml, mutants were tested in LB kanamycin and LB kanamycin with rifampicin at 2.5 and 1.0 µg/ml (see Figures 3.5 and 3.6).

3.6.2 Differential rifampicin responses in *S. typhimurium* and *E. coli*.

The strains containing the four promoters that were down-regulated by rifampicin in *S. typhimurium* (*fliA, flhBA, cirA* and *STM1328*) were also down-regulated in the *E. coli* mutants and their two isogenic parents with two exceptions: M182 Δ*hns* with *fliA::luxCDABE* and *flhBA::luxCDABE* reporters. With the flagellar promoters, *E. coli* M182 displayed RIDR whereas the Δ*hns* strains displayed no response to rifampicin suggesting that RIDR of these promoters in *E. coli* is dependent on H-NS. In *Salmonella*, the *hns* mutant had a similar response to rifampicin as its isogenic parent, suggesting that RIDR of the flagellar genes is independent of H-NS. This difference in H-NS regulation may be due to differential regulation between *S. typhimurium* and *E. coli* or due to different *hns* mutations in the two organisms.

The strains containing the *STM3595, ucpA* and *talA* were up-regulated by rifampicin in *S. typhimurium* but down-regulated in response to rifampicin in both *E. coli* parental strains (MC4100 and M182) and their isogenic mutant derivatives with two exceptions: *E. coli* M182 Δ*hns* containing the *ucpA::luxCDABE* and *talA::luxCDABE* reporter plasmids showed RIUR. This suggests that rifampicin responsive positive regulators of these promoters are missing in *E. coli* and are present in *S. typhimurium*. This absence of RIUR may also be due to weaker binding of *E. coli* regulators to *S. typhimurium* DNA, the absence of *S. typhimurium* specific regulators and/or lower ppGpp levels in *E. coli* (due to the *relA1* mutation discussed below).

One promoter, *spvAB::luxCDABE*, showed RIUR in one *E. coli* parent strain and some of its isogenic derivatives. As found in *S. typhimurium, E. coli* MC4100, MC4100 *arcA*::Tn*10* and MC4100 *narP*::Tn*10*d *narL*::Tn*10* displayed RIUR of *spvAB::luxCDABE* suggesting that the RIUR of *spvAB* did not require *S. typhimurium* specific activators. MC4100 *lrp*::Tn*10*, MC4100 *fis*::Tn*10* and MC4100 *rpoS*::Tn*10* displayed RIDR suggesting that RIUR of these promoters was dependent on Lrp, Fis and σ^S. Results also showed that basal expression levels of *spvAB* were elevated in the *lrp* mutant and reduced in the *fis* and *rpoS* mutants suggesting that
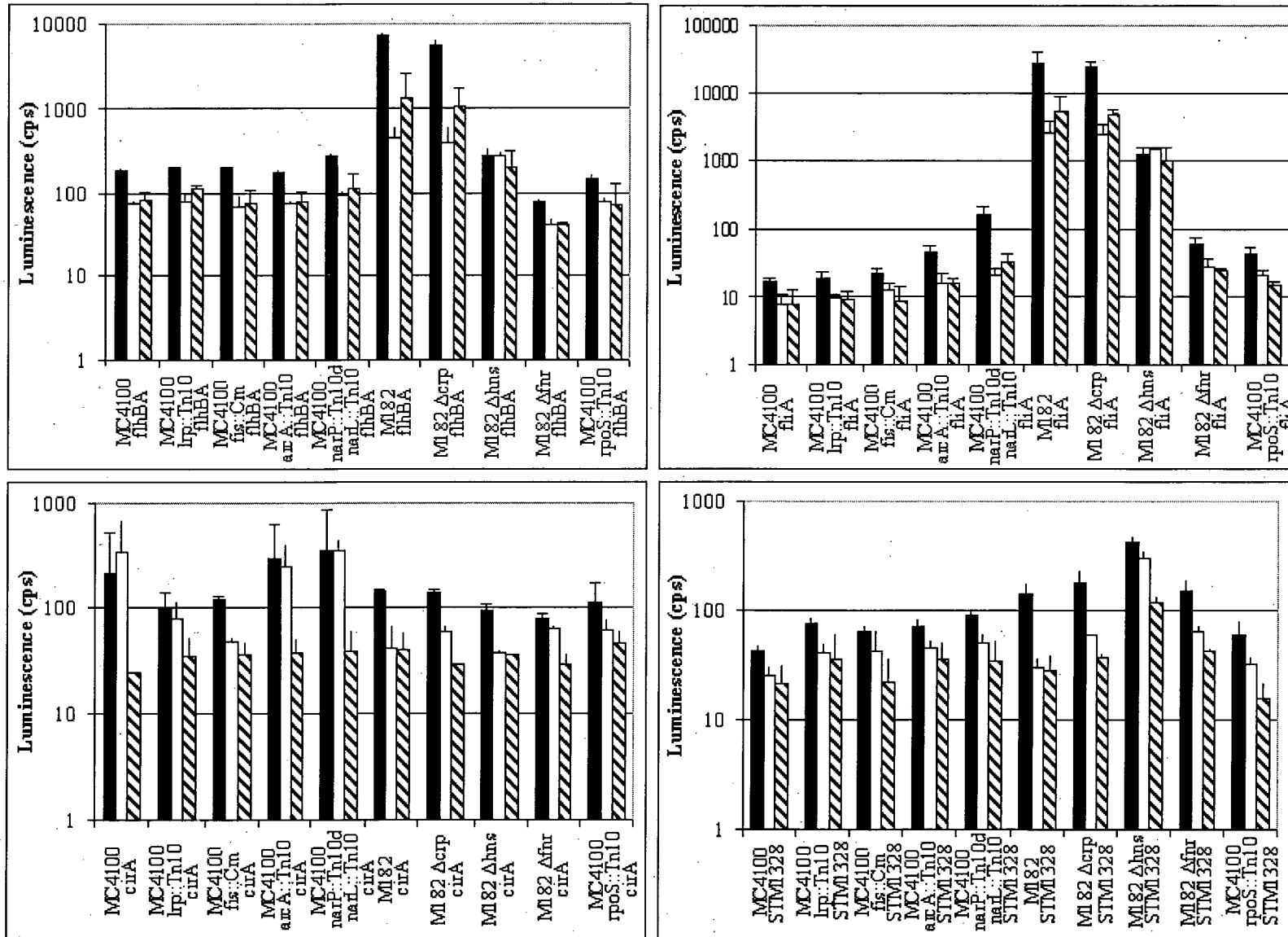
Figure 3.5. Luminescence of *E. coli* MC4100, M182 and isogenic mutants in response to rifampicin. Strains were grown in LB with kanamycin and 0 µg/ml (black bars), 1.0 µg/ml (open bars) or 2.5 µg/ml (striped bars) of rifampicin.
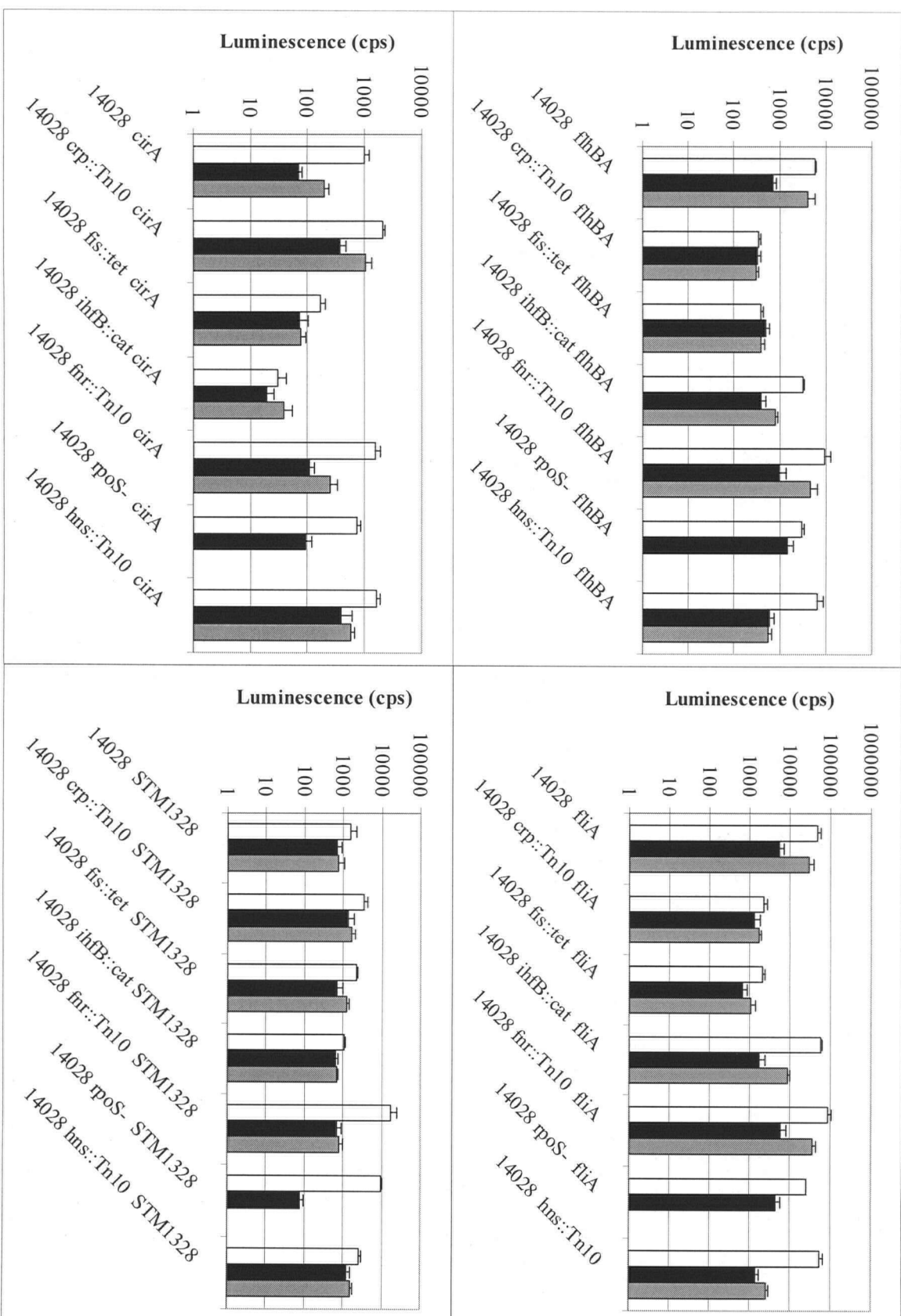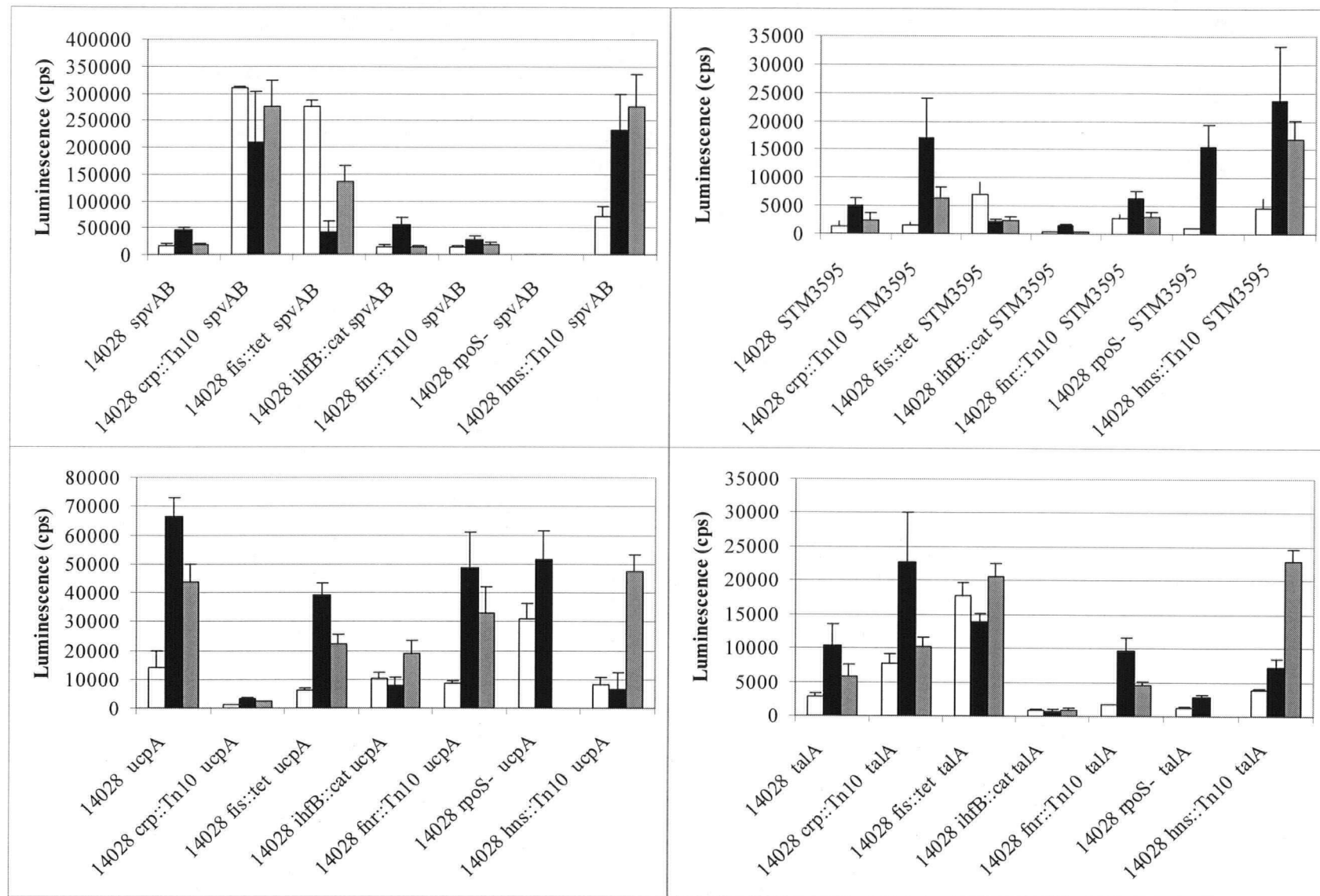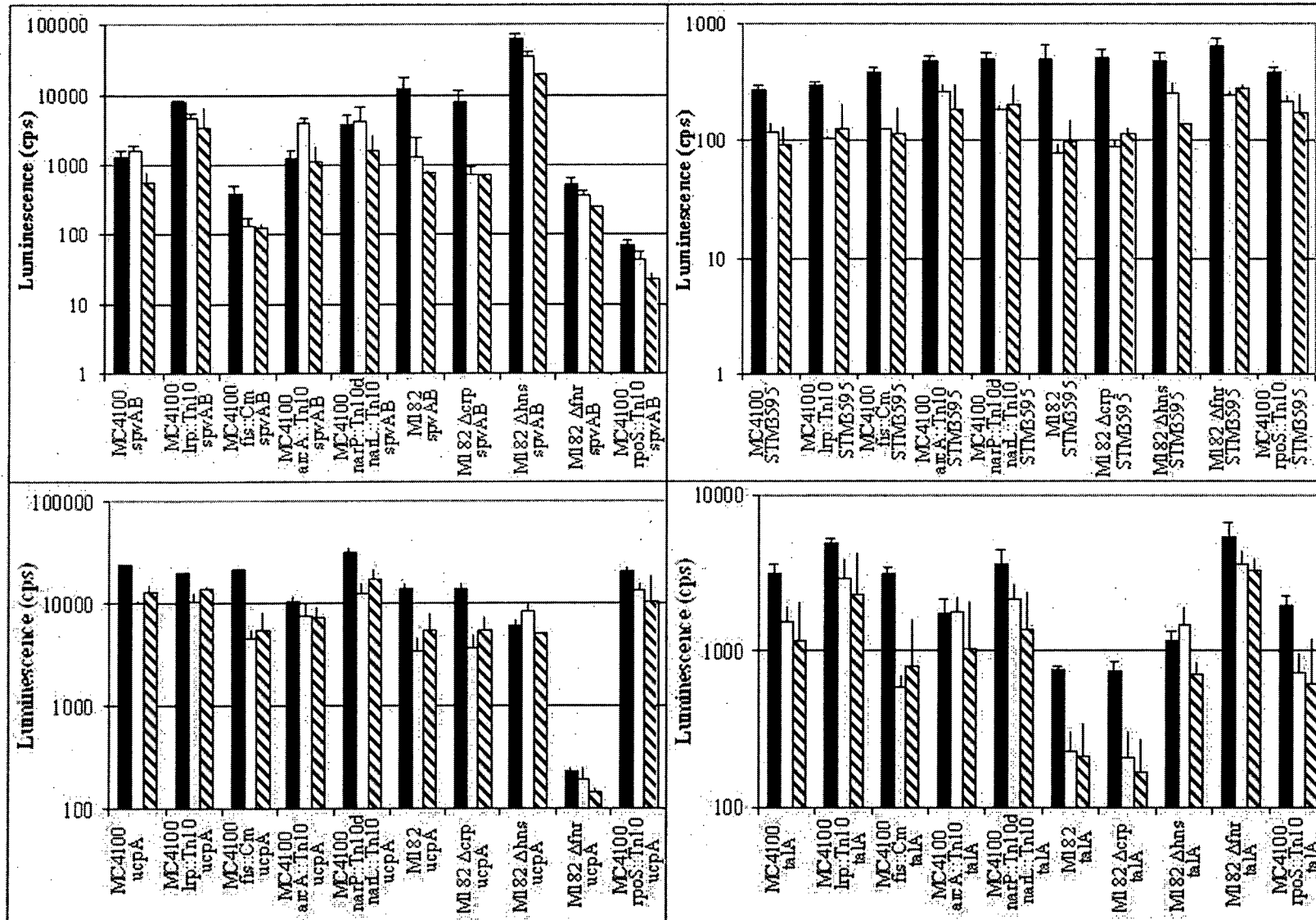
Figure 3.6. Luminescence of *E. coli* MC4100, M182 and isogenic mutants in response to rifampicin. Strains were grown in LB with kanamycin and 0 μg/ml (black bars), 1.0 μg/ml (open bars) or 2.5 μg/ml (striped bars) of rifampicin.

Lrp repressed and that Fis and σ^S activated *spvAB* transcription under control conditions. On the other hand, *E. coli* M182 and its Δcrp, Δhns and Δfnr derivatives displayed RIDR of the *spvAB* reporter. The obvious, relevant difference between the two parent strains is a *spoT1* mutation. MC4100 and M182 both possess a *relA1* mutation whereas M182 has an additional *spoT1* mutation resulting in low ppGpp levels in MC4100 and nonexistent levels of ppGpp in M182 suggesting that ppGpp is required for RIUR of *spvAB::luxCDABE*.

In general, *lux* expression was lower in *E. coli* than in *S. typhimurium*, this could be due to weaker binding of *E. coli* regulators to *S. typhimurium* DNA, the absence of *S. typhimurium* specific regulators, differential regulation of homologous genes (187) and/or lower ppGpp levels in the *E. coli* strains used. Luminescence of the *fliA* and *flhBA* reporters was low in *E. coli* MC4100 and its derivatives due to a mutation in *flhD*, the flagellar master regulator, present in those strains.

3.7 Up-regulation of SPI-2 *lacZ* reporters by rifampicin.

Since the transcription of genes encoding many virulence factors were up- or down-regulated by rifampicin, a group of *S. typhimurium* virulence gene reporters were obtained from Dr. B. Coombes (UBC) and tested for RITM and for responsiveness to other antibiotics. These reporters are single-copy chromosomal integrations of *lacZ* at the SPI-2 genes *sseA*, *sseD* and *sseE*. When measured on M9 glucose plates, the SPI-2 genes were up-regulated not only by rifampicin and polymyxin B (Figure 3.7) but also many other antibiotics (Table 3.4). Although done on solid media with *lacZ* reporters and not on liquid media using *lux* reporters, the SPI-2 *lacZ* reporters showed similar responses to rifampicin as did other virulence gene *lux* reporters. Furthermore, the pattern of antibiotic induced up-regulation of *sseA* seemed to differ from that of *sseD* and *sseE*, suggesting that *sseD* and *sseE* were co-regulated, possibly constituting one operon, independently of *sseA* (see Table 3.4).

3.8 Bioinformatic analysis of insert sequences from rifampicin responsive clones.

In order to examine the possibility of a nucleotide sequence associated with RITM, the insert nucleotide sequences from the 33 reporter plasmids isolated in Section 3.4 were analyzed with two motif finding programs. Insert sequences were analyzed with MEME, a computational

Table 3.4. Response of *sse::lacZ* reporters to various antibiotics on solid media.

	Erythromycin 15 µg	Gentamicin 10 µg	Tetracycline 30 µg	Ciprofloxacin 5 µg	Imipenem 10 µg	Triclosan 2 µg	Colistin 10 µg	Fosfomycin 50 µg	Tobramycin 10 µg	Streptomycin 10 µg	Clindamycin 2 µg	Spectinomycin 50 µg	Rifampicin 10 µg	Chloramphenicol 30 µg	Polymyxin B 300 U	Trimethoprim 5 µg
sseA::lacZ	0	+	++	+++	+++	0	+	+++	0	0	0	0	+++	+	+++	++
sseD::lacZ	0	+	++	0	+++	+	+++	+++	+	0	0	+	++	+	+++	+
sseE::lacZ	0	+	++	0	+++	+	+++	+++	+	0	0	+	+++	+	+++	++

Induction of *lacZ* reporters were scored by the intensity of blue which was indicative of the cleaved X-gal substrate: 0 = no response, + = weak response, ++ = intermediate response and +++ = strong response. Amounts listed indicate the amount of drug in the filter disc. Note that the parent strain, *S. typhimurium* SL1344, is streptomycin resistant and thus no inhibition zone was observed. Differential patterns between *sseDE* and *sseA* are highlighted.

motif discovery tool (5) and a Gibbs motif sampler (178) using several different settings for motif width and frequency. Twenty-two consensus sequences of various lengths were obtained. Several consensus sequences had weak homology to known transcription factor binding sites when analyzed using the nBLAST algorithm available on the DPInteract database (155) (see Appendix 7.4). The corresponding transcription factors and sigma factors to the binding sites were σ^S, Fur, IHF, OxyR, BetI, and Ada. Perhaps it is not surprising that there was only weak similarity to known binding sites as none of the regulators mutated were found to be involved in RITM of all the promoters tested. In some cases the weak homology to a binding site for a known regulator may represent partial regulation but not regulation of RITM. Inability to find a consensus sequence may also suggest that the majority of the promoters are indirectly affected by rifampicin through intermediate regulator(s) or unknown regulatory mechanisms. For example, rifampicin may be affecting one regulator that represses and activates other regulators that then cause RITM.
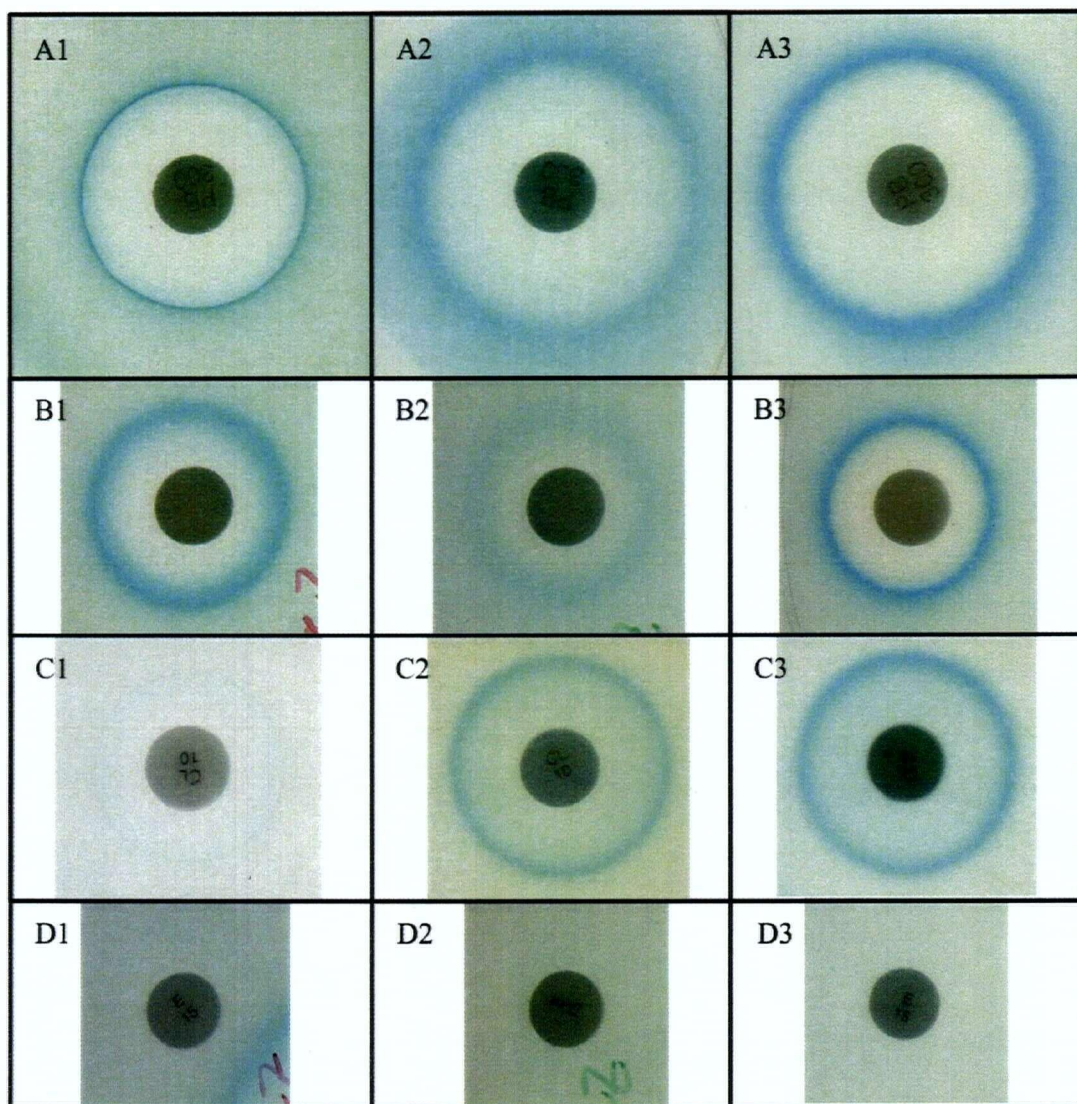
Figure 3.7. Response of *S. typhimurium* SL1344 SPI-2 *lacZ* reporters to various antibiotics. Sensi-discs or premade filter discs containing the antibiotics: polymyxin at 300 Units (A), rifampicin at 10 µg (B), colistin at 10 µg (C) and erythromycin at 15 µg (D) were placed on top of soft agar overlays containing *sseA::lacZ* (column 1), *sseD::lacZ* (column 2) and *sseE::lacZ* (column 3) reporter strains. Overlays were poured over M9 plates supplemented with histidine, glucose and X-gal. The amount of transcription can be determined by the intensity of blue that is indicative of cleaved X-gal (see Section 2.11 for details).

Chapter 4. Discussion.

4.1 Differential regulation of *S. typhimurium* virulence regulons.

Examination of the sequences in Section 3.4 revealed that rifampicin differentially affected the transcription of many *S. typhimurium* virulence genes according to their involvement in two distinct regulons. Virulence genes associated with intracellular growth in macrophages, *slyA* (18, 23, 100, 169), *spvAB* (56, 57, 99, 101), and SPI-2 genes (68) showed RIUR. Genes involved in intestinal invasion, those associated with the TTSS encoded on SPI-1 and its secreted effectors (177), showed RIDR. These genes included *invF*, *sopA* and *sopB*. This differential regulation in distinct environments and between types/routes of infections is often observed and commented on in studies of SPI-2 (87, 186). Rifampicin may be mimicking cues that cause switching of virulence modes from penetration of epithelial cells to growth and survival in macrophages that occurs when the salmonellae move from the intestine into macrophages during systemic infection.

The PhoP/Q system has been shown to positively influence the transcription of genes involved in systemic infection. These include *slyA* and *spvAB* and PhoP has been shown to bind to the *slyA* promoter region (129, 163). Transcription of *spvB* (61, 115) and SPI-2 genes (32) have also been shown to be PhoP dependent. When analyzed at the protein level using 2DGE, SlyA has been shown to be required for the expression of UcpA in magnesium limiting conditions (169). (The *ucpA* gene was identified as being up-regulated by rifampicin. See Table 3.1.) In addition, a putative SlyA binding site consensus was found upstream of *xseB*, and may be associated with the divergently transcribed RUR gene *thiI* (170). Other genes involved with virulence that display RIUR include *ybjX* (123) and *htrB* (84, 151) which are involved with lipid A biosynthesis, and *yijP*, a protein required for invasion of brain microvascular endothelial cells by *E. coli* K1 (70). Since *ybjX*, *htrB* and *yijP* show stimulation patterns similar to other characterized RUR genes, they may also be SlyA and/or PhoP dependent. In summary, these results suggest that RIUR of virulence genes associated with systemic infection could be mediated through activation of either the PhoP/Q system or SlyA.

The PhoP/Q system could also be implicated in down-regulation of *S. typhimurium* invasion genes. HilA is thought to be the primary regulator of intestinal virulence determinants encoded

within SPI-1 (177). HilA has many activators and repressors (8, 13, 14, 40); this includes PhoP which has been shown to have a negative influence on *hilA* expression (6, 143). In addition to activating transcription of the SPI-1 genes, HilA also positively influences SPI-4 transcription (2) which also displays RIDR. It has been suggested that PhoP does not directly interact with *hilA*, but influences *hilA* through Pag (PhoP activated gene), which has been shown to inhibit *hilA* transcription (39). Since genes that are indirectly repressed by PhoP/Q through HilA (*invF*, *sopA*, *sopB* and *STM4257* which is the first gene in SPI-4) are down-regulated by rifampicin, rifampicin may be causing down regulation of *hilA* transcription by activation of PhoP/Q.

4.2 Down-regulation of motility and invasion genes.

In addition to down-regulating virulence genes involved in invasion, rifampicin also down-regulated genes involved in motility. This may be mediated through FliZ which has been shown to activate *hilA* transcription (76, 104). The *fliAZY* operon provides a link between motility and virulence co-activation as *fliA* transcription is required for class III flagellar gene transcription (3) as well as class II gene activation by *fliZ* (74) and *fliA* (91). There is also evidence that motility is regulated by PhoP and PhoQ. Class I and II flagellar protein expression was found to be down-regulated in a *phoP⁻* strain when analyzed by 2DGE (1). Rifampicin may be indirectly or directly causing activation of the PhoP/Q system, which may then activate *pag* transcription. In turn, Pag may down regulate the *fliAZY* operon, causing less activation of *hilA* and of the class II flagellar gene transcription. Less activation of *hilA* may cause further down regulation of the invasion virulence genes. Neither *phoP* nor *phoQ*, which are positively auto-regulated (53), were present in the list of genes showing RIUR or RIDR that resulted from screening the *lux* library (Table 3.1).

Motility has been shown to be an important determinant for host cell invasion and the two functions are often co-regulated, *e.g. Listeria monocytogenes* (36), *Vibrio anguillarum* (135, 138) and *Campylobacter jejuni* (173, 191). *S. typhimurium* is no different (78, 108, 171); chemotactic genes were found in the original screen which identified the key regulators of *S. typhimurium* virulence (95). One proposed rationale for coordinated gene expression is that bacterial pathogens may need chemotactic abilities early in the infection process to guide them to a suitable location, *i.e.* the intestinal wall, for optimal virulence gene expression (107). If

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rifampicin is mimicking intracellular cues that bacteria use for appropriate signaling of virulence genes, it would be necessary to co-down-regulate genes involved in invasion and motility during the systemic phase of infection.

4.3 The rifampicin effects are similar to those induced by cationic peptides and bile.

The patterns of RITM are similar to the stimulons of cationic microbial peptides (CAMP) (4) and bile (148, 149). All three stimuli cause generalized transcriptional repression of the SPI-1 genes, their effectors and motility genes in *S. typhimurium*. One commonality between these three stimuli is their antibacterial property. Both rifampicin and CAMP (*e.g.* polymyxin B) are broadly used antibiotics while bile has been recently shown to have antibacterial activity (58). Other than their antibacterial properties it is unclear what relationship there is between the three molecules that could account for their similar transcription profiles; both host produced CAMPs 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.

Structurally there are no commonalities between the three molecules (see Figure 4.1). The structural comparison is complicated by the fact that CAMP themselves are structurally diverse (only one is shown in Figure 4.1). The CAMPs tested by Bader *et al.* showed similar effects on transcription: polymyxin, a cyclic peptide; C18G, an α-helical peptide and protegrin-1, a β-sheet peptide (4). Furthermore, the active component of bile for the induction of bile and antimicrobial resistance genes through the transcription factor MarR has been identified as deoxycholate (147), a salt present in bile. It is unclear if deoxycholate causes down-regulation of invasion and motility genes. Thus, while the effects of these three inhibitors are similar, it is not likely due to structural similarity.

In addition to down-regulating invasion and motility genes, CAMP and bile are suggested activators of the PhoP/Q system. CAMP and bile have been shown to regulate *pag* genes, *prg* genes, CAMP resistance (181) and bile resistance genes (182) in a PhoP dependent manner, but it has not been determined whether down-regulation of invasion and motility is PhoP dependent (182). Thus, CAMP and bile may act through PhoP/Q to down-regulate motility and invasion genes.
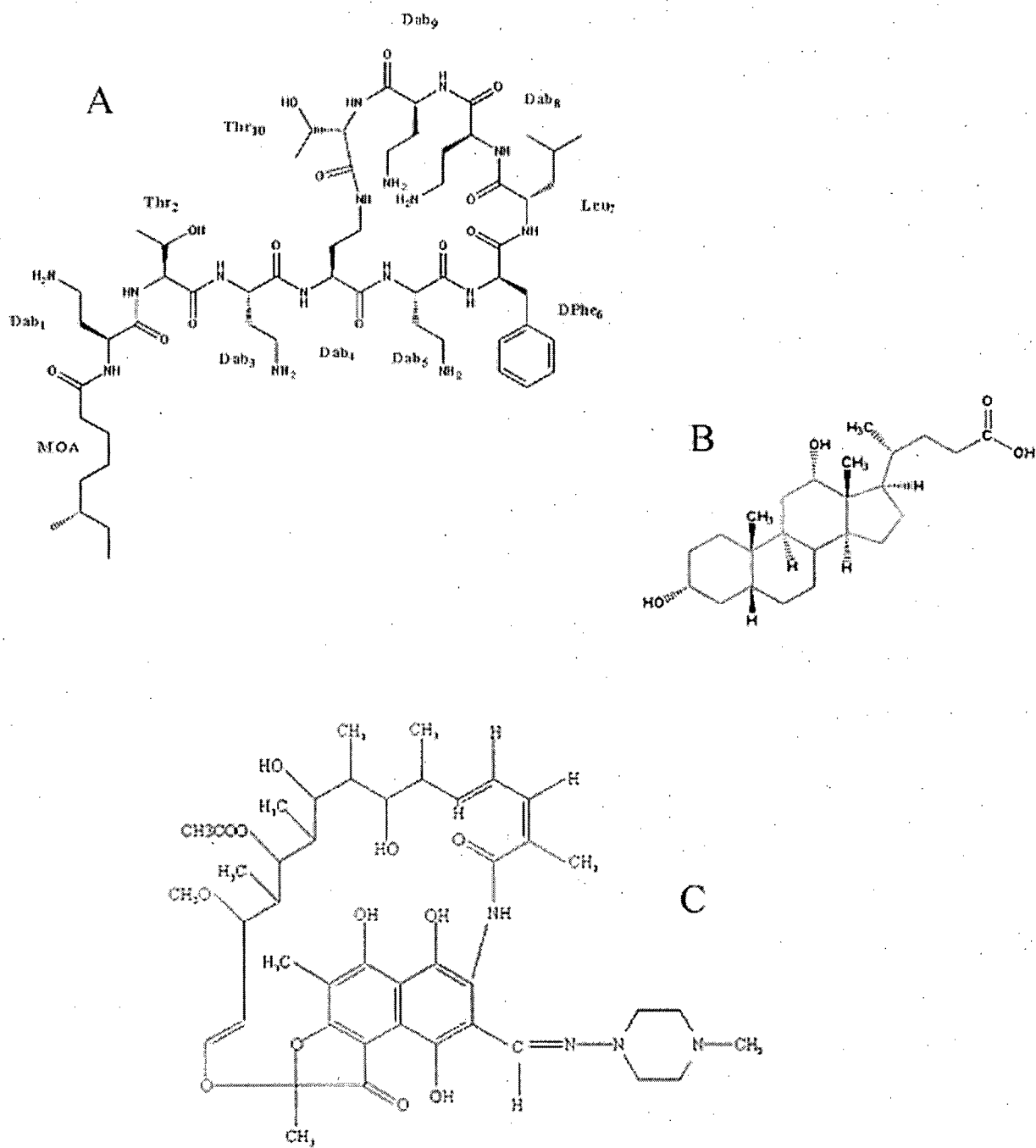
Figure 4.1. Structures of three antibacterial compounds that induce down-regulation of motility and invasion genes. (A) polymyxin B1 (181), (B) deoxycholate and (C) rifampicin (44) down-regulate motility and invasion genes in *S. typhimurium.* (MOA, 6-methyl-octanoic acid or 6-methyl heptanoic acid; Dab, diaminobutyric acid)

4.4 Possible mechanisms of rifampicin induced transcription modulation.

There are several possible mechanisms for RITM: i) Rifampicin may "fine-tune" transcription directly at the level of RNA polymerase, the target for rifampicin's antibacterial activity, for all RRPs. ii) The activity of an unknown regulator may be altered by binding rifampicin and may have an indirect effect through a regulatory cascade on RRPs or iii) a combination of i and ii.

In support of i), we have shown that *rpoB* (encodes the β subunit of RNAP) mutations conferring rifampicin resistance reduced or abolished rifampicin activation of promoters (data not shown). These *rpoB* studies were done using an *E. coli* K-12 strain and using a limited number of promoters, two metabolic promoters and a *Pseudomonas* quorum sensing promoter. Examination of other promoters, in particular those that are *Salmonella* specific or regulated by *Salmonella* specific regulators (e.g. HilA, SlyA) and involved in virulence such as *invF* or *ucpA* have not been completed and would make important extensions to this work.

In mechanism ii), rifampicin could be modulating transcription through a TF. This may involve rifampicin binding to the TF and altering the activity of the TF. The binding of rifampicin to targets other than RNAP is not unprecedented. As mentioned earlier, rifampicin has been reported to elicit transcription changes through binding and activation of the human glucocorticoid (20) and PXR receptors (15). Rifampicin could bind and activate a receptor such as PhoQ, which would activate its response regulator, PhoP, which in turn could activate various repressors and activators to elicit RITM.

Alternatively, rifampicin may stimulate at TFs such as PhoP to bind RNAP. Although differential trypsin digestion patterns of PhoQ upon addition of Mg^{+2} suggested that Mg^{+2} induces conformational changes in PhoQ (47) the mechanism of PhoQ activation by Mg^{+2} is unclear. The interaction between PhoP~P with RNAP is also unclear. Several "PhoP" boxes have been identified upstream of PhoP regulated genes by EMSA and DNase I footprinting (97, 163) suggesting that PhoP elicits its regulatory effects by stabilizing RNAP-DNA interactions in the regulatory regions of the DNA. However, it is possible that PhoP or another regulator may be directly binding RNAP and modulating transcription directly at the RNAP active site in addition to stabilizing RNAP –DNA interactions at the PhoP boxes.

A potential model for a SM mediated binding of a TF to RNAP is provided by DksA. DksA is a transcription factor that binds directly to RNAP to regulate rRNA transcription (141). The crystal structure of DksA showed a coiled coil domain which was hypothesized to protrude into the RNAP secondary channel to coordinate a ppGpp bound Mg^{+2} ion with the Asp residues, thereby stabilizing the ppGpp-RNAP complex (144). Examination of the crystal structure of *T. aquaticus* RNAP in complex with rifampicin shows that the rifampicin binding site is only 12 Å away from the Mg^{+2} binding site (21) making ppGpp and rifampicin only 12 Å apart as well. Interestingly, in a *S. typhimurium* $\Delta relA \Delta spoT$ mutant unable to synthesize ppGpp, transcription of *hilA*, *invF*, and other SPI-1 encoded factors are down-regulated when measured by *lacZ* fusions (146). This is similar to the effects of rifampicin with respect to SPI-1 invasion genes. However, rifampicin up-regulated *spvR* and *spvAB*, while expression in the ppGpp mutant is down-regulated (146). This suggests that if rifampicin is involved in an interaction between ppGpp and RNAP at the RRPs studied in this thesis, it is not the only mechanism involved. It remains a possibility that Mg^{+2} with or without the aid of a transcription factor such as PhoP may modulate the ppGpp–RNAP interaction at the active site to regulate RITM.

Furthermore, direct interactions between rifampicin and RNAP may not be directly responsible for transcription modulation at all RRPs. Rifampicin-RNAP interactions may only modulate expression levels of one or few regulators. Altered levels of a regulator such as PhoP would have similar effects on the PhoP signaling pathway as those proposed for altering the activity of PhoQ. Thus, a combination of direct interactions between rifampicin and RNAP causing changes in transcription of a few TFs that are RRPs may elicit RITM.

4.5 Regulation of promoters in the absence of rifampicin.

With respect to the dependence of RRP transcription on the global transcription regulators tested, most of the results presented here confirm the findings of others. The data with respect to current literature is discussed below:

i. *fliA::luxCDABE* and *flhBA::luxCDABE*

Previous reports have shown that H-NS and CRP both activate transcription of flagellar genes. In this study, basal expression levels from both *fliA* and *flhBA* reporters were significantly reduced in the *crp⁻* strain but unaffected by the *hns-6* mutation. When measured using *lacZ*

fusions, *hns⁻* and *crp⁻* mutations reduced *flhD* and *fliA* transcription and displayed aflagellation (and hence lack of motility) in *E. coli* (10) and *S. typhimurium* (10, 88, 91, 167). EMSA, DNase I footprinting and *in vitro* transcription assays using *E. coli* proteins have confirmed CRP and H-NS binding upstream of *flhD*, the corresponding sites and activation, respectively (167). However, the studies presented here with the FlhD positively regulated genes, *fliA* and *flhB*, indicate that activation of the flagellar genes is CRP dependent but independent of H-NS. It has been shown that *flhD* transcription in some *hns* alleles is not affected or only partially reduced (10, 67). Also, a *hns⁻* strain has been reported to have flagella but to be non-motile due to lack of motor function with levels of *flhD* transcription similar to the wild type strain (88). In general, the effect of *hns* mutations has been reported to be highly allele-specific (180). Considering the allele-specific phenotypes of H-NS mutants, it is possible that the *hns6::Tn10* allele used in our study may be a *hns* allele that does not affect class I and class II flagellar gene transcription as in the aforementioned studies (10, 88). In addition, basal expression levels of both flagellar gene reporters in the *fis⁻* strain were significantly reduced confirming other EMSA, *lacZ* reporter and DNA microarray studies showing that Fis binds and directly activates transcription of *flhD*, the master flagellar regulator, *fliA*, the class III sigma factor and *fliC*, a class III flagellar gene (82).

ii. *STM1328::luxCDABE*

FNR negatively influenced *STM1328* transcription. This has not been reported previously. FNR is a redox sensing transcriptional regulator which activates genes involved in anaerobic respiration and represses genes involved in aerobic respiration (85). *STM1328* may be a gene involved in anaerobic respiration. σ^S negatively influenced *STM1328* transcription. Since *rpoS* encodes a sigma factor it is possible that σ^S is required for transcription of a repressor of *STM1328*.

iii. *spvAB::luxCDABE*

CRP negatively regulated the *spv* operon supporting previous *spvB::lacZ* experiments (130). As CRP is a transcriptional activator, CRP could be required to transcribe a repressor of *spvRAB*. Fis also negatively influenced *spvAB* transcription. In a DNA microarray analysis, *spvAB* expression was not influenced by a *fis* mutation (82). The discrepancy between the microarray study and the results presented here could be explained in several ways: a) the cells for the *lux* assay were grown microaerobically (growth in microtitre plates without shaking) and the DNA

microarray used cells grown aerobically; or b) *lux* assays provide data over a time course, so that differences at all phases of growth can be observed. mRNA for the microarray study was harvested after 1 and 4 hrs of growth and Fis regulation of *spvA* in other growth phases could have been missed.

H-NS negatively influenced *spvAB* transcription and has been shown in other reports to repress both *spvR*, the transcriptional activator of the *spvAB*, and *spvAB* transcription in both *S. typhimurium* (131) and *E. coli* (154). σ^S positively affected *spvAB* transcription agreeing with experiments which shows that σ^S is required for expression of *spvRAB::cat*, *spvAB::lacZ* (90) and *spvRAB::lacZ* (41) reporters in *S. typhimurium*. σ^S dependence has also been shown using *spvA* probes against total RNA in *S. typhimurium* (90).

IHF has been shown to have a 2-fold positive influence on *spvB::lacZ* expression (111) but our results showed that IHF had no effect on *spvAB* transcription. This may be due to strain to strain variation. *S. typhimurium* 14028 was used here while Marshall *et al.* (111) used *S. typhimurium* χ3340.

iv. *STM3595::luxCDABE*

Fis negatively influenced *STM3595* transcription; this suggested that Fis was a repressor of *STM3595*. However, this result is not in agreement with the microarray study mentioned in section 4.5.iii, perhaps for similar reasons as described above.

v. *ucpA::luxCDABE*

CRP positively affected *ucpA* transcription supporting *ucpA::lacZ* reporter data displaying the positive influence of CRP on *ucpA* transcription (166). It has been shown that mutants in both *ihfA* and *ihfB* activate transcription of a *ucpA::lacZ* reporter 1.9 fold in a CRP-dependent manner (166). The results obtained here showed that an *ihfB* mutation had little effect on basal levels of expression; it is unclear why the results differ.

vi. *talA::luxCDABE*

Fis negatively affected *talA* expression and as in 4.5.iii and 4.5.iv the microarray study done by A. Kelly *et al.* (82) conflicts with this result.

4.6 Regulators involved with rifampicin induced transcription modulation.

The results in Section 3.5.4 showed that some regulators influenced RITM but that no one regulator was involved for all RRPs. In the following cases, the respective regulator appeared to

be involved in RITM. CRP and Fis in the case of *spvAB*, Fis in the case of *STM3595*, and Fis in the case of *talA* could be working co-operatively with a regulator or group of regulators such as SlyA, PhoP and HilA (that were not mutated here) to repress or activate transcription in response to rifampicin. For example, in the case of *spvAB*, rifampicin may activate PhoQ, which in turn phosphorylates PhoP and in turn activates *slyA* transcription. CRP and Fis may both be needed for efficient binding of SlyA to the regulatory region of *spvAB* to cause up-regulation of *spvAB* transcription.

4.7 Distinct rifampicin induced transcription profiles between *E. coli* and *S. typhimurium*. Only one of the four promoters that displayed RIUR in the parental *S. typhimurium* also displayed RIUR in a parental *E. coli* strain (*spvAB::luxCDABE*). *spvAB* displayed RIUR in one *E. coli* parent strain, MC4100, and some of its isogenic derivatives (MC4100 *arcA*::Tn*10* and MC4100 *narP*::Tn*10*d *narL*::Tn*10*) but not in *E. coli* M182. Lack of RIUR may not be surprising if one considers that many of the suspected intermediate regulators of RIUR suggested in Section 4.1 and 4.2 such as HilA and InvF are *Salmonella* specific and are located on the *Salmonella* pathogenicity islands. It has also been shown that homologous genes in *S. typhimurium* and *E. coli* can be differentially regulated even if genus specific regulators are not involved (187).

The regulators involved in RIUR of the *spv* genes appeared to be present and sufficient for RIUR in both organisms as RIUR occurs in both. In support of this, there were no previous reports indicating that the *spv* genes are regulated by proteins encoded on SPIs. Possible regulators of *spvAB* discussed in Section 4.1, which may also be involved in RIUR, would be PhoP/Q and SlyA. (PhoPQ and SlyA are both present in *E. coli* K-12 (53, 105).) Another regulator of *spvAB* that could be involved in RIUR is *spvR* which encodes a positive regulator of the *spvABCD* operon located directly upstream of *spvABCD* in the *Salmonella* virulence plasmid (109). (*spvR*, *spvA* and part of *spvB* are present in the *lux* reporter plasmid *spvRAB::luxCDABE*.) The results also suggested that under control conditions (without rifampicin) Lrp repressed *spvAB* transcription and that Fis and σ^S activates *spvAB* transcription (Figure 3.6). This agrees with previous EMSA and *lacZ* experiments indicating that Lrp binds and represses *spvR* expression (111) and that σ^S is required for *spvR* and *spvA* transcription (as

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discussed in Section 4.5.iii). *E. coli* M182 and its Δ*crp*, Δ*hns* and Δ*fnr* derivatives displayed RIDR of the *spvAB* reporter. The *spvAB* reporter may not have been activated due a lack of ppGpp in *E. coli* M182 as was mentioned in Section 3.6.2. This is supported by studies which show that *spvA* transcription requires ppGpp (146).

4.8 Advantages and disadvantages of promoter-*lux* reporters and library screening.

The comparison between mutant and parent levels of transcription discussed in Section 4.5, agreed fairly well with previous reports. It appears that *lux* reporters are sensitive reporters that are well suited to these types of studies. Significantly higher changes in luminescence can be observed between mutant and parent strains when compared to changes in *lacZ* activity *i.e.* 20-fold change in luminescence in the case of *crp*⁺ and *crp*⁻ strain with the *spvAB* reporter and a 4-fold change in Miller units in the corresponding *lacZ* experiments (130). As *lux* reporter studies provide a greater amplification of differences in transcription than other reporter systems, weaker contacts between regulator and promoter that give borderline results (around 2-fold) in other reporters such as *lacZ* can be determined with more confidence using *luxCDABE*. Lux reporters are also preferable over other reporters as no exogenous substrate is required.

Reporter libraries have other advantages compared to DNA microarray technology. They avoid many of the common problems associated with microarray technology: short bacterial mRNA half-lives (some as short as 30s), lysing, sampling, RNA extraction, cross-hybridization, dye incorporation and cost (29). Reporter libraries are also not limited to measurements at discrete time points and have the advantage of permitting monitoring of real-time gene expression. This is particularly important when measuring time-sensitive effects. However, as *lux* is so sensitive there are often large discrepancies between the absolute luminescence values when readings are taken from liquid cultures from two independent agar colonies or between fresh and stored liquid seed cultures. Although the fold increase or decrease in response to a stimulus was not always consistent, the direction of up-regulation or down-regulation was consistent. It was found that to obtain consistent absolute value replicates, control and test cultures must be inoculated from the same freshly grown seed culture. It is likely that this is also the case with other technologies such as microarrays.

Disadvantages of using a promoter-*lux* library screening method appears to be sequence coverage and the luciferase reaction. As quite a few genes encoding regulators were identified as RRPs (SlyA, InvF, FliA) it may be surprising that more of the genes regulated by these proteins, such as many of the class III flagellar genes, (which are regulated by FliA) did not appear in the list of RRPs obtained from screening the promoter-*lux* library with rifampicin. This may reflect screening methods or a lack of coverage in the original 6528-clone library. As mentioned in Section 3.1, luminescence measurements during preliminary screens were taken at discrete time points and not continuously in order to decrease clone numbers to a more manageable size. This may have inadvertently removed the "missing" genes from the pool of positive clones. The rescreening of clones discussed in Section 3.2 that was done to reduce the number of clones for further study may have also removed "missing" genes from our final list of RRPs. Furthermore, as seen in Fig. 2.1 the LuxCDABE catalyzed reaction requires the bacterial cell to provide $FMNH_2$, flavin mononucleotide, and molecular oxygen. These requirements could be energetically taxing to cells. Overall, *lux*-reporter libraries have the advantage of real-time measurements, convenience and cost, but require rigorous screening methods and may be energetically taxing to the cell.

4.9 Future Experiments.

Further research to elucidate the mechanism and properties of RITM should include the following five aspects: (1) identification of the transcription start site for a smaller selection of promoters (2) RT-PCR of the aforementioned promoters to confirm *lux* reporter results (3) determination of whether PhoP/Q is a regulator involved in RITM by testing for RITM in low and high Mg^{+2} (4) identification of other regulators involved in RITM by random and targeted mutagenesis (5) testing the effects of rifampicin on motility and invasiveness.

To confirm the identity of the gene and promoter in the reporter construct associated with RITM the transcription start site should be measured for rifampicin repressed genes (such as *invF*, *sopB*, *fliA* and *flgK)* and rifampicin stimulated genes (such as *STM3595*, *slyA*, *yijP* and *spvAB*). This could be accomplished by primer extension or cloning of RT-PCR products using mRNA extracted from cells carrying the constructs treated with and without rifampicin. The minimal promoter sequence required for RITM should be determined using PCR amplified fragments of

varying lengths surrounding the transcription start site. Fragments would be subcloned back into a promoterless-*lux* vector and tested for RITM by luminescence production. If the minimal fragment required for RITM is simply the −10 and −35 element, this would be evidence that rifampicin is mediating its effects directly at RNAP and not through intermediate regulators which bind upstream of the −35 element. One could also use qPCR to confirm RITM of genes in *S. typhimurium* 14028 with primers designed from information obtained above.

To examine the role of PhoP/Q, one could test *S. typhimurium* 14028 containing the relevant reporter constructs from above for transcription modulation in low and high Mg^{+2}. If rifampicin is activating PhoP through PhoQ, constructs should show similar behavior in low Mg^{+2} as in rifampicin. If rifampicin is stimulating PhoQ, then adding rifampicin to cells grown in low Mg^{+2} should have no effect as PhoP is already active and phosphorylated. If rifampicin is not able to cause RITM in high Mg^{+2}, this may suggest that PhoP is required for RITM. If low Mg^{+2} and rifampicin do not regulate any of the constructs similarly, a *phoP* mutant should not affect RITM.

To identify regulators involved in RITM, known regulators of RRPs could be examined, *e.g.* HilA, SlyA and PhoP. Deletion mutants of each gene could be made and the strains inspected for loss of RITM. Deletions of possible regulators could be constructed using the phage λ *Red* recombinase system, transformed with the relevant reporter construct from above and measured for loss of RITM by luminescence. To search for novel regulators, random mutagenesis could be done via transduction with a P22HT*int* lysate from a Tn*10 S. typhimurium* 14028 library into strains containing reporter constructs made above and screened for loss of RITM (Note: the screen for loss or gain of luminescence is simplified by use of promoters that are highly activated or repressed).

To determine if low concentrations of rifampicin affect the motility and pathogenicity of *S. typhimurium,* motility plates could be used and invasion assays carried out in increasing concentrations of rifampicin. If rifampicin reduces motility and invasiveness, motility and invasiveness of the regulator deficient mutants identified above could be examined for loss of the rifampicin induced phenotype.

Chapter 5. Conclusion.

The presence of sub-MIC concentrations of rifampicin resulted in differential regulation of the transcription of *S. typhimurium* virulence genes. Rifampicin up-regulated genes associated with intracellular growth in macrophages and down-regulated those involved in intestinal invasion and motility. Testing of eight rifampicin responsive promoters in *S. typhimurium* 14028 CRP, FNR, IHF, FIS and σ^S mutants showed that for most of the promoter-mutant combinations RITM was independent of the respective regulator. In a few cases, RITM did appear to be dependent on the regulator tested and in these cases the regulator tested may work co-operatively with other rifampicin responsive regulatory mechanisms that have not been examined to elicit RITM. The mechanism of RITM is likely a combination of the following: i) rifampicin is "fine-tuning" transcription directly at the level of RNA polymerase, the traditional target for rifampicin's antibacterial activity and ii) rifampicin affects a regulator that has not been identified and rifampicin is having an indirect effect through a regulatory cascade on RRPs.

When rifampicin is ingested orally for clinical purposes, rifampicin may mimic intestinal cues such as those elicited by CAMPs and bile that are normally present in the human gut and could promote switching of virulence modes from enteropathogenic to systemic infection. CAMPs and bile are host produced and host beneficial defensive compounds in the intestine. Rifampicin, CAMP and bile compounds may work to the host's advantage and cause inappropriate down-regulation of invasion and motility genes and prevent invasion of epithelial cells thus decreasing *Salmonella* virulence. Thus, rifampicin could be used clinically not only for inhibition of bacterial growth but also for reduction in bacterial pathogenicity.

This research may have an impact on the use of certain antibiotics. If virulence functions prove to be activated by certain antibiotics, their continued use should be considered. Alternatively, if an antibiotic causes misregulation or down-regulation of virulence genes then it could be used to treat a bacterial infection although the drug does not inhibit growth. A good example of this are the macrolide antibiotics which are being used in diffuse panbronchiolitis and cystic fibrosis infections, even though they do not reduce bacterial load; they have been shown to have immunomodulatory effects (160, 164) and to inhibit expression of virulence determinants (189).

My research may add another dimension to antibiotic use and may contribute to the more rational, efficacious use of antibiotics. Antibiotics will not only kill or slow bacterial growth but also decrease the pathogenicity of the bacteria. Studying the signaling properties of antibiotics will help understand how other SMs affect gene expression and their potential as therapeutic agents, antimicrobial or otherwise and may also elucidate the "true" purpose of antibiotics in nature as signaling molecules and not as weapons for inter-microbial competition.

Chapter 6. References.

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Chapter 7. Appendices[1]

7.1 Goh *et al.*: publication resulting from screening the *S. typhimurium* 14028 promoter-*lux* library with rifampicin and erythromycin.

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[1] A version of this chapter has been published. Goh, E.B., Yim, G. *et al.* (2002) Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. PNAS 99(26):17025-17030.

Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics

Ee-Been Goh*[1], Grace Yim*, Wayne Tsui*, JoAnn McClure[‡], Michael G. Surette[‡], and Julian Davies*[§]

*Department of Microbiology and Immunology, University of British Columbia, 300-6174 University Boulevard, Vancouver, BC, Canada V6T 1Z3; and
[‡]Department of Microbiology and Infectious Disease, University of Calgary, Calgary, AB, Canada T2N 4N1

Antibiotics such as erythromycin and rifampicin, at low concentrations, alter global bacterial transcription patterns as measured by the stimulation or inhibition of a variety of promoter–*lux* reporter constructs in a *Salmonella typhimurium* library. Analysis of a 6,500-clone library indicated that as many as 5% of the promoters may be affected, comprising genes for a variety of functions, as well as a significant fraction of genes with no known function. Studies of a selection of the reporter clones showed that stimulation varied depending on the nature of the antibiotic, the promoter, and what culture medium was used; the response differed on solid as compared with liquid media. Transcription was markedly reduced in antibiotic-resistant hosts, but the presence of mutations deficient in stress responses such as SOS or universal stress did not prevent antibiotic-induced modulation. The results show that small molecules may have contrasting effects on bacteria depending on their concentration: either the modulation of bacterial metabolism by altering transcription patterns or the inhibition of growth by the inhibition of specific target functions. Both activities could play important roles in the regulation of microbial communities. These studies indicate that the detection of pharmaceutically useful natural product inhibitors could be effectively achieved by measuring activation of transcription at low concentrations in high-throughput assays using appropriate bacterial promoter–reporter constructs.

M icrobes such as bacteria and fungi produce a bewildering array of low-molecular-weight organic molecules that have many biological activities; their roles in nature are largely unknown, although many have been suggested (1–4). In addition, it is well established that bacteria are exposed to and respond to many different extracellular signals in the environment (5). Antibiotics are the most extensively studied of these molecules, and their use in the therapy of infectious disease since the 1940s has revolutionized medicine, leading to many life-saving treatments. Numerous small molecules (SMs) with other biological activities (e.g., antiviral, antifungal, antitumor, and immunosuppressive) have also been isolated, creating an enormous market for natural products as therapeutics (6). However, there have been comparatively few studies of the potential roles of SMs in nature, apart from the recent identification of a diversity of molecules as autoinducers of quorum sensing, a process in which a specific chemical signal (autoinducer) triggers a variety of biological functions when microbial populations attain certain cell densities (7). It is popularly assumed that the majority of SMs with inhibitory (antibiotic) activity are important as weapons in intermicrobial competition. Nonetheless, many antibiotics have been shown to possess biological activities other than inhibition (3, 8), and this prompted us to examine the possibility that they might act as chemical signals to modulate metabolic processes in bacteria at low concentrations (9, 10). We demonstrate that SMs, at concentrations below the minimal inhibitory concentrations (MICs), stimulate or depress bacterial gene expression at the transcription level, as detected by their effects on bacterial promoter–reporter constructs.

Materials and Methods

Bacterial Strains and Growth Conditions. Strains used in the study are listed in Table 1. Cultures were grown aerobically in Luria–Bertani (LB) broth at 30 or 37°C. When appropriate, kanamycin (50 μg/ml), tetracycline (20 μg/ml), erythromycin (50 and 500 μg/ml), and rifampicin (50 and 200 μg/ml) were added. All antibiotics were obtained from Sigma or from the laboratory collection.

Solid Media Assay. Overnight LB (BD Biosciences, Sparks, MD) cultures from single colonies of reporter strains were diluted 100-fold, inoculated into 0.7% agar, and overlaid on LB plates. Etest strips (AB Biodisk, Solna, Sweden), Sensi-discs (BD Biosciences), or antibiotic-sensitivity discs made in our laboratory were placed on the overlay. Etest strips contain precisely graduated concentrations of antibiotics that permit the accurate determination of MICs where the lower end of the inhibition zone intersects the strip (see Fig. 1 *A* and *C*). Plates were incubated at 30°C or 37°C overnight and luminescence (relative light units) was detected with a Berthold USA (Oakridge, TN) LB980 photon camera.

Liquid Media Assay. Two-fold serial dilutions of antibiotics were made in the wells of black clear-bottom or white 96-well plates (Thermo Labsystems, Helsinki). Overnight liquid cultures of reporter strains were diluted from 1:100 to 1:300 in LB and added to the wells containing antibiotics. OD_{620} and luminescence from each well were recorded at 37°C in a Wallac 1420 Victor multilabel counter (Perkin–Elmer) or a Tecan Spectra-Fluor Plus (Tecan, Durham, NC).

Screening for Promoters Activated by Subinhibitory Concentrations of Antibiotics. *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) strain ATCC 14028 was used in this study. A random promoter library was constructed by cloning genomic restriction endonuclease fragments into the expression vector pCS26 upstream of a promoterless *luxCDABE* operon (J. Bjarnason, C. M. Southward, and M.G.S., unpublished data). The library consisted of 6,528 clones (17 × 384 microtiter plates) exhibiting promoter activity under different growth conditions. *Salmonella* clones were cultured aerobically at 37°C in LB containing kanamycin (25 μg/ml). Erythromycin was added to selected cultures at a concentration of 1–30 μg/ml, and rifampicin at a concentration of 0.2–5 μg/ml. Screening was conducted by using black 384-well solid-bottom plates. A 384-pin replicator (V&P Scientific, San Diego) was used to inoculate 384-well plates from overnight cultures. The plates were incubated at 37°C and light production was measured in a multilabel

Table 1. Bacterial strains and plasmids employed in this study

Strain or plasmid	Characteristics	Source and/or ref.
Escherichia coli		
N281	Erythromycin-resistant mutant *rplV* of AB301	A. E. Dahlberg (11)
K802NR	Nalidixic acid (*gyrA*), rifampicin-resistant (*rpoB*) mutant of K802	J.D.
7120	*lexA3, tifsf1A1, ind⁻* derivative of W3110	C. A. Gross
CA8306	Δ*cya* derivative of CA8000	R. J. Redfield (12)
13703	Δ*dnaKJ* derivative of MG1655	C. A. Gross (13)
13751	*dnaJ313* derivative of MG1655	C. A. Gross (13)
CF1652	Δ*relA261*::kan derivative of MG1655	M. Cashel (14)
CF1693	Δ*spoT207*::cml derivative of CF1652	M. Cashel (14)
RH90	*rpoS359*::Tn*10* derivative of MC4100	R. Hengge-Aronis (15)
RH100	Δ(*nlpD-rpoS*)360 *zfi3251*::Tn*10* derivative of MC4100	R. Hengge-Aronis (15)
S. typhimurium		
WG	Rifampicin-resistant (*rpoB*) derivative of 14028	J.D.
SA2386	*pyrE123, recAl* derivative of 14028	*Salmonella* Stock Center
SA3670	*recA, srl202*::Tn*10* derivative of 14028	*Salmonella* Stock Center
Plasmids		
pSB401	*luxI'*::*luxCDABE, luxR, tc*ᴿ	B. Ahmer (16)
pSB536	*ahyl'*::*luxCDABE, ahyR, ap*ᴿ	B. Ahmer (17)
pSB1075	*lasl'*::*luxCDABE, lasR, ap*ᴿ	B. Ahmer (16)
pBA428	*rck*::*luxCDABE*	B. Ahmer (18)
pCS26	Low-copy cloning vector	M.G.S. (19)

counter at 6 and 24 h. Clones showing differential expression of 3× or greater were chosen and rearrayed into 384-well plates. A second round of screening was done in a similar manner, except that light readings were taken at 2, 4, 6, 8, and 24 h; additional readings of OD$_{620}$ were taken to account for possible growth effects. Clones showing differential expression of 3× or more were rearrayed into 96-well plates. These clones give a consistent positive antibiotic response when reassayed. As with any high-throughput method, some false positives were selected initially; however, these were identified and eliminated by screening potential positives by using a more rigorous second screen. In the screens reported here the initial number of false positives was between 5% and 10%. Consistent, reproducible responses were obtained for true positives in the final rescreening. (Details of library construction and screening methodology will be published elsewhere.) Plasmid DNA was isolated from positive clones by using the Concert Miniprep system (Life Technologies, Rockville, MD) and sequenced by using a vector primer pZE06 5′-AATCATCACTTTCGGGAA-3′ (Qiagen Operon, Alameda, CA). Sequencing was carried out by the Marine Biotechnology Lab, National Research Council of Canada (Halifax, NS). The promoters were identified by comparison to the GenBank database by using the National Center for Biotechnology Information (NCBI) standard nucleotide-nucleotide BLASTN program (www.ncbi.nlm.nih.gov/BLAST/) and analyzed by using VECTOR NTI software (Informax, Bethesda).

Results

Activation of *lux* Genes Using Quorum-Sensing Promoters. Initially, the effects of antibiotics on transcription from different plasmid-borne quorum-sensing promoters of the *luxI* type (Table 1) were examined by using *lux* reporter constructs with and without the *luxR* element. This testing was done by placing Etest strips on bacterial lawns and examining light production in a luminometer; the use of Etest strips provided a simultaneous indication of the concentration dependence of promoter activation and MIC (20). As shown in Fig. 1 *A* and *B*, erythromycin, an antibiotic inhibitor of bacterial protein synthesis, activated transcription (as measured by light production) at concentrations significantly lower than the MIC. A similar result was obtained with the RNA polymerase-inhibitor rifampicin (Fig. 1 *C* and *D*).

A variety of other antibiotics were found to be active in stimulating different promoter–*lux* constructs in similar Etest studies. Patterns of activation differed in liquid as compared with solid media, depending on the strain and the antibiotic being used (Table 2). Antibiotics with distinct modes of action (inhibition of transcription, translation, cell-wall synthesis, or metabolic reactions) were active, suggesting that transcriptional modulation is a common bacterial response to SMs. However, not all antibiotics were active in these tests; for example, a number of β-lactams (including penicillin), certain protein synthesis inhibitors (some aminoglycosides), and the gyrase B inhibitors, coumermycin and novobiocin, showed no response. However, the "inactive" compounds may well be active against other promoters or hosts that have not been tested. It should be noted that inhibition of the growth of the tester strain was not a requirement for transcriptional modulation by SMs, as indicated by both Etest and growth curves in liquid media (see Fig. 6); thus strong responses were detected with SMs that had little or no inhibitory activity against the bacterial strains tested.

Antibiotic Activation on a Global Scale. The global effects of antibiotics on transcription in bacteria were examined against 6,500 isolates of a "promoter-clone" library of *S. typhimurium*, which was constructed by cloning *Sau*3A fragments of *S. typhimurium* DNA into a plasmid vector upstream of a promoterless *luxCDABE* cluster and screened automatically by using different antibiotic concentrations in liquid medium (see *Materials and Methods*). The results of two such surveys using erythromycin and rifampicin are shown as scatter plots in Figs. 2 and 3. The patterns show clearly that these two antibiotics, at subinhibitory concentrations, activate (points above the diagonal) or inhibit (below the diagonal) many different promoters in *S. typhimurium*. Nucleotide sequence analyses showed that many different genes are activated by low concentrations of antibiotics, including those involved in transport, virulence, DNA repair, and numerous unidentified functions. In addition, different classes of antibiotics modulate the function of different promoters, presumably by a variety of mechanisms, as discussed below.

Response of Antibiotic-Resistant Hosts. To obtain information on the mechanism of induction, a number of bacterial strains

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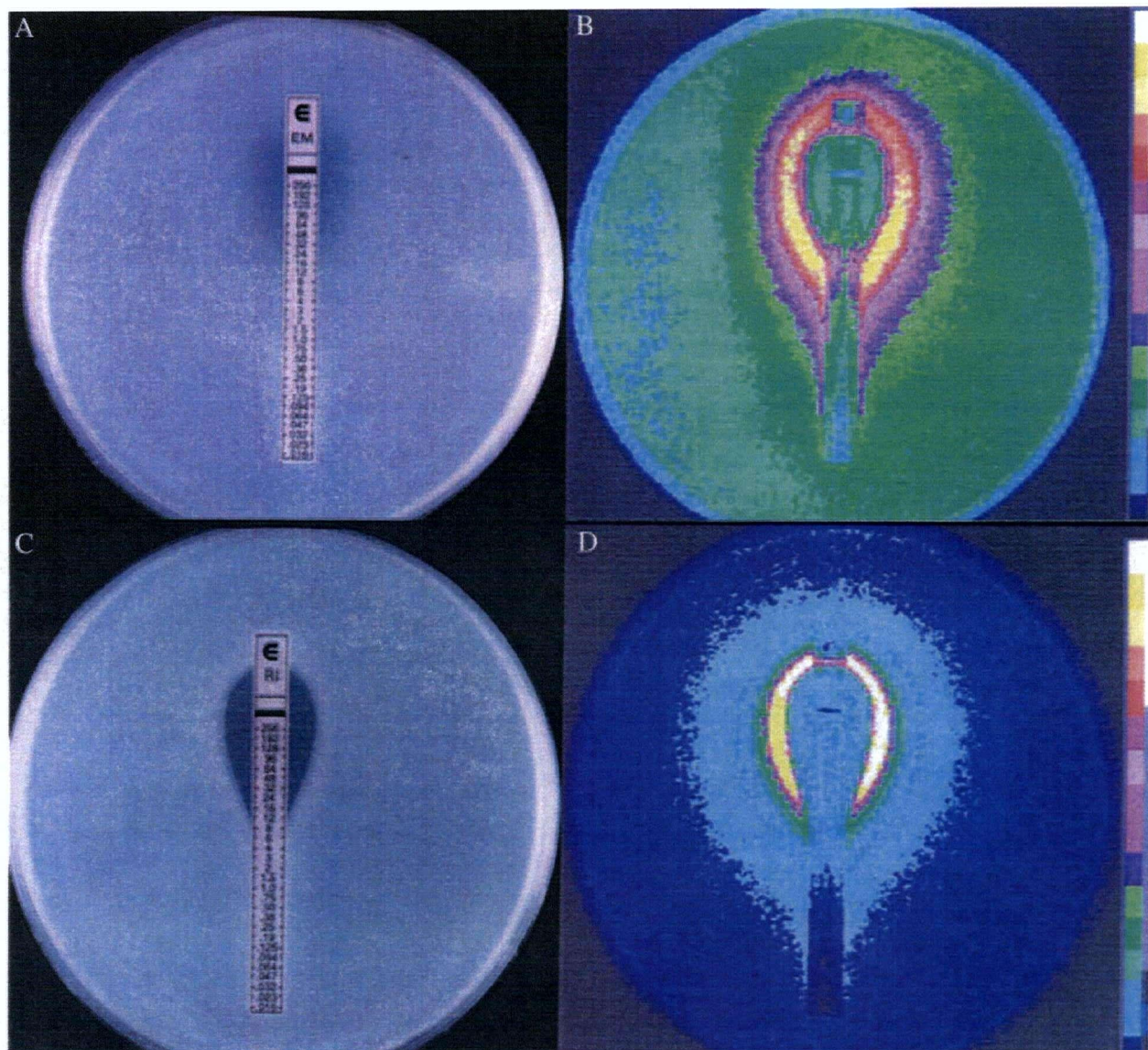
Fig. 1. Comparison between growth inhibition and promoter activation (light production) by erythromycin (*A* and *B*) and rifampicin (*C* and *D*) over a range of different concentrations as measured with Etest strips placed on bacterial cell overlays. (*B* and *D*) Luminescence. The *lux*–reporter constructions used are listed in Table 1. The bottom of the inhibition zone (where it intersects with the Etest strip) gives a measure of the MICs, which are 32 μg/ml (erythromycin) and 12 μg/ml (rifampicin).

resistant to the active antibiotics were examined. In Fig. 4 we show the responses of erythromycin-resistant and rifampicin-resistant mutants, in which the inhibitory targets of the drugs are altered. For erythromycin, mutants in ribosomal protein L22 with attenuated binding of the antibiotic to the 50S ribosome were used (11). For rifampicin, mutants in the *rpoB* gene for the RNA polymerase β-subunit (the binding site for the antibiotic), were used (13). Significantly reduced activation of transcription by erythromycin was seen in *eryR* mutants. The rifampicin-resistant strain showed no response to rifampicin. As expected, a *rifR* mutation had no effect on activation by erythromycin and the *eryR* mutants responded normally to rifampicin.

Mutants Defective in Stress Responses Show Antibiotic-Induced Modulation. Numerous cell stress responses are mediated through the association of alternative σ factors with the transcription complex (21). We introduced several "responsive" promoter–*lux* constructs (including *luxR* promoters) into bacterial hosts de-

fective in a variety of stress responses and examined the modulation effects of antibiotics. Mutations in *rec* and *lex* (SOS), *dnaJ* and Δ*dnaKJ* (heat shock response), and Δ*relA* Δ*spoT* (universal stress response) did not significantly reduce the antibiotic stimulatory effects. We also examined the effect of mutations in *rpoS*, which is considered to be the general regulator of a variety of stress responses (22). No influence on antibiotic activation was observed (results not shown). In addition, SM activation was normal in a strain lacking cAMP (*cya*), a global regulatory molecule in bacteria.

Characterization of Promoter Responses. The above studies show that promoters may differ in their response to antibiotics. This finding was confirmed by testing a selection of the active *S. typhimurium* promoter–*lux* reporters for their activity in the presence of different structural classes of antibiotics, with different modes of action. The results in Fig. 5 indicate that any given promoter may be activated to a different extent, depending

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Table 2. Activation of promoter–*lux* fusions by different antibiotics on solid and in liquid media

Promoter-*lux* fusions

Antibiotics	lasRI' (solid)	lasRI' (liquid)	luxRI' (solid)	luxRI' (liquid)	rck (solid)	rck (liquid)	ahyRI' (solid)	ahyRI' (liquid)
Polymixin	+	-	++	+++	+	-	+	-
Colistin	++	-	+	+	+	-	+++	-
Erythromycin	+++	+	++	+++	++	+	+++	+
Clindamycin	+	-	+++	+++	-	-	+	++
Imipenem	+	-	+	-	-	-	++	-
Fosfomycin	+	-	+	-	+	-	++	-
Rifampicin	+++	-	++	+++	+++	+++	-	++

Bacterial cultures (*E. coli*: *lasRI'*, *luxRI'*, and *ahyRI'* or *S. typhimurium*: *rck*) were tested on LB agar overlays or LB cultures as described in *Materials and Methods*. Amounts of antibiotics applied in solid assays ranged from 15 to 2 µg, and concentrations in liquid assays ranged from 19.53 to 0.3125 µg/ml. Values above the diagonal, solid medium; below the diagonal, liquid medium; –, no light production; +++, strong activation.

on the antibiotic being used (we assume that this is true for those promoters that are repressed). These variations appear to be a function of the antibiotic mode of action. In some cases the discrimination is subtle, because 14- and 16-membered macrolide inhibitors of translation, which are known to block the peptide exit tunnel of the ribosome, activate different promoters (results not shown).

The kinetics and concentration dependence of promoter activation were examined in liquid cultures, and Fig. 6 shows that positive responses can be detected in most cases at antibiotic concentrations lower than the MIC for the *E. coli* strain being used. The stimulatory activity reaches a maximum level near the MIC, where transcription levels increased some 2- to 10-fold. At concentrations greater than the MIC, promoter activation was considerably reduced. The activity of different antibiotics was also dependent on the phase of bacterial growth.

Discussion

We show that antibiotics with different chemical structures and modes of inhibitory action activate or repress a wide variety of promoters in *S. typhimurium* at low concentrations; similar effects have been obtained with *E. coli* and *Pseudomonas aeruginosa* (results not shown). As examples, erythromycin (an inhibitor of translation) and rifampicin (an inhibitor of transcription) modulate (activate or repress) transcription of a significant proportion of genes ($\approx$5%) in *S. typhimurium* and *E. coli*. Studies with other inhibitors, including trimethoprim (targeting dihydrofolate reductase) and fosfomycin (which blocks cell-wall synthesis), confirm that compounds with different structures and modes of action exert effects on bacterial transcription at subinhibitory concentrations (results not shown). Thus, it appears that many antibiotic inhibitors, when used at low concentrations, have in common the ability to activate or repress gene transcription, which is distinct from their inhibitory effects. Interestingly, the two contrasting responses occur with the binding of the antibiotics to their "normal" targets, because resistant mutants affecting binding to the cell targets (ribosomes and RNA polymerase, respectively, in the case of erythromycin and rifampicin) showed significantly reduced responses.

What biochemical mechanisms are responsible for these effects? We suggest that all macromolecular processes are coupled to the transcription machinery such that even minor (non-growth-retarding) effects of the binding of SMs to a macromolecular target lead to alteration of the rate of mRNA production. Because this result occurs with a variety of SMs active at different sites on the ribosome (chloramphenicol, aminoglycosides, macrolides, and tetracycline), or in cell-wall synthesis (some β-lactams and fosfomycin), the transcription machinery must have the means to sense these subtle conformational or stoichiometric changes and respond by specific up- or down-regulation. In the case of rifampicin, which acts on the RNA polymerase β-subunit, a direct interaction must operate. We cannot eliminate the possibility that some antibiotics have occult binding sites whose effects are detected only by the sensitive assay system being used, or that the antibiotics activate one or
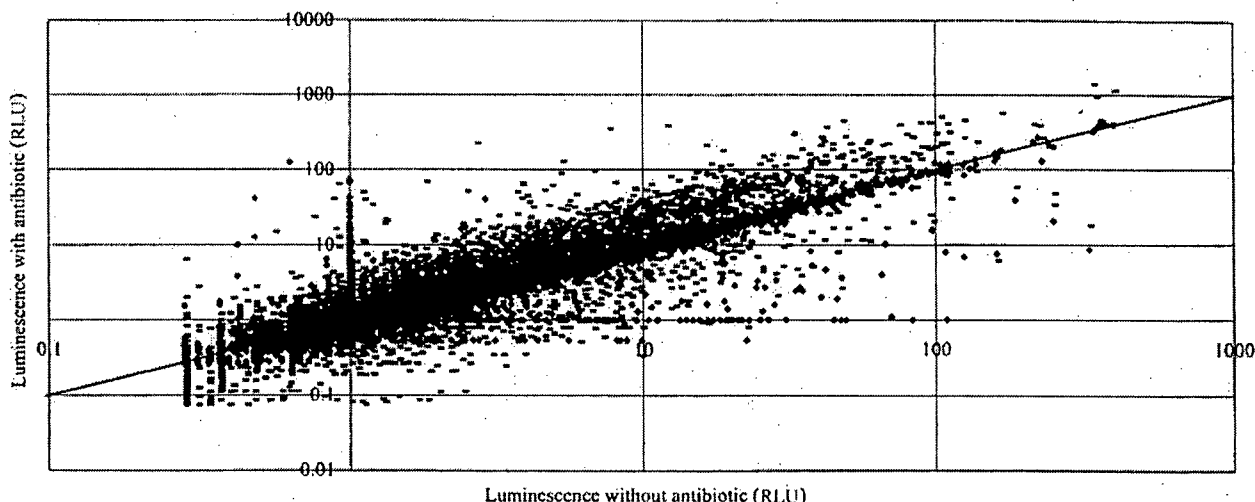
Fig. 2. Combined scatter plot of the actions of rifampicin at 1 µg/ml and erythromycin at 5 µg/ml determined by using a 6,500-clone *S. typhimurium* random promoter–*lux* library. RLU, relative light units. Incubation in microtiter plate liquid cultures was for 24 h at 37°C. Points above the diagonal indicate promoter-activated strains and points below the diagonal indicate clones in which promoter activity was repressed. (■, erythromycin; ◆, rifampicin.)
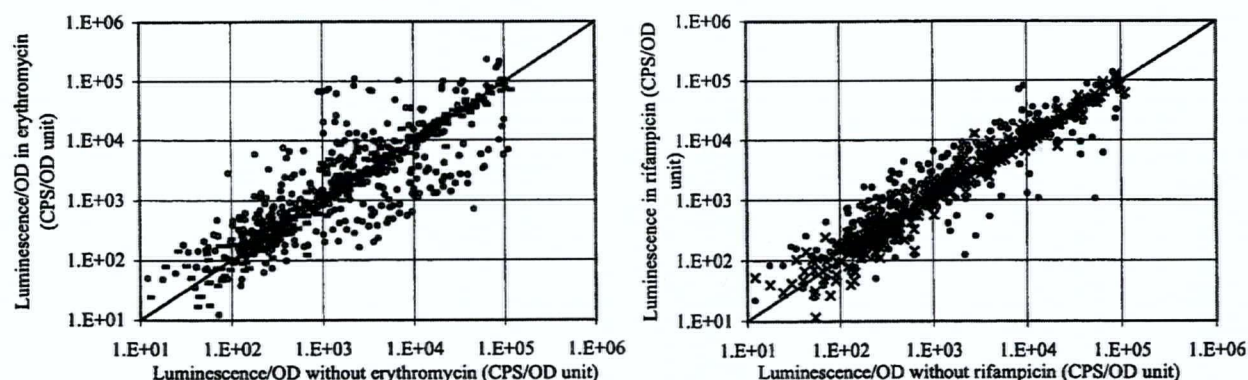
Fig. 3. (*Left*) Scatter plots of the reassay of selected clones activated by erythromycin at 1 μg/ml (-) and 30 μg/ml (●). (*Right*) Reassay of selected clones activated by rifampicin at 0.2 μg/ml (×) and 2.5 μg/ml (●). CPS, counts per second. OD is at 620 nm; 1.E+01 = 10^1, etc.

more metabolic networks (stress responses) through functional interactions with their "normal" targets.

We have shown that stress responses such as SOS and the universal response have no significant effect on SM activation, but it is conceivable that hitherto-unidentified stress responses, bacterial regulons, or signal transduction processes are responsible for the effects observed. The isolation and genetic analysis of mutants that do not respond to antibiotics should throw some light on this matter. In addition, it is known that bacterial metabolism is a complex network of interacting pathways, and negative effects on one pathway often lead to compensatory adjustments in other pathways (a form of homeostasis), as shown by expression profiling studies (23, 24). This result may occur through coordinate changes in transcription rates, which would be reflected as an apparent activation or repression of promoter activity. In prokaryotes, regulation of transcription in response to external signals is rapid and efficient. At present, such explanations for the global transcriptional changes observed in the presence of low antibiotic concentrations cannot be excluded.

In summary, antibiotics of different structure and known inhibitory activity show extensive stimulation or inhibition of a large number of promoters when target bacteria are exposed to subinhibitory concentrations of the drugs. A variety of chromosomal gene promoters are activated, including those involved in virulence, metabolic, and adaptive functions; others remain to be identified. The extent and magnitude of the effects observed suggest that the transcriptional modulation by commonly used antibiotics could lead to negative consequences during the treatment of bacterial infections in human hosts. The upregulation of quorum-sensing systems by low concentrations of antibiotics would lead to the precocious activation of bacterial regulons, including the production of virulence factors in pathogens (10). Such untoward activity against host cells and tissues may contribute to the deleterious side reactions that accompany antibiotic therapy. Low concentrations of antibiotics remaining in the host after the treatment of infection could contribute to the physiological state normally described as the postantibiotic effect. In addition, subinhibitory concentrations of antibiotics may disrupt the ecology of the normal flora by transcriptional modulation, as described here.

Concerning the role(s) of SMs in the chemical ecology of the environment, our studies indicate that SMs may be significant elements in the dynamics of bacterial communities in nature, contributing both competitive and interactive responses. Inhibition occurs when high concentrations are attained, transcriptional changes occur at low concentrations. Many of the promoters identified in our studies regulate genes of unknown function; it is possible that a number of these are associated with processes that would not be important under laboratory conditions.

The SMs identified as antibiotics may have evolved to play two distinct roles in natural microbial communities (2). Antibiotics are a complex class of molecules, differing from other small molecule effectors such as amino acid derivatives. They do not have any enzyme-substrate activity (apart from their resistance enzymes, which may regulate their effects on transcription); in fact, antibiotic resistance genes are frequently present in strains that do not make antibiotics (25). Numerous examples of metabolic interdependence in microbial consortia are known (5), and within communities SMs may influence population structure and dynamics, and interspecies stimulation because of SMs appears to be common between streptomycetes (26). The modulatory effects of SMs suggest many possibilities as modal-
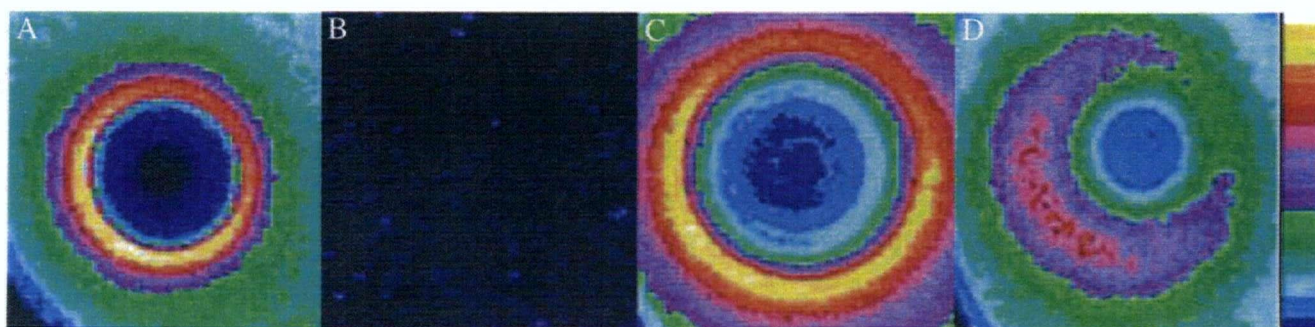
Fig. 4. The effect of mutations to resistance to erythromycin (*rplV*) and to rifampicin (*rpoB*) in *S. typhimurium* on antibiotic inhibition and promoter activation on solid media. (*A*) Rifampicin-sensitive. (*B*) Rifampicin-resistant. (*C*) Erythromycin-sensitive. (*D*) Erythromycin-resistant.
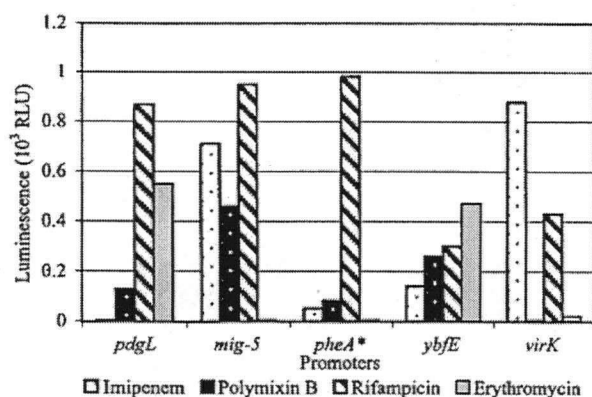
Fig. 5. The antibiotic specificity of the transcription activation of different promoters. Overlays of *S. typhimurium* promoter–*lux* fusions on LB agar were exposed to discs containing antibiotics (10 μg of imipenem, 10 μg of polymixin B, 10 μg of rifampicin, or 15 μg of erythromycin). Light production was measured as described in *Materials and Methods*. RLU, relative light units. *pheA** is a presumptive identification.

Fig. 6. Concentration dependence of promoter activation (*plasI'*::*luxCDABE*) in a rifampicin-sensitive (K802; MIC 12 μg/ml) and a rifampicin-resistant (K802NR; MIC >256 μg/ml) *E. coli* host.

ities of microbial communication. Even those SMs that are ineffective inhibitors (e.g., erythromycin and rifampicin) can enter cells of Gram-negative bacteria and bind to macromolecular targets in concentrations sufficient to exert the transcriptional responses demonstrated here. The same is likely to be true for a wide range of other SMs and could include both prokaryotic and eukaryotic cells.

At first sight, the differences in response seen when bacteria are exposed to SMs in liquid or on solid media may appear unusual, but it is well known that colonies growing on agar plates have distinct community structures containing bacteria in different physiological states; the generation times of bacteria are different in sessile compared with planktonic growth. The most striking differences are seen when bacteria form community structures known as biofilms on solid supports (5). Differences in SM-induced changes may be considered a further manifestation of the two states.

Finally, the effects of SMs at low concentrations on transcription may provide the basis for novel approaches to the identification of biologically active SMs from natural sources for use as pharmaceutical agents. Employing transcriptional responses to test microbial or plant extracts for their abilities to interact with intracellular targets would be a very sensitive measure of biological activity that is readily adaptable to high-throughput methods. Stress-response promoter–reporters have been described for this purpose (27, 28), but greater discrimination could be attained with a broader range of promoters, as described here. Because active small molecules exert their transcriptional effects by binding to specific intracellular targets, testing compounds against defined panels of promoter–reporter constructs should provide the means for the identification of primary cellular targets important to mechanism of action studies.

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Appendix 7.2. Results of partial nucleotide sequencing from Goh *et al.*

Hit Start	Hit stop	Accession number	Gene	Anti-biotic	Res ponse	Notes
6163	6876	AE008858	bfr or bfd	erm/rif	down	bfd is a regulatory or redox component complexing with bfr in iron storage and mobility
16029 (225)	16430 (634)	AE008749	csgD	erm/rif	down	putative transcriptional regulator- LuxR/UhpA family of csgAB operon
3148	3627	AE008872	cysE	rif	down	serine acetyltransferase
9300	8612	AE008751	flgK	rif	down	flagellar biosynthesis, hook-filament junction protein 1
2249 (64)	2625 (440)	AE008787	fliA	rif	down	sigma F (sigma 28)
10455 (67)	10040 (482)	AE008826	iroB	rif	down	putative glycosyl transferase
2890	2202	AE008757	osmE	erm/rif	down	transcriptional activator of ntrL gene
4351	4799	AE008786	otsB	erm/rif	down	trehalose-6-phosphate phosphatase
8346	7942	AE008901	proP	erm/rif	down	MFS family, low affinity proline transporter
20292 (79)	20531 (319)	AE008795	stcC in gene	rif	down	
14385	14798	AE008754	STM 1249	rif	down	putative periplasmic protein
1654	1146	AE008755	stm1261	erm/rif	down	putative cytoplasmic protein
6044	5498	AE008774	STM1672	rif	down	putative cytoplasmic protein
14825	14317	AE008782	stm1851	erm/rif	down	putative cytoplasmic protein
19021	18773	AE008802	stm2287 in gene	erm/rif	down	sn-glycerol-3-phosphate dehydrogenase
12052	12351	AE008849	tdcB/C	rif	down	threonine dehydratase
8520 (69)	8156 (435)	AE008811	tktB	erm/rif	down	transketolase 2, isozyme
6553	6425	AE008752	tmk	rif	down	thymidylate kinase
6525	6405	AE008886	trkH in gene	rif	down	
7731 (72)	7212 (593)	AE008843	trna(pheV)	rif	down	
14586	13903	AE008913	tsr	rif	down	methy-accepting chemotaxis protein I, serine sensor receptor
17301	17571	AE008718	ybaL	rif	down	putative CPA2 family transport protein
2790	3047	AE008728	ybfE	rif	down	lexA regulated, putative SOS response
17568	17009	AE008727	ybfM in gene	erm/rif	down	putative outer membrane protein
3804	4087	AE008740	ybjX	rif	down	homolog of virK
379	1	AE008777	yciG	erm/rif	down	putative cytoplasmic protein
13244	12705	AE008775	yciW	rif	down	putative cytoplasmic protein
7015	7417	AE008833	ygbJ	erm/rif	down	3-hydroxyisobutyrate dehydrogenase
18450	18804	AE008836	ygdI or gcvA	erm/rif	down	putative lipoprotein
8166	7871	AE008898	yjbJ	erm/rif	down	putative cytoplasmic protein
6801	7383	AE008908	yjgA	rif	down	putative cytoplasmic protein

18262	17838	AE008769	yncD in gene	rif	down	
1582 (70)	1921 (409)	AE008848	yqjH	erm/rif	down	putative transporter
15225	14797	AE008860	yrfF	rif	down	putative inner membrane protein
2531	2008	AE008718	aefA in gene	rif	up	
12300	12495	AE008727	nagB	rif	up	glucosamine-6-phosphate deaminase
313 (263)	32 (555)	AE008714	phoB	rif	up	response regulator in 2-component system with PhoR (CreC)
18376	18109	AE008762	slyA	erm/rif	up	transcriptional regulator for hemolysin (MarR family)
10665	11140	AE008747	sopB	rif	up	outer protein: homologous to ipgD of Shigella
3983	3826	AE008774	STM 1671	rif	up	putative bacterial regulatory helix-turn-helix proteins, araC family
17252	16583	AE008754	STM1252	rif	up	putative cytoplasmic protein
17412	16814	AE008781	stm1833	rif	up	putative inner membrane protein
2033	1640	AE008827	virK	rif	up	virulence gene
15538	14853	AE008741	yca/dorM	rif	up	putative MFS family transport protein
20063 (295)	20349 (581)	AE008866	yhjG	rif	up	putative inner membrane protein
12322	12778	AE008892	yijP	rif	up	putative integral membrane protein
16841	16395	AE008909	yjgK	rif	up	putative cytoplasmic protein
1663	1821	AE008782	yobG	rif	up	putative inner membrane protein
8173	7528	AE008797	yohK or J	rif	up	putative transmembrane protein

Appendix 7.3. Supplemental information regarding ORFs encoded on insert sequences of rifampicin responsive clones

Primary locus name	Putative identification:	Comment:	Coordinates:	DNA Molecule:	E.coli homolog and (predicted) operon	E.coli promoter sequence in RegulonDB?
STM3084	putative regulatory protein, gntR family	product=putative regulatory protein, gntR family note=Paralog of E. coli regulator for uxu operon (AAC77280.1); Blastp hit to AAC77280.1 (257 aa), 39% identity in aa 8 - 247	3247898 to 3247098	chromosome		
STM3595	putative phosphatase	product=putative phosphatase	3767435 to 3766164	chromosome		
STM2066/sopA	Secreted effector protein of Salmonella dublin	product=Secreted effector protein of Salmonella dublin note=Paralog of E. coli orf, hypothetical protein (AAC77008.1); Blastp hit to AAC77008.1 (442 aa), 24% identity in aa 194 - 432, 20 503320494dentity in aa 59 - 151, 23 0dentity in aa 34 - 71, 32 173642208dentity in aa 19 - 50		chromosome		
STM4255	putative cytoplasmic protein	product=putative cytoplasmic protein note=hypothetical protein	4476003 to 4476119	chromosome		
STM4256/ssb	ssDNA-binding protein controls activity of RecBCD nuclease	ssDNA-binding protein controls activity of RecBCD nuclease	4476286 to 4476816	chromosome	ssb	Y
STM4257	putative inner membrane or exported	product=putative inner membrane or exported note=S. typhimurium hypothetical protein spi4_A (gi|7467268); first gene in spi4 Infect Immun. 1998 Jul;66(7):3365-71	4477857 to 4478489	chromosome		
STM4258	putative methyl-accepting chemotaxis protein	product=putative methyl-accepting chemotaxis protein note=S. typhimurium hypothetical protein spi4_A (gi|7467268)	4478486 to 4479874	chromosome		
STM1956/fliA	sigma F (sigma 28) factor of RNA polymerase	class II flagellar gene, transcription of late flagellar genes (class 3a and 3b operons)	2045466 to 2044747	chromosome	fliA	
STM2946/cysH	3-phosphoadenosine 5-phosphosulfate (PAPS) reductase	product=3-phosphoadenosine 5-phosphosulfate (PAPS) reductase note=S. typhimurium phosphoadenosine phosphosulfate reductase involved in sulfate assimilation. (SW:CYSH_SALTY)	3088923 to 3088189	chromosome	cysJIH	Y

STM1311/osmE	transcriptional activator of ntrL gene; NtrL is glutamine dependant NAD+ synthase	transcriptional activator of ntrL gene; NtrL is involved in pyridine nucleotide cycling	1391165 to 1391506	chromosome	osmE	
STM4454/treB		pseudogene; frameshift; -10_signal complement(4698331..4698339) /evidence=not_experimental -35_signal complement(4698350..4698358) /evidence=not_experimental	STM4454	chromosome	treB	Y
PSLT039/spvB	Salmonella plasmid virulence: hydrophilic protein	product=Salmonella plasmid virulence: hydrophilic protein	29966 to 28191	plasmid		
PSLT040/spvA	Salmonella plasmid virulence: outer membrane protein	product=Salmonella plasmid virulence: outer membrane protein	30915 to 30148	plasmid		
PSLT041/spvR	Salmonella plasmid virulence: regulation of spv operon	product=Salmonella plasmid virulence: regulation of spv operon, lysR family	32320 to 31427	plasmid		
STM1248		pseudogene; frameshift	1333542..1333717	chromosome		
STM1183/flgK	flagellar biosynthesis, hook-filament junction protein 1	product=flagellar biosynthesis, hook-filament junction protein 1 note=S. typhimurium flagellar hook-associated protein 1 (HAP1). (SW:FLGK_SALTY); class III flagellar gene	1265468 to 1267129	chromosome	flgK	
STM1328	putative outer membrane protein	product=putative outer membrane protein	1408077 to 1407118	chromosome		
STM4118/yijP	putative Integral membrane protein	product=putative Integral membrane protein note=Ortholog of E. coli orf, hypothetical protein (AAC76937.1); Blastp hit to AAC76937.1 (577 aa), 89% identity in aa 1 - 577	4335042 to 4333309	chromosome	yijP	Y

STM1091/sopB	Salmonella outer protein: homologous to ipgD of	product=Salmonella outer protein: homologous to ipgD of Shigella note=S. typhimurium invasion gene D protein (gi|2582385); nothing similar in E.coli	1179916 to 1178231	chromosome		
STM1093	putative cytoplasmic protein	product=putative cytoplasmic protein note=hypothetical protein	1180529 to 1180419	chromosome		
STM0425/thiI	sulfur transfer protein (from cys to ThiS	product=sulfur transfer protein (from cys to ThiS and from IscS to U8-tRNA) note=S. typhimurium thiamine biosynthesis protein thiI. (SW:THII_SALTY); gene immediately upstream, xseB, is transcribe divergently and has slyA binding site (i.e. site is BW slyA and xseB); Ecocyc:ThiI is required for the synthesis of the thiazole moiety of thiamine and plays a role in the conversion of uridine to 4-thiouridine at position 8 in tRNA.	477868 to 479316	chromosome	yajK	Y
STM1778/lolB	outer membrane lipoprotein	product=outer membrane lipoprotein note=S. typhimurium outer-membrane lipoprotein LOLB precursor. (SW:LOLB_SALTY) -96.1% similarity to E.coli hemM=b1209	1876024 to 1876647	chromosome	hemM_ychB operon	Y
STM2199/cirA	outer membrane porin, receptor for colicin I	note=Ortholog of E. coli outer membrane receptor for iron-regulated colicin I receptor; porin; requires tonB; Orthologue of E. coli cirA (CIRA_ECOLI); Fasta hit to CIRA_ECOLI (663 aa), 88% identity in 663 aa overlap; Ecocyc:Cir is a member of the Outer Membrane Receptor (OMR) family of porins. Cir is a TonB (TonB=integral membrane protein, thought to channel energy of PMF to OMR) dependent iron-siderophore complex uptake receptor. The substrate spectrum of Cir is very similar to that of Fiu. Cir transports monomers, dimer, and linear trimers of 2,3-dihydorxybenzoylserine. In addition Cir is a receptor for colicins IA, IB, and V and microcins E492, H47, and M.	2298542 to 2296551	chromosome	two promoters of cirA: a)cirp1 b)cirp2	Y
STM1597/ydcW	putative aldehyde dehydrogenase	product=putative aldehyde dehydrogenase note=Ortholog of E. coli putative aldehyde dehydrogenase (AAC74526.1); Blastp hit to AAC74526.1 (474 aa), 83% identity in aa 1 - 474; Putative betaine aldehyde dehydrogenase	1688279 to 1686834	chromosome	b1440_ydcT_b1442_b1443_ydcW	Y

STM2287/	putative cytoplasmic protein	product=putative cytoplasmic protein note=Paralog of E. coli putative sulfatase / phosphatase (AAC75329.1); Blastp hit to AAC75329.1 (403 aa) elaD(b2269), 30% identity in aa 27 - 294, 29 503321556dentity in aa 297 - 363	2394795 to 2395748	chromosome	b2269_2380 664	
STM1155/htrB	lauroyl/myristoyl acyltransferase involved in lipid A biosynthesis	product=lauroyl/myristoyl acyltransferase involved in lipid A biosynthesis note=Ortholog of E. coli heat shock protein (AAC74138.1); Blastp hit to AAC74138.1 (306 aa), 83% identity in aa 1 - 306; Ecocyc: htrb=waaM=lpxL; waaN=msbB=lpxM is required for the toxicity of lipidA and LPS Mol micro. 1998 29(2):571-579;	1243536 to 1242616	chromosome	htrB	Y
STM1154/yceE	putative MFS family transport protein	product=putative MFS family transport protein note=Ortholog of E. coli putative transport protein (AAC74137.1), mdtG=b1053; Blastp hit to AAC74137.1 (408 aa), 27% identity in aa 1 - 404 (b1051); Ecocyc:Overexpression of the cloned yceE gene in a drug-sensitive background strain resulted in a two-fold increase in resistance to deoxycholate and a four-fold increase in resistance to fosfomycin.	1242461 to 1241247	chromosome	yceE	Y
STM1153/msyB	acidic protein suppresses mutants lacking function of protein export	product=acidic protein suppresses mutants lacking function of protein export note=Ortholog of E. coli acidic protein suppresses mutants lacking function of protein export (AAC74135.1); Blastp hit to AAC74135.1 (125 aa), 92% identity in aa 2 - 125	1241165 to 1240791	chromosome	b1052_msy B	Y
STM2899/invF	invasion protein	product=invasion protein note=S. typhimurium invasion protein INVF. (SW:INVF_SALTY)	3043932 to 3043282	chromosome		
STM1913/flhA	flagellar biosynthesis possible export of flagellar proteins	product=flagellar biosynthesis; possible export of flagellar proteins note=S. typhimurium flagellar biosynthesis protein FLHA. (SW:FLHA_SALTY)	2010290 to 2008212	chromosome	flhB_flhA_f lhE	Y
STM1914/flhB	putative part of export apparatus for flagellar	product=putative part of export apparatus for flagellar proteins note=S. typhimurium flagellar biosynthetic protein FLHB. (SW:FLHB_SALTY)	2011434 to 2010283	chromosome		
STM1915/cheZ	chemotactic response CheY protein phophatase	product=chemotactic response; CheY protein phophatase note=S. typhimurium chemotaxis protein CHEZ. (SW:CHEZ_SALTY)	2012272 to 2011628	chromosome	tar_tap_che R_cheB_ch eY_cheZ	Y

STM1916/cheY	chemotaxis regulator, transmits chemoreceptor signals to flagelllar	product=chemotaxis regulator, transmits chemoreceptor signals to flagelllar motor components note=S. typhimurium chemotaxis protein CHEY. (SW:CHEY_SALTY)	2012672 to 2012283	chromosome	tar_tap_che R_cheB_ch eY_cheZ	Y
STM1917/cheB	methyl esterase, response regulator for chemotaxis (cheA	product=methyl esterase, response regulator for chemotaxis (cheA sensor) note=S. typhimurium protein-glutamate methylesterase. (SW:CHEB_SALTY)	2013739 to 2012690	chromosome	tar_tap_che R_cheB_ch eY_cheZ	Y
STM3248/garR/y haE	tartronate semialdehyde reductase (TSAR)	product=tartronate semialdehyde reductase (TSAR) note=Ortholog of E. coli putative dehydrogenase (AAC76159.1); Blastp hit to AAC76159.1 (299 aa), 97% identity in aa 4 - 299	3417343 to 3416453	chromosome	yhaU_yhaF _yhaE_yha D	Y
STM3249/garL;y haF	2-Dehydro-3-Deoxy-Galactarate Aldolase	product=2-Dehydro-3-Deoxy-Galactarate Aldolase note=Ortholog of E. coli orf, hypothetical protein (AAC76160.1); Blastp hit to AAC76160.1 (256 aa), 90% identity in aa 1 - 256	3418139 to 3417369	chromosome	yhaU_yhaF _yhaE_yha D	Y
-		hypothetical protein, % similarity to E.coli yhaU	3418322 to 3418441	chromosome	yhaU_yhaF _yhaE_yha D	Y
STM2445/ucpA	putative oxidoreductase	product=putative oxidoreductase note=Ortholog of E. coli putative oxidoreductase (AAC75479.1); Blastp hit to AAC75479.1 (285 aa), 92% identity in aa 23 - 285	2557390 to 2556599	chromosome	yfeF	Y
STM1444/slyA	transcriptional regulator for hemolysin (MarR family)	product=transcriptional regulator for hemolysin (MarR family) note=S. typhimurium transcriptional regulator SLYA (salmolysin) (cytolysin SLYA). (SW:SLYA_SALTY)	1519880 to 1520320	chromosome	slyA	
PSLT102/traS	conjugative transfer: surface exclusion	product=conjugative transfer: surface exclusion	82685 to 83179	plasmid		
PSLT103/traT	trat complement resistance protein precursor. {salmonella	Unclassified: Role category not yet assigned	83284 to 83946	plasmid		
STM2474/tktB	transketolase 2, isozyme	product=transketolase 2, isozyme note=Ortholog of E. coli transketolase 2 isozyme (AAC75518.1); Blastp hit to AAC75518.1 (667 aa), 92% identity in aa 1 - 666	2583500 to 2585500	chromosome	tktB	

STM0389/yaiA	putative cytoplasmic protein	product=putative cytoplasmic protein note=Paralog of E. coli orf, hypothetical protein (AAC73492.1); Blastp hit to AAC73492.1 (63 aa), 93% identity in aa 1 - 62	441417 to 441608	chromosome	yaiA	
STM0940/ybjX	Homolog of virK	product=Homolog of virK/STM2781 note=Ortholog of E. coli putative enzyme (AAC73964.1); Blastp hit to AAC73964.1 (330 aa), 57% identity in aa 15 - 329; STM2781 product=virulence gene; homologous sequence to virK in Shigella note=Paralog of E. coli putative enzyme (AAC73964.1); Blastp hit to AAC73964.1 (330 aa), 39% identity in aa 47 - 329	1018483 to 1017515	chromosome	ybjX	
STM1833	putative inner membrane protein	product=putative inner membrane protein note=Ortholog of E. coli orf, hypothetical protein (AAC74890.1); Blastp hit to AAC74890.1 (152 aa), 88% identity in aa 1 - 152, yobD=b1820	1931276 to 1931746	chromosome		
STM0047/lspA	prolipoprotein signal peptidase (Spase II)	product=prolipoprotein signal peptidase (SPase II) note=Ortholog of E. coli prolipoprotein signal peptidase (SPase II) (AAC73138.1); Blastp hit to AAC73138.1 (164 aa), 92% identity in aa 1 - 164	56689 to 57189	chromosome	ribF-ileS-lspA-slpA-lytB operon;	
STM0048/slpA	FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase)	product=FKBP-type peptidyl-prolyl cis-trans isomerase (rotamase) note=Ortholog of E. coli probable FKBX-type 16KD peptidyl-prolyl cis-trans isomerase (a rotamase) (AAC73139.1); Blastp hit to AAC73139.1 (149 aa), 91% identity in aa 1 - 149	57344 to 57793	chromosome	ribF-ileS-lspA-slpA-lytB operon;	
STM0049/lytB	Mevalonate-independent isoprenoid biosynthetic enzyme/Penicillin tolerance protein	similar to Escherichia coli control of stringent response; involved in penicillin tolerance (AAC73140.1); regulates the activity of guanosine 3',5'-bispyrophosphate synthetase I; RelA		chromosome		
STM2473/talA	transaldolase A	product=transaldolase A note=Ortholog of E. coli transaldolase A (AAC75517.1); Blastp hit to AAC75517.1 (316 aa), 89% identity in aa 1 - 316; Ecocyc:There are two closely related transaldolases in E. coli. Transcription is induced by the CreBC two-component system by minimal media growth conditions.	2582530 to 2583480	chromosome	talA_tktB	Y

Appendix 7.4.1. Summary of nucleotide consensus sequences with similarity to known transcription factor binding or termination sites. Consensus sequences were derived from bioinformatic analysis of insert nucleotide sequences obtained from rifampicin responsive clones

Consensus sequence	Regulator	Gene	Motif In DPInteract
AAATTA TTAAAA ATAATT ATAATA TTAAT	rpoS #44	csgB A	....|....| ....|....| ....|....| ....|....| 10 20 30 40 -AAATTATTA AAAATAATT- ---ATA ATA TTAAT-- tgagttatta aaaatatttc cgcagacata ctttccatcg
	rpoS #6 and rpoD #21	csgB A	....|....| ....|....| ....|....| ....|....| ....|....| ....| 10 20 30 40 50 ---------- -------AAA TTATTAAAAA TAATT----- ---ATAATAT TAAT- aatacaacgc gcgggtG-AG TTAttaaaaa tatttccgca gacatacttt ccatc
	fur #18	fbpA-Ngo	....|....| ....|....| ....|....| .... 10 20 30 AAATTATTAA AAATAATTAT AAT-ATTAAT ---- aaatt-taaa aaataattat ttgcatttat aaaa
	ihf #89	S.typh carA B	.....|....| ....|....| ....|....| ....|.. 10 20 30 AAATTATTAA AA-AT--AAT TATAATATTA AT----- atatttttaa tatattgatt tttaaaattt ttttgtc
TTTATA ATATAT TCCTGA AAACTA AATTT	oxyR #13	Q11 (SEL- EX)	....|....| ....|....| ....|....| ....|....| ....|....| .. 10 20 30 40 50 TTTATAATAT ATTCCTGAAA ACTAAATTT- ---------- ---------- -- ------ATAG TTTTCAGGAA ACTTA-TCGA TCCGCAAAAC CTATCGATCA TC
AAAAAA TTAAAC ACATTT ATATAA	betI #1	betI-betT	....|....| ....|....| ....|....| ....|....| ....|....| 10 20 30 40 50 AAAAAATTAA -ACACATTTT ATATAATAAT TATAAAT ATTTAATTTAAAA ---caattaa gacacatTTT ATATTGAACG TCCAATGAAt aaccgcttta ata
TAATTA TAAAATA TTTAATT TAAAA	ada #4	ada-Eco	....|....| ....|....| ....|....| ....|....| ....|....| ....|. 10 20 30 40 50 -------AAA AAATTAAA-C ACATTTTATA TAATAATTAT AAATATTTAATTTAAA tcagcgaaaa aaattaaagc gca----aga ttgtt----- ----------------
	ada #2	ada	....|....| ....|....| ....|....| ....|....| ....|....| 10 20 30 40 50 --AAAAAATT AAA-CACATT TTATATAATA ATTATAAATA TTTAATTTAA AA gaaaaaaatt aaagcgca-- --agattgtt ---------- ----------
AAAAAA TTGAAT AAAT	rtp_Bsu #2	TerII	....|....| ....|....| ....|....| ....|....| ....|....| 10 20 30 40 50 ---------- -------AAA AAATTGAATA AAT------ ---------- ttcttctgtc actgacaaca ctatgtacta aatattcaat tttcttttat
	rtp_Bsu #8	TerII-W23	....|....| ....|....| ....|....| ....|....| ....|....| 10 20 30 40 50 ---------- -------AAA AAATTGAATA AAT------ ---------- tccttctatc actgagaaca ctatgtacta aatattcaat tttattctat
	ihf #105	psp	....|....| ....|....| ....|....| ....|....| ... 10 20 30 40 ---------- ---------- -------AAA AAATTGAATA AAT aatcagatct ttataaatca aaaagataaa aaattg---- ---

Note: Sequences which are identical in both consensus sequences obtained from motif finding programs and known transcription factor binding or terminator (Ter) sites found in RegulonDB are highlighted.

Appendix 7.4.2. Summary of 22 nucleotide consensus sequences obtained from insert sequence of rifampicin responsive clones

Motif	Program	G+C% Content	IR or DR (min width=3, max gap=10)	Length (bp)	DPInteract
Gcggcgaattt	Meme Motif 1	54.6	DR	11	no hits
Ctgcaaaaact	Meme Motif 2	36.4	none	11	no hits
Cgctggcg	Meme Motif 3	87.5	IR	8	no hits
Anngcgncggcgatantgncg	Gibbs nm=2	52.4	IR, DR	21	no hits
Anngngncggcgataatgnc	Gibbs nm=20	45.0	DR	20	no hits
GCTGGCG	10 72 30w any Motif 1	85.7	none	7	no hits
CGGGCTGGATTTTCTCTTCCTG	10 72 30w any Motif 2	54.6	IR, DR	22	no hits
AAATTATTAAAAATAATTATAATATTAAT	10 72 30w any Motif 3	0.0	IR, DR	29	5 hits
CCGCCCAGGCTGGCGC	20 48 30w any Motif 1	87.5	IR, DR	16	no hits
TTTATAATATATTCCTGAAAACTAAATTT	20 48 30w any Motif 2	13.8	IR, DR	29	1 hit
GCTGGAAAAACTGATGGACGA	20 48 30w any Motif 3	47.6	DR	21	0 hits
GCTGAATGAACTGCCGGGCG	18 24 20w motif 1	65.0	IR, DR	20	0 hits
AATTAAAAAAATGA	18 24 20w motif 2	7.1	IR, DR	14	0 hits
CCGGCTGCGAATATT	18 24 20w motif 3	53.3	IR	15	0 hits
CGTCCGCCAAATCGGCGGTG	10 72 20w Motif 2	70.0	IR, DR	20	0 hits
TAAAAAAATGATTATTTACC	10 72 20w Motif 3	15.0	IR, DR	20	0 hits
CTGGTCGACGCCCAGGACGCCGCCGTGAAGATTGCCGGCGA	20 48 20w Motif 1	70.7	IR, DR	41	0 hits
AAAAAATTAAACACATTTTATATAATAATTATAAATATTTAATTTAAAA	20 48 20w Motif 2	4.1	IR, DR	49	3 hits
AATCGCTGGCGCTG	20 48 20w Motif 3	64.3	IR, DR	14	0 hits
ACGGCGCCAGCG	18 100 20w any Motif 1	83.3	IR, DR	12	0 hits
AAAAAATTGAATAAAT	18 100 20w any Motif 2	6.3	IR, DR	16	3 hits
TTTCGCGCAGCGCCGG	18 100 20w any Motif 3	75.0	IR, DR	16	0 hits