

Regulation of Temporal Expression of IHF

In *Legionella pneumophila*

by

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Abstract

The Gram-negative bacillus *Legionella pneumophila*, possesses a distinct dimorphic life cycle that alternates between the replicative form (RF) and the infectious cyst-like form (CLF). It is proposed that this CLF, when inadvertently inhaled by an individual, manifests as an atypical pneumonia termed Legionnaires' disease. Studies elsewhere have determined that *L. pneumophila* IHF (LpIHF), a heterodimeric protein encoded by *ihfA* and *ihfB*, plays a key role in the morphological differentiation into CLF. This study was undertaken to elucidate the regulation of LpIHF expression as well as the regulation of targeted genes associated with morphological differentiation by LpIHF. Through green fluorescent protein reporter assays and electrophoretic mobility shift assays, it was shown that both *ihfA* and *ihfB* expression is negatively autoregulated by LpIHF and is positively activated by the *L. pneumophila* stationary sigma factor RpoS (LpRpoS). In addition, LpIHF positively regulates expression of the non-coding RNAs RsmY and RsmZ.

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Table of Contents

Abstract	i
Acknowledgements	ii
List of Tables	vii
List of Figures	viii
List of Abbreviations	xi
Chapter 1: Introduction	1
1.1 <i>Legionella pneumophila</i> life cycle/background	1
1.2 <i>Legionella pneumophila</i> global regulatory cascade	5
1.3 Integration Host Factor (IHF)	10
1.4 RpoS	16
1.5 RsmY and RsmZ	19
1.6 Study aims	22
Chapter 2: Materials and Methods	25
2.1 Bacterial strains and plasmids	25
2.2 Culture conditions	38
2.2.1 <i>E. coli</i> culture conditions	38
2.2.2 <i>L. pneumophila</i> culture conditions	38
2.3 Genome Extraction	39
2.4 PCR	40
2.4.1 Cycling conditions	40
2.4.2 Agarose gel electrophoresis	45
2.5 Cloning of insert DNA into plasmid	45

2.6	Constructed plasmid transformation or electroporation into <i>E. coli</i> cells.....	47
2.6.1	Preparation of rubidium chloride chemically competent <i>E. coli</i> DH5 α cells.....	47
2.6.2	Transformation of ligations into rubidium chloride chemically competent <i>E. coli</i> DH5 α cells.....	48
2.6.3	Preparation of electrocompetent <i>E. coli</i> DY330 and DY331 cells.....	49
2.7	PCR colony confirmation of ligated plasmid construct in <i>E. coli</i>	49
2.8	Sequencing.....	50
2.9	Constructed plasmid electroporation into <i>L. pneumophila</i> cells.....	51
2.9.1	Preparation of electrocompetent <i>L. pneumophila</i> cells.....	51
2.9.2	Electroporation of constructed plasmids into <i>L. pneumophila</i> cells.....	51
2.10	Fluorometer assays.....	52
2.11	Protein purification.....	53
2.11.1	Growth and protein induction.....	53
2.11.2	Preparation of beads and French press.....	54
2.11.3	Gravity column and dialysis.....	54
2.11.4	HiTrap TM Heparin HP column and concentration.....	55
2.11.5	Western blot analysis of eluted recombinant protein.....	56
2.11.6	Determination of the concentration of protein and DNA in samples.....	57
2.12	Electrophoretic mobility shift assay.....	58
2.13	Double knockout strategy ($\Delta ihf\alpha letA$).....	59
2.14	Triple knockout strategy ($\Delta ihf\alpha ihf\beta rpoS$ & $\Delta ihf\alpha ihf\beta letA$).....	59
Chapter 3:	Results	61

3.1	Bioinformatic pattern searches.....	61
3.1.1	Computational bioinformatic pattern search of IHF binding sites in the upstream promoter regions of <i>ihfα</i> and <i>ihfβ</i> in <i>L. pneumophila</i>.....	61
3.1.2	Computational bioinformatic pattern search of RpoS binding sites in the upstream promoter regions of <i>ihfα</i> and <i>ihfβ</i> in <i>L. pneumophila</i>.....	64
3.2	Creation of <i>ihfα</i> and <i>ihfβ</i> truncated promoter plasmid constructs.....	64
3.3	Expression of GFP based on the regulation of the truncated promoter constructs....	80
3.3.1	Expression profiles of <i>ihfα</i> and <i>ihfβ</i> in Lp02 wild-type and $\Delta ihf\alpha ihf\beta$.....	83
3.3.2	Microplate expression profiles of <i>ihfα</i> and <i>ihfβ</i> in Lp02 wild-type, $\Delta ihf\alpha ihf\beta$, $\Delta rpoS$ and $\Delta rpoS ihf\alpha$.....	93
3.3.2.1	Microplate expression profile of <i>ihfα</i> in Lp02 wild-type.....	93
3.3.2.2	Microplate expression profile of of <i>ihfα</i> in Lp02 $\Delta ihf\alpha ihf\beta$.....	96
3.3.2.3	Microplate expression profile of of <i>ihfα</i> in Lp02 $\Delta rpoS$.....	96
3.3.2.4	Microplate expression profile of <i>ihfα</i> in Lp02 $\Delta rpoS ihf\alpha$.....	101
3.3.2.5	Microplate expression profile of <i>ihfβ</i> in Lp02 wild-type.....	101
3.3.2.6	Microplate expression profile of <i>ihfβ</i> in Lp02 $\Delta ihf\alpha ihf\beta$.....	106
3.3.2.7	Microplate expression profile of of <i>ihfβ</i> in Lp02 $\Delta rpoS$.....	109
3.3.2.8	Microplate expression profile of <i>ihfβ</i> in Lp02 $\Delta rpoS ihf\alpha$.....	109
3.4	Protein Purification of LpIHF and LpRpoS.....	114
3.5	Electrophoretic mobility shift assays (EMSA).....	121
3.5.1	Binding of Recombinant pre-HiTrapTM LpIHF protein with upstream promoter regions of <i>ihfα</i> and <i>ihfβ</i>.....	121
3.5.2	Binding of Recombinant post-HiTrapTM LpIHF protein with upstream promoter regions of <i>ihfα</i> and <i>ihfβ</i>.....	131

3.6 RsmY and RsmZ	136
3.6.1 Microplate expression profiles of <i>rsmY</i> and <i>rsmZ</i> in Lp02 wild-type, <i>Δihfaihfβ</i> , <i>ΔletA</i>	137
3.6.2 EMSA of LpIHF on <i>rsmY</i> and <i>rsmZ</i> promoter regions.....	145
Chapter 4: Discussion	149
4.1 Regulation of <i>ihfα</i> expression by LpIHF and LpRpoS.....	150
4.2 Regulation of <i>ihfβ</i> expression by LpIHF and LpRpoS.....	152
4.3 Proposed mechanistic model of LpIHF regulation by LpIHF and LpRpoS.....	154
4.4 Regulation of <i>rsmY</i> and <i>rsmZ</i> by LpIHF.....	155
4.5 Summary.....	157
4.6 Future directions.....	160
Chapter 5: Appendix	161
5.1 Double knock out strategy (Lp02 <i>ΔihfaletA</i>).....	161
5.2 Triple knockout (Lp02 <i>ΔihfaihfβrpoS</i> & Lp02 <i>ΔihfaihfβletA</i>).....	161
5.3 Construction of pDuet vector with <i>ihfα</i> and <i>infβ</i> insertions.....	163
References	165

List of Tables

Table 2.1 Catalogue of bacterial strains used in this study.....	26
Table 2.2 Catalogue of plasmids used in this study.....	35
Table 2.3 Description of amplification conditions constructs assembled in this study....	41
Table 2.4 List of restriction enzymes used to digest various amplicons and vectors for plasmid construction in this study.....	46

List of Figures

Figure 1.1 Morphological features of post-exponential <i>L. pneumophila</i> cells.....	2
Figure 1.2 Representative schematic of the <i>L. pneumophila</i> global regulatory cascade....	8
Figure 1.3 IHF consensus sequences identified in various bacteria.....	13
Figure 1.4 Comparison of conserved palindromic sequences.....	23
Figure 2.1 Genetic map of the promoterless GFP reporter vector pBH6119.....	37
Figure 3.1 Identification of putative IHF consensus sequences within the promoter regions of <i>ihfα</i> and <i>ihfβ</i>	62
Figure 3.2 Putative LpIHF binding sites.....	65
Figure 3.3 Comparison of partially matched binding sites identified within the <i>ihfα</i> and <i>ihfβ</i> promoter regions.....	67
Figure 3.4 Promoter regions of <i>ihfα</i> and <i>ihfβ</i>	70
Figure 3.5 Ligated <i>ihfα</i> constructs within the promoterless GFP vector pBH6119.....	72
Figure 3.6 Ligated <i>ihfβ</i> constructs within the promoterless GFP vector pBH6119.....	74
Figure 3.7 Gradient PCR of β 3.....	76
Figure 3.8 β 3 Phusion TM PCR.....	78
Figure 3.9 Individual colony PCR of β 3.....	81
Figure 3.10 Fluorescence detected from <i>L. pneumophila</i> Lp02 containing vector control pBH6119 and <i>P_{ihfα}</i> promoter DNA-GFP constructs.....	85
Figure 3.11 Fluorescence detected from <i>L. pneumophila</i> Lp02 Δ <i>ihfαihfβ</i> containing vector control pBH6119 and <i>P_{ihfα}</i> promoter DNA-GFP constructs.....	87
Figure 3.12 Fluorescence detected from <i>L. pneumophila</i> Lp02 containing vector	

control pBH6119 and <i>P_{ihff}</i> promoter DNA-GFP constructs.....	89
Figure 3.13 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta ihfaihf\beta$ containing vector control pBH6119 and <i>P_{ihff}</i> promoter DNA-GFP constructs.....	91
Figure 3.14 Fluorescence detected from <i>L. pneumophila</i> Lp02 containing <i>P_{ihfa}</i> promoter DNA-GFP constructs.....	94
Figure 3.15 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta ihfaihf\beta$ containing <i>P_{ihfa}</i> promoter DNA-GFP constructs.....	97
Figure 3.16 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta rpoS$ containing <i>P_{ihfa}</i> promoter DNA-GFP constructs.....	99
Figure 3.17 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta rpoSihfa$ containing <i>P_{ihfa}</i> promoter DNA-GFP constructs.....	102
Figure 3.18 Fluorescence detected from <i>L. pneumophila</i> Lp02 containing <i>P_{ihff}</i> promoter DNA-GFP constructs.....	104
Figure 3.19 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta ihfaihf\beta$ containing <i>P_{ihff}</i> promoter DNA-GFP constructs.....	107
Figure 3.20 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta rpoS$ containing <i>P_{ihff}</i> promoter DNA-GFP constructs.....	110
Figure 3.21 Fluorescence detected from <i>L. pneumophila</i> Lp02 $\Delta rpoSihfa$ containing <i>P_{ihff}</i> promoter DNA-GFP constructs.....	112
Figure 3.22 Recombinant LpIHF after purification, dialysis and concentration without implementation of the HiTrap TM Heparin HP column in the purification protocol.....	115
Figure 3.23 Recombinant LpIHF after purification, dialysis and concentration with implementation of the HiTrap TM Heparin HP column in the purification protocol.....	117

Figure 3.24 Recombinant LpRpoS after purification and concentration.....	119
Figure 3.25 Binding of recombinant LpIHF with upstream promoter region of <i>magA</i>	122
Figure 3.26 Lack of binding of recombinant LpIHF with internal segment of <i>lpg2112</i>	124
Figure 3.27 Binding of recombinant LpIHF to truncated <i>ihfα</i> and <i>ihfβ</i> promoter regions (α 1 – α 4 & β 1 – β 4).....	127
Figure 3.28 Binding of recombinant LpIHF to truncated <i>ihfα</i> and <i>ihfβ</i> promoter regions (α 5 – α 8 & β 5 – β 7).....	129
Figure 3.29 Binding of recombinant LpIHF to truncated <i>ihfα</i> promoter region.....	132
Figure 3.30 Binding of recombinant LpIHF to truncated <i>ihfβ</i> promoter region.....	134
Figure 3.31 Binding site comparisons.....	138
Figure 3.32 Fluorescence detected from <i>L. pneumophila</i> Lp02 wild-type, Δ <i>ihfαihfβ</i> , and Δ <i>letA</i> mutant strains containing <i>P_{rsmY}</i> promoter DNA-GFP constructs.....	141
Figure 3.33 Fluorescence detected from <i>L. pneumophila</i> Lp02 wild-type, Δ <i>ihfαihfβ</i> , and Δ <i>letA</i> mutant strains containing <i>P_{rsmZ}</i> promoter DNA-GFP constructs.....	143
Figure 3.34 Binding of recombinant LpIHF with upstream promoter regions of (A) <i>rsmY</i> and (B) <i>rsmZ</i>	147
Figure 4.1 Inclusion of LpIHF within the global regulatory cascade.....	158

List of Abbreviations

2D	two dimensional
α	alpha
A	adenine
<i>A. castellanii</i>	<i>Acanthamoeba castellanii</i>
Amp	ampicillin
APS	ammonium persulfate
β	beta
<i>B. abortus</i>	<i>Brucella abortus</i>
BaIHF	IHF protein from <i>B. abortus</i>
BCYE	buffered charcoal yeast extract
BCV	<i>Brucella</i> -containing vacuole
bp	base pair
BSA	bovine serum albumin
BYE	buffered yeast extract
C	cytosine
CaCl ₂	calcium chloride
CIP	calf intestinal alkaline phosphatase
CLF	cyst-like form
Cm	chloramphenicol
CO ₂	carbon dioxide
°C	degrees celsius
Δ	inactivation of gene
dH ₂ O	distilled water

dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EcIHF	IHF protein from <i>E. coli</i>
EcRpoS	RpoS protein from <i>E. coli</i>
EDTA	ethylenediaminetetraacetic acid
Em	emission
EMSA	electrophoretic mobility shift assay
EtOH	ethanol
Ex	excitation
Fe	iron
FRT	Flp recombinase targets
g	gram
G	guanine
Gent	gentamicin
GFP	green fluorescent protein
h	hour
HCl	hydrochloric acid
HeLa	Henrietta Lacks
IHF	integration host factor
IPTG	Isopropyl- β -D-galactoside
kb	kilo base pair
KCl	potassium chloride
kDa	kiloDalton

KO	knockout
KOH	potassium hydroxide
kV	kilo volts
L	litre
<i>L. pneumophila</i>	<i>Legionella pneumophila</i>
LB	Luria-Bertani
LCV	Legionella-containing vacuole
LpIHF	IHF protein from <i>L. pneumophila</i>
LpRpoS	RpoS protein from <i>L. pneumophila</i>
µg	microgram
µL	microlitre
µm	micrometer
µM	micromolar
MCS	multiple cloning site
mg	milligram
min	minute
mL	millilitre
mm	millimeter
mM	millimolar
MnCl ₂	magnesium chloride
MOPS	4-morpholinepropanesulfonic acid
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
ncRNA	noncoding ribonucleic acid
ng	nanogram

Ni-NTA	nickel-nitrilotriacetic acid
nm	nano meter
OD	optical density
<i>ori</i>	origin of replication
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
<i>P. fluorescens</i>	<i>Pseudomonas fluorescens</i>
<i>P. syringae</i>	<i>Pseudomonas syringae</i>
PAGE	polyacrylamide gel electrophoresis
PaRpoS	RpoS protein from <i>P. aeruginosa</i>
PCR	polymerase chain reaction
PF	primer forward (5')
PHBA	poly-B-hydroxybutyrate
Phu	phusion enzyme
pmol	picamol
ppGpp	guanosine 3',5'-bispyrophosphate
PR	primer reverse (3')
RbCl ₂	rubidium chloride
Rbs	ribosome binding site
RF	replicative form
RFU	relative fluorescence units
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction

s	second
<i>S. enterica</i>	<i>Salmonella enterica</i> servovar Typhimurium
SDS	sodium dodecyl sulfate
SeIHF	IHF protein from <i>S. enterica</i>
SeRpoS	RpoS protein from <i>S. enterica</i>
spp.	Species
T	thymine
T4SS	type four secretion system
TAE	tris base, acetic acid, EDTA buffer
Taq	<i>Thermus aquaticus</i> polymerase enzyme
TE	tris base, EDTA buffer
Thy	thymidine
TTSS	type three secretion system
V	volts
<i>V. cholerae</i>	<i>Vibrio cholerae</i>
VcIHF	IHF protein from <i>V. cholerae</i>
VcRpoS	RpoS protein from <i>V. cholerae</i>

Chapter 1: Introduction

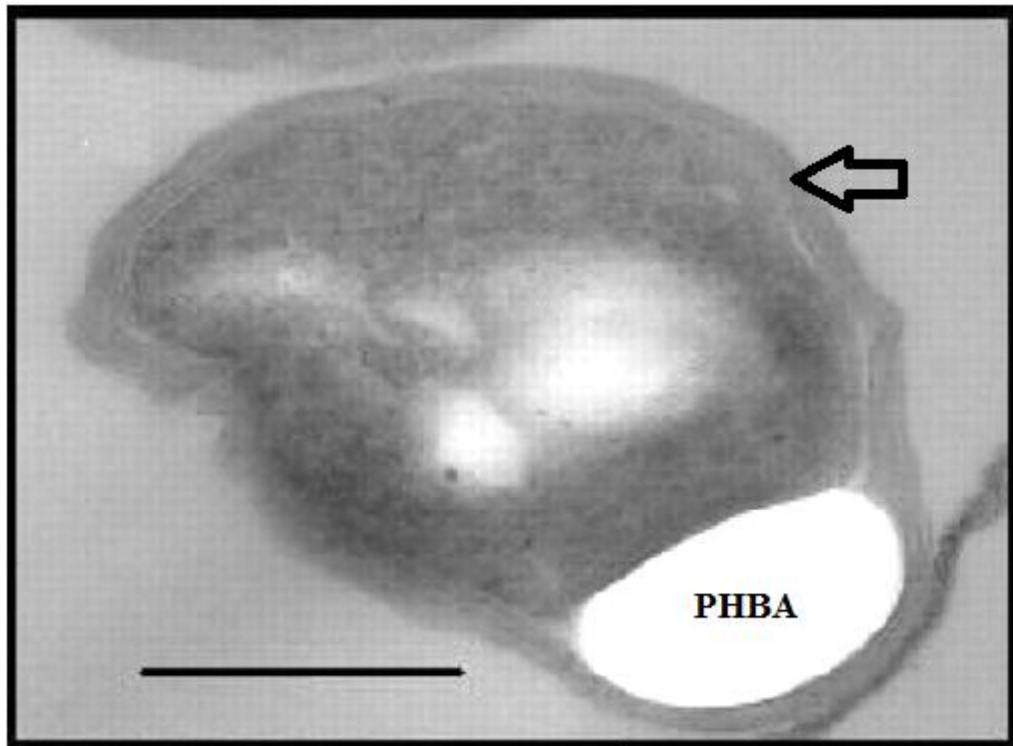
1.1 *Legionella pneumophila* life cycle/background

Legionella pneumophila is a Gram-negative bacillus that is typically found in fresh water as an intracellular parasite of protozoa (Albert-Weissenberger *et al.*, 2007). Within the protozoan host cell, *L. pneumophila* exhibits a distinct dimorphic lifecycle that alternates between vegetative replicative form (RF) and cyst-like form (CLF) (Garduno *et al.*, 2002). Free-swimming *L. pneumophila*, as the CLF, are engulfed via phagocytosis by the protozoan and establish a protective vacuole (replicative vacuole) around themselves to provide protection from lysosomal digestion (Molofsky and Swanson, 2004). Initially within the replicative vacuole conditions are favourable and nutrients are available. These conditions initiate *L. pneumophila* to morphologically change into the RF by repressing transmission traits and activating pathways to promote replication (Molofsky and Swanson, 2004). Transmission electron micrographs of the dimorphic forms revealed the RFs as typical Gram-negative rods (Garduno *et al.*, 2002). In addition, the RFs are found to be unflagellated (Albert-Weissenberger *et al.*, 2007). When nutrients become limited the RFs begin to change into the CLF in order to infect another potential host (Molofsky and Swanson, 2004). The CLFs are flagellated, virulent, and very motile (Albert-Weissenberger *et al.*, 2007). Transmission electron micrographs revealed the CLFs to be irregularly shaped coccoids featuring unique characteristics that included thickened cell walls, multiple membrane laminations and poly- β -hydroxybutyrate (PHBA) inclusions (Figure 1.1 A) (Morash *et al.*, 2009). These distinct morphological attributes along with metabolic dormancy of the CLF ensure its

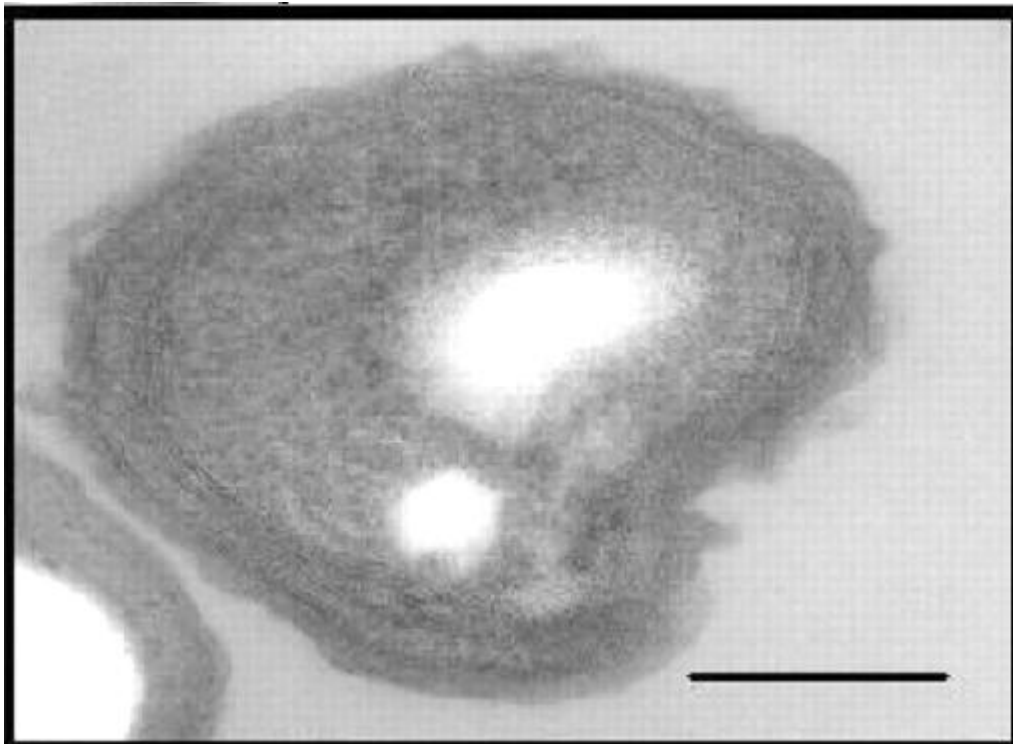
Figure 1.1: Morphological features of post-exponential *L. pneumophila* cells.

Transmission electron microscopic images of HeLa cells 48 h postinfection with *L. pneumophila* (A) Lp02 and (B) Lp02 Δ ihfaihf β (Morash *et al.*, 2009). The arrow identifies the multiple membrane laminations. Scale bar 100nm for images. Used with permission from American Society for Microbiology, October 27, 2011.

A



B



survival in unfavourable conditions until the next encounter with a suitable protozoan host cell.

L. pneumophila is not only a fresh water protozoan parasite, but also is an intracellular pathogen of human alveolar macrophages causing the atypical pneumonia Legionnaires' disease. The first recognized outbreak of Legionnaires' disease occurred in Philadelphia, PA in 1976 in which 29 individuals died among the 180 persons attending the American Legion convention; therefore the infection was labelled as Legionnaires' disease (Abu Kwaik *et al.*, 1998; Albert-Weissenberger *et al.*, 2007). In addition to natural fresh water sources, *L. pneumophila* has also been found in anthropogenic locations that employ freshwater such as cooling towers, air conditioners, water heaters, hot tubs, and humidifiers (Palmer *et al.*, 1993; Woo *et al.*, 1992). From these sources and possibly others, contaminated aerosols are released into the air where *L. pneumophila* (as CLFs) enters the human body upon inhalation (Woo *et al.*, 1992). It is proposed that CLFs, when aerosolized in water droplets and inadvertently inhaled by susceptible individuals, infect alveolar macrophages manifesting as the atypical pneumonia (Berk *et al.*, 1998; Garduno *et al.*, 2002). This hypothesis is supported by the fact that CLFs are highly-resistant to the effects of detergents and antibiotics, and are hyper-infectious as shown by cell-based infection models (Garduno *et al.*, 2002). Eradication of *L. pneumophila* from water is difficult as the CLFs are resistant to chlorine and hot water, and are viable in water for up to 14 months (Garduno *et al.*, 2002). Individuals at high risk of contracting the disease are very often elderly or immunocompromised (Albert-Weissenberger *et al.*, 2007).

Upon inhalation, *L. pneumophila* enters the lungs where they are engulfed via phagocytosis within alveolar macrophages (Albert-Weissenberger *et al.*, 2007). *L. pneumophila* remains within the phagosome that functions as a replicative vacuole or LCV (*Legionella*-containing vacuole) (Albert-Weissenberger *et al.*, 2007), where, with the use of the Dot/Icm type IV secretion system, delivers more than 40 ‘effector’ proteins into the host cytosol to modulate host processes (*i.e.* remodelling of host cell environment) (Isberg *et al.*, 2009; Tieden *et al.*, 2007). Individual functions of the effector molecules remain largely unknown, though a few have been identified (Isberg *et al.*, 2009). Some of which include; vacuolar remodelling, endosome-lysosome fusion avoidance, and endoplasmic reticulum recruitment (Ensminger and Isberg, 2009). Within the LCV, *L. pneumophila* changes into the RF and begins to replicate (Albert-Weissenberger *et al.*, 2007). In amoebae, when nutrients become limited the RFs begin to change into the CLF in order to infect another potential host; in human alveolar macrophages this is not the case (Molofsky and Swanson, 2004; Albert-Weissenberger *et al.*, 2007). Macrophage lysis is induced before the CLFs are appreciably formed, thus communicable transmission of *L. pneumophila* to other individuals is unlikely (Garduno *et al.*, 2002).

1.2 *Legionella pneumophila* global regulatory cascade

The regulatory network governing the morphological differentiation of RF into CLF is not well understood. CLF formation in *L. pneumophila* occurs post-exponentially and is coordinated with virulence traits described for stationary phase bacteria such as

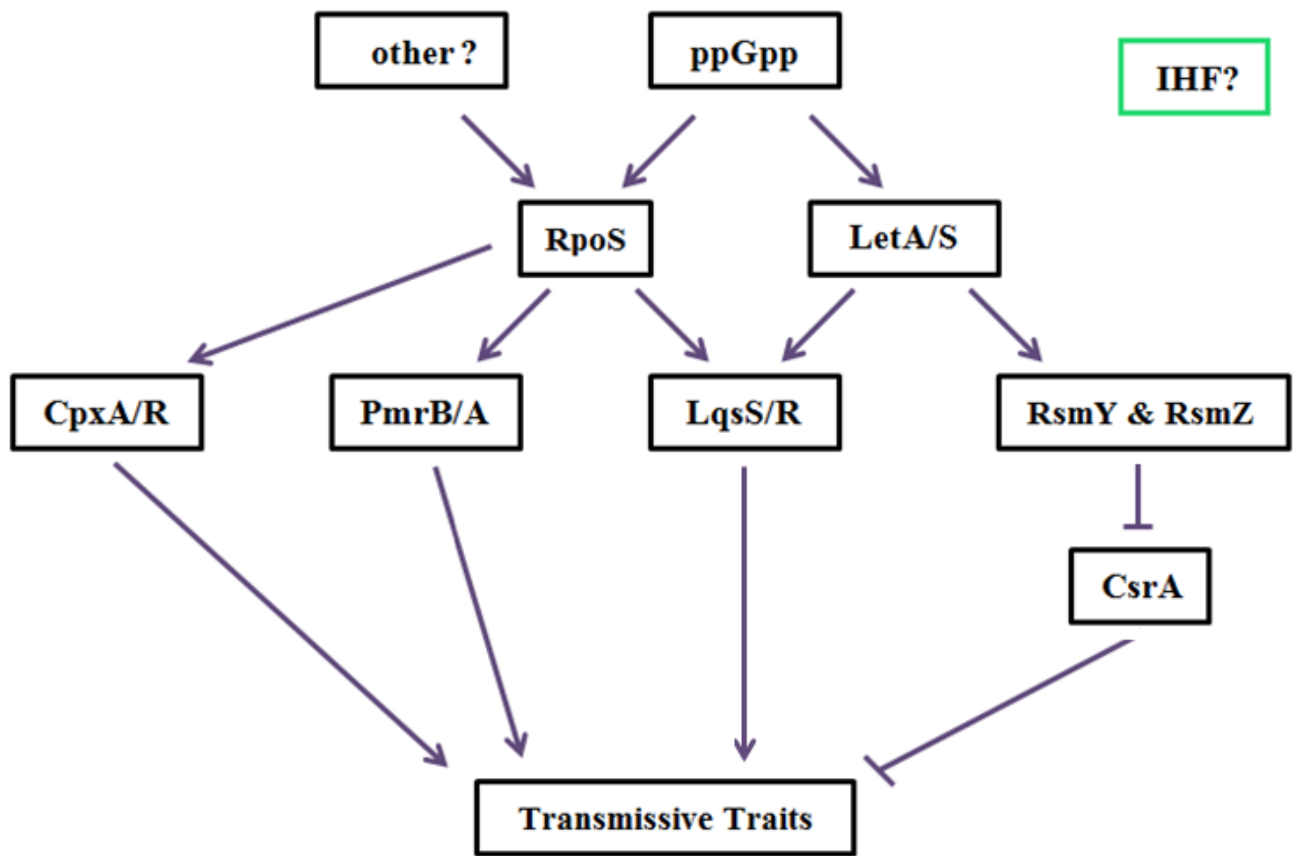
increased infectivity, motility, osmotic resistance and cytotoxicity (Morash *et al.*, 2009). A conserved regulatory cascade centering on three components has been revealed to be involved in gene expression during post-exponential/stationary phase for several pathogenic bacteria (Molofsky and Swanson, 2004; Rasis and Segal, 2009). These three components include a two-component system, genes encoding small non-coding RNAs (ncRNAs) positively regulated by the two-component system, and a protein involved in post-transcriptional repression of stationary phase mRNAs, which is sequestered and inhibited by the small ncRNAs (Rasis and Segal, 2009).

When *L. pneumophila* enters post-exponential phase an unknown signal thought to be a starvation signal related to ppGpp (guanosine 3',5'-bispyrophosphate) formation results in autophosphorylation of LetS (Molofsky and Swanson, 2004; Sahr *et al.*, 2009). LetS, part of the two-component system LetS/LetA (homologue in *P. aeruginosa*: GacS/GacA), is a sensor kinase that when autophosphorylated, subsequently phosphorylates the response regulator LetA (Hammer *et al.*, 2002; Rasis and Segal, 2009). Once phosphorylated, LetA binds to the consensus sequence, TNAGAAATTTCTNA, located upstream of two genes which encode small ncRNAs: RsmY and RsmZ (Sahr *et al.*, 2009; Rasis and Segal, 2009). This binding in turn induces both *rsmY* and *rsmZ* expression (Sahr *et al.*, 2009; Rasis and Segal, 2009). Both RsmY and RsmZ, once transcribed, then bind and sequester the CsrA protein (homologous to RsmA in *P. aeruginosa*) (Sahr *et al.*, 2009; Rasis and Segal, 2009). With CsrA sequestered, the mRNAs it was bound to are now free to be translated (mRNA responsible for transmissive phase traits) or decayed (mRNA responsible for replicative traits) (Sahr *et al.*, 2009). It should be noted that traits associated with morphological

differentiation appears to overlap those associated with virulence. Other transcription factors have been identified to also be involved in the regulatory cascade such as the stationary sigma factor LpRpoS, which affects the transcription of the *cpxR* and *pmrA* genes (Rasis and Segal, 2009; Hovel-Miner *et al.*, 2009). These genes encode for the response regulators of the two component signal transduction systems CpxR/A and PmrA/B, respectively (Rasis and Segal, 2009). Both of which directly affect the transcription of Icm/Dot substrates (responsible for the Dot/Icm type IV secretion system and the ‘effector’ proteins) (Rasis and Segal, 2009; Hovel-Miner *et al.*, 2009; Morash *et al.*, 2009). The post-exponentially expressed protein OxyR is responsible for activating genes associated with protection against oxidative stress (LeBlanc *et al.*, 2008). The response regulator LqsR is responsible for transitioning from CLF to RF where it is thought to play a role in cellular division, promoting interactions between *L. pneumophila* and the phagocytes, and is known to regulate the expression of virulent genes (Tiaden *et al.*, 2007). LqsR is part of the two component system LqsS/R, previously identified to be controlled by both LpRpoS and LetA (Tiaden *et al.*, 2007) (Figure 1.2).

Recently, the integration host factor (IHF) has been shown to play a role in the regulation of genes associated with differentiation of CLFs (Morash *et al.*, 2009). Though it has been determined that IHF is responsible for complete differentiation into the CLF it is unknown how IHF is regulated and which genes IHF targets in order to complete this differentiation.

Figure 1.2: Representative schematic of the *L. pneumophila* global regulatory cascade. The placement of IHF into this cascade is yet to be determined. See text for details.



1.3 Integration Host Factor (IHF)

IHF is a heterodimeric protein consisting of an alpha and a beta subunit and has been identified in many bacteria, some of which include *L. pneumophila*, *Escherichia coli*, *Brucella abortus*, *Vibrio cholerae*, and *Salmonella enteric* servovar Typhimurium (Morash *et al.*, 2009; Aviv *et al.*, 1994; Sieira *et al.*, 2004; Stonehouse *et al.*, 2008; Mangan *et al.*, 2006). IHF is highly conserved structurally and functionally among prokaryotes and has been shown to be involved in all aspects of transcriptional regulation, particularly those concerning cell differentiation and virulence (Morash *et al.*, 2009). Several examples will be discussed below.

In *E. coli*, IHF (EcIHF) has multiple functions, some of which include site-specific recombination, DNA replication, and expression of multiple genes (Freundlich *et al.*, 2006). It is thought that since the action of IHF binding DNA results in the DNA being bent, IHF's main role may be architectural such that it can promote or inhibit interactions between the DNA of interest and other regulatory factors/proteins (Rice *et al.*, 1996). In *E. coli* IHF recognizes a 13-bp consensus sequence WATCAANNNTTR (W: A/T and R: A/G) and when bound covers a region of approximately 35 bp (Rice *et al.*, 1996). In addition the expression of EcIHF is found to be negatively autoregulated (Aviv *et al.*, 1994).

In *L. pneumophila*, IHF (LpIHF) was found to be most abundant post exponentially and to be concentrated within the CLF (Morash *et al.*, 2009). To date the only gene identified to be regulated by LpIHF is *magA* (Morash *et al.*, 2009). MagA is associated with morphological differentiation into CLF and therefore serves as a useful late-stage developmental marker (Garduno *et al.*, 2002; Morash *et al.*, 2009). To

determine whether LpIHF had a role in differentiation and/or virulence in *L. pneumophila*, a morphological analysis of an LpIHF deletion mutant in mammalian cells was performed (Morash *et al.*, 2009). The analysis indicated incomplete differentiation of the bacteria into the CLF as transmission electron microscopic images of the LpIHF mutant strain showed a lack of PHBA inclusions, decreased membrane laminations, and thinner cell walls (Figure 1.1 B) (Morash *et al.*, 2009). In addition, reduced infectivity was observed with the LpIHF mutant strain in comparison to the parental strain in mammalian cell infection studies (Morash *et al.*, 2009). The study also revealed that the LpIHF mutant strain was unable to successfully grow in the *Acanthamoeba castellanii* host cell (Morash *et al.*, 2009). Therefore, it was concluded that LpIHF must play an important role in both the morphological change and virulence in *L. pneumophila*.

Similarly to *L. pneumophila*, *Brucella abortus* is a Gram-negative intracellular pathogen (Sieira *et al.*, 2004). The *virB* operon encodes for a type IV secretion system (T4SS) which is used to interact with and export effector molecules into the host cell (Sieira *et al.*, 2004). *B. abortus* is able to survive and replicate within a *Brucella*-containing vacuole (BCV) in a host cell, otherwise known as the replicative vacuole (Sieira *et al.*, 2004). T4SS expression is activated at the beginning of stationary phase (post-exponential phase) and has been shown to be essential for virulence and maturation of the BCV into an intracellular replication niche (Sieira *et al.*, 2004). An electrophoretic mobility shift assay (EMSA) of the promoter region of *virB* (P_{virB}) with crude extracts of *B. abortus* revealed a putative transcription factor which bound between -201 and -130 (Sieira *et al.*, 2004). Through protein purification and tandem mass spectrometry the protein was identified as IHF (BaIHF) (Sieira *et al.*, 2004). Footprinting analysis

revealed that BaIHF protected a 51-bp DNA region (-188 to -137) and within this region, two sequences which partially matched the *E. coli* IHF consensus sequence were identified (Figure 1.3 A) (Sieira *et al.*, 2004). The two sequences overlapped with one another and together covered a region of 20 bp (Sieira *et al.*, 2004). Replacement of this 20-bp region with a non-related sequence resulted in abolishment of complex formation thus indicating that BaIHF recognizes this specified region with is similar to that of *E. coli* binding site (Sieira *et al.*, 2004). Moreover, *VirB* expression experiments of P_{virB}-*lacZ* fusions revealed that BaIHF regulated the activity of P_{virB} during intracellular and vegetative growth (Sieira *et al.*, 2004). In addition, a mutant stain containing a 20-bp BaIHF binding site replacement resulted in failure of *virB* operon expression during the initial stages of macrophage infection and resulted in severe intracellular multiplication defects (Sieira *et al.*, 2004). Thus, BaIHF plays a major role in promoting virulence of *B. abortus*.

In *Vibrio cholerae*, IHF (VcIHF) affects the expression levels of two main virulence factors *tcpA* and *ctx* (Stonehouse *et al.*, 2008). Inactivation of *V. cholera ihfA* and *ihfB* (genes encoding VcIHF) resulted in reduction of *tcpA* and *ctx* expression levels and prevented production of both the toxin-coregulated pilus and the cholera toxin, respectively (Stonehouse *et al.*, 2008). EMSA and foot printing assays revealed that VcIHF bound directly to the promoter region of *tcpA* (centered at position -162) but did not bind the promoter region upstream of *ctx*. Like *B. abortus*, the VcIHF consensus site partially matched the *E. coli* IHF consensus sequence (Figure 1.3 B) (Stonehouse *et al.*, 2008). Based on the lack of pilus and toxin production, as well as the reduction in the expression levels of the virulence factors *tcpA* and *ctx*, it can be concluded that, like in

Figure 1.3: IHF consensus sequences identified in various bacteria. (A) 20-bp region within P_{virB} of *Brucella abortus* which contains two overlapping IHF binding sites. (B) IHF binding site located within P_{tcpA} of *Vibrio cholera*. (C) IHF binding site with *Pseudomonas syringae*. Binding sites partially match the *E. coli* IHF consensus sequence (WATCAANNNTTR where W: A/T and R: A/G). Sequences are identified as bolded font.

A

WATCAANNNTTR
:: : : : : : :
GTATATAAGATTTTGT**TAAAAAGAATTT**TCTAATAGAACCAATACA
CATATATTCTAAAACAATTTTT**CTTAAAAGATTAT**CTTGGTTATGT
: : : : : : : : : :
RTTNNNNAACTAW

B

WATCAANNNTTR
: : : : : : : : : :
AATCATTGAATT

C

WATCAANNNTTR
: : : : : : : : : :
WNNCARNWNNTTR

L. pneumophila and *B. abortus*, VcIHF plays a major role in the virulence of *Vibrio cholera* (Stonehouse *et al.*, 2008).

A gene expression profile of *Salmonella enterica* serovar Typhimurium revealed that IHF (SeIHF) protein levels peaked during transition into stationary phase (Mangan *et al.*, 2006). *S. enterica* requires the use of its type three secretion systems (TTSS) for cell invasion and survival within the macrophage (Mangan *et al.*, 2006). It was discovered that the SeIHF protein was required for the expression of all three TTSS and their effector proteins (Mangan *et al.*, 2006). In addition a SeIHF knock out revealed failure of the stationary-phase regulon to become fully activated resulting in cellular processes to shut down (Mangan *et al.*, 2006). SeIHF in *S. enterica* is required for expression of genes involved in both virulence and initiation of stationary phase (Mangan *et al.*, 2006).

Lastly an IHF binding site was identified in the *Pseudomonas syringae* genome and similarly to *Brucella abortus* and *Vibrio cholera* the binding site partially matches the *E. coli* IHF consensus sequence (Figure 1.3 C) (Arvizu-Gomez *et al.*, 2011). IHF in *P. syringae* has also been shown to be involved in the expression of virulence factors (Arvizu-Gomez *et al.*, 2011). This study revealed that IHF interacted with the promoter region of the *phtD* operon (Arvizu-Gomez *et al.*, 2011). This operon was reported to be part of the regulation system involved in phaseolotoxin synthesis (Arvizu-Gomez *et al.*, 2011).

Taken together, IHF binding sites matching the *E. coli* IHF consensus sequence have been identified in a variety of Gram-negative bacteria. Although considerable effort has been put into the identification of genes regulated by IHF, little is known of the

regulation of IHF expression which to date is limited to *E. coli* IHF (as discussed in sections 1.3 and 1.4).

1.4 RpoS

RpoS is a highly conserved global regulator of stationary-phase physiology among Gram-negative bacteria (Bachman and Swanson, 2004). RpoS is a sigma subunit of RNA polymerase; upon entering stationary phase or in response to stress, RpoS can replace the vegetative sigma factor RpoD in order to target transcription of required genes (Hengge-Aronis, 2002; Hovel-Miner *et al.*, 2009). In bacterial pathogens including *Salmonella enterica* and *Vibrio cholerae*, the RpoS regulon is involved not only in stress resistance but also in the regulation of virulence genes (Hovel-Miner *et al.*, 2009).

In *E. coli*, RpoS (EcRpoS) is regarded as the general stress sigma factor as it senses and responds to an array of stress signals by targeting transcription of specified genes thus protecting the cell (Hengge-Aronis *et al.*, 2002). Some of the many types of stress include: oxidative, potentially lethal heat shock, low pH, and hyperosmolarity (Hengge-Aronis, 2002). In addition EcRpoS also targets expression of genes resulting in changes in overall morphology including the cell envelope (Hengge-Aronis, 2002). Upon transition into stationary phase, the EcRpoS transcript has been shown to increase up to five to tenfold (Hengge-Aronis *et al.*, 2002). Promoter-*lacZ* fusion assays revealed that EcRpoS plays a role in the regulation of EcIHF expression as shown by reduced *himA* and *himD* (genes which encode for the alpha and beta subunits of EcIHF) promoter activities in a $\Delta rpoS$ mutant strain (Aviv *et al.*, 1994).

In *L. pneumophila*, RpoS (LpRpoS) ensures the transcription of genes required for survival in stressful conditions as well as in stationary phase (Bachman and Swanson, 2004). When *L. pneumophila* enters post-exponential phase, LpRpoS induces motility and promotes replication of the intracellular bacteria within the LCV in the amoeba host cell (Bachman and Swanson, 2004). Specifically, LpRpoS positively affects the transcription of *cpxR* and *pmrA* genes, which directly affect the transcription of Icm/Dot components which make up the T4SS and the effector molecules used to remodulate the host cell environment (Hovel-Miner *et al.*, 2009). LpRpoS is also known to be a key regulator of the transcription of the *lqsR* gene, which promotes interactions between *L. pneumophila* and the phagocytes, and cytotoxicity. In addition to LpIHF, LpRpoS is also known to regulate *magA* as revealed by EMSA and green fluorescent reporter assays (Morash *et al.*, 2009).

Elsewhere the function of RpoS as a regulator of stress response genes, which overlaps to some extent genes encoding virulence traits, appears to be conserved among Gram-negative bacteria. RpoS (VcRpoS) in *Vibrio cholerae*, was revealed to be involved in both stress response and virulence (Yildiz and Schoolnik, 1998). Similar to *E. coli*, a VcRpoS mutant strain was shown to be less resistant to oxidative stress, hyperosmolarity and starvation, thus indicating its role in stress response (Yildiz and Schoolnik, 1998). A 2D gel electrophoresis assay revealed that during stationary-phase growth, VcRpoS regulates the expression of 25 or more genes responsible for the stress response (Yildiz and Schoolnik, 1998). In addition it was also revealed that a $\Delta rpoS$ mutant strain exhibited a decrease in intestinal colonization, within the suckling mouse host, when

compared to the wild-type, by four to fivefold (Merrell *et al.*, 2000). Both experiments indicate VcRpoS to play a role in virulence (Merrell *et al.*, 2000).

Similar to *V. cholerae*, RpoS (SeRpoS) in *Salmonella enteric* serovar Typhimurium has been revealed to be involved in both stress response and virulence (Nickerson and Curtiss, 1997). Its role in stress response includes environmental conditions which include oxidative stress, starvation, and low pH (Nickerson and Curtiss, 1997). RT-PCR analysis revealed that the transcription levels of SeRpoS increase when inside the macrophage (Khan *et al.*, 2006). In addition, SeRpoS was discovered to control the expression of genes required for systemic infection (Nickerson and Curtiss, 1997). Interestingly, a SeRpoS mutant strain was still able to infect macrophages; however the strain was discovered to be less cytotoxic (Khan *et al.*, 2006).

In *Pseudomonas aeruginosa*, RpoS (PaRpoS) expression levels are found to increase significantly at the onset of stationary phase (Litifi *et al.*, 2008). Moreover, maximal expression is observed for genes regulated by PaRpoS during stationary phase (Litifi *et al.*, 2008). PaRpoS was revealed to be responsive to stress as a mutant strain was sensitive to starvation conditions (Suh *et al.*, 1999). But mainly PaRpoS appears to play a major role in the virulence of *P. aeruginosa* (Suh *et al.*, 1999). Colonization of *P. aeruginosa* requires mediation via the type IV fimbriae (Suh *et al.*, 1999). When *rpoS* was mutated a twitching assay revealed that the zone of motility decreased by 30 – 40% with shape of the zone being altered from its normal circular shape to elliptical (Suh *et al.*, 1999). In addition, the swarming motility appeared to be altered. Lastly the mutant strain was shown to produce 50% less exotoxin A (Suh *et al.*, 1999).

In the intracellular pathogen *Brucella* spp, it was proposed that RpoS may trigger entry into stationary phase and possibly also regulate *virB* (responsible for induction of the T4SS) expression in *Brucella* spp. (Dozot *et al.*, 2006). However, *Brucella suis* and *Brucella melitensis*, known to contain the gene *virB* like in *Brucella abortus*, were found not to contain a RpoS homologue, instead a strain mutated in the *rpoH*-like heat shock sigma factor revealed reduced expression of the major virulence factor, T4SS (Delory *et al.*, 2006).

Overall RpoS is post exponentially expressed and a major regulator of virulence traits in many Gram-negative bacteria. So far it has only been revealed that in *E. coli* EcIHF is regulated by EcRpoS, since IHF is also post exponentially expressed and is also found to function in virulence traits, as well as morphological, it may be worth investigating RpoS as a possible regulator of IHF in other Gram-negative bacteria.

1.5 RsmY and RsmZ

Repressors of stationary phase metabolites (rsm) are ncRNAs that regulate multiple genes and their processes post-transcriptionally (Dubey *et al.*, 2005). In *E. coli*, Rsms encoded by CsrB and CsrC are distinguished by their conserved GGA motifs found in the loop regions of their secondary structures (Romeo, 1998; Dubey *et al.*, 2005). The purpose of these motifs is to bind and sequester a target protein (Dubey *et al.*, 2005). CsrA, an RNA binding protein and the main target of Rsms, has a vital role which includes activation of exponential phase functions and repression of stationary phase functions (Romeo, 1998). When post-exponential phase is reached RsmY and RsmZ

both sequester CsrA by binding to the conserved GGA motif thus resulting in CsrA inactivation which subsequently allows translation of previously bound mRNAs, enabling stationary phase functions and decaying of mRNAs required for exponential phase (Dubey *et al.*, 2005; Weilbacher *et al.*, 2003). Activation of *csrB* and *csrA* expression is positively regulated by UvrY (Tomenius *et al.*, 2005). UvrY is the response regulator from the two component system BarA/UvrY, which is known to be strongly associated with virulence (Tomenius *et al.*, 2005).

In *L. pneumophila*, a bioinformatic search for repeated GGA motifs identified two ncRNA genes, *rsmY* and *rsmZ*, homologous to *csrB* and *csrC* (Sahr *et al.*, 2009). Structural analysis via the MFOLD program revealed that the putative structures of RsmY and RsmZ contained GGA motifs at the loops (Sahr *et al.*, 2009). RT-PCR revealed expression of *rsmY* and *rsmZ* increased from exponential phase to post exponential phase by 3.1 and 6.8 fold respectively (Sahr *et al.*, 2009). A search revealed a similar palindromic motif to the *E. coli* UvrY consensus sequence, to be located within the promoter region of both *rsmY* and *rsmZ*, TNAGAAATTTCTNA (Sahr *et al.*, 2009). An ortholog of the two component system BarA/UvrY in *E. coli* is the LetS/LetA two component system in *L. pneumophila* (Sahr *et al.*, 2009). An EMSA revealed LetA to bind to a 35-bp region containing this motif for both *rsmY* and *rsmZ*, indicating that LetA regulates the expression of *rsmY* and *rsmZ* (Sahr *et al.*, 2009). Moreover, RsmY and RsmZ interacted with CsrA as indicated by EMSAs (Sahr *et al.*, 2009). It was determined that *rsmY* and *rsmZ* play a physiological role in *L. pneumophila* as a $\Delta rsmY rsmZ$ mutant was less effective at infecting the protozoan and intracellular replication was severely reduced (Sahr *et al.*, 2009). Single mutants of either *rsmY* or

rsmZ revealed no significant difference of replication and virulence from the wild-type parental strain. A $\Delta letA$ mutant also displayed reduced the levels of intracellular replication, but not as drastically as in the *rsmYZ* double mutant and therefore *rsmY* and *rsmZ* may potentially be under the control of an additional regulator (Sahr *et al.*, 2009).

In *Pseudomonas* spp. GacS/GacA are orthologs to the LetS/LetA and BarA/UvrY systems in *L. pneumophila* and *E. coli*, respectively (Sahr *et al.*, 2009). The GacA protein binds and directly activates the expression of both *rsmY* and *rsmZ* (Humair *et al.*, 2010). A bioinformatic search with the *E. coli* IHF consensus sequence revealed two possible IHF binding sites located within the *rsmZ* promoter region (around -100 and -40) of *Pseudomonas fluorescens* (Humair *et al.*, 2010). Based on the fact that the IHF protein among enteric bacteria is structurally and functionally conserved, an EMSA assay was conducted using *E. coli* IHF protein and the *P. fluorescens rsmZ* promoter region (Humair *et al.*, 2010). The EMSA revealed that the *E. coli* IHF formed a complex with high-affinity binding to the *rsmZ* promoter fragment (Humair *et al.*, 2010) suggesting the possibility of IHF as a regulator of *rsmZ* expression in *P. fluorescens* (Humair *et al.*, 2010).

The *rsmY* promoter was also found to contain a conserved palindromic sequence TGTAAGcNNNNtCtTACA which displays similarity to the sequence element found in the promoter upstream of *rsmZ* (Valverde *et al.*, 2003). This conserved sequence element found within the promoter of *rsmZ* was found to be essential for expression of *rsmZ* and regulation by GacA indicating the possibility of GacA and IHF sharing similar binding sites that included the conserved sequence (Heeb *et al.*, 2002). Comparison of the *E. coli* IHF binding site consensus sequence with the *P. fluorescens* conserved palindromic

sequence revealed them to be almost identical with one mismatch (Figure 1.4 A). In addition, comparison of the *P. fluorescens* sequence to the *L. pneumophila* LetA consensus sequence, which is similar to the *E. coli* UvrY consensus sequence, revealed that they also share high similarity with one mismatch (Figure 1.4 B).

In summary, the aforementioned two component systems LetS/LetA (*L. pneumophila*), BarA/UvrY (*E. coli*), and GacS/GacA (*Pseudomonas* spp.) are found to be highly conserved as global regulators of stationary-phase traits which also include virulence and pathogenicity phenotypes (Bachman and Swanson, 2004; Sahr *et al.*, 2009). Homologs of these two component systems are also found in other Gram-negative bacteria such as *Vibrio cholerae* (VarS/VarA) and *Salmonella enterica* (SirA/BarA) which were shown to also regulate the expression of ncRNAs which in turn sequester CsrA (Lenz *et al.*, 2005; Teplitski *et al.*, 2006).

1.6 Study aims

The study aims are to: (1) determine if the expression of IHF in *L. pneumophila* is autoregulated and regulated by RpoS as observed with *E. coli* and (2) determine if IHF regulates the expression of RsmY and RsmZ.

Figure 1.4: Comparison of conserved palindromic sequences. (A) The *P. fluorescens* conserved sequence TGAAGcNNNNtCtTACA found within the *rsmY* promoter similar to the sequence element found within the *rsmZ* promoter compared with the *E. coli* IHF consensus sequence WATCAANNNTTR. Similar with the exception of one mismatch. (B) The *L. pneumophila* LetA consensus TNAGAAATTTCTNA (similar to the UvrY consensus sequence) compared with the *P. fluorescens* conserved sequence TGAAGcNNNNtCtTACA. Both sets of alignments are similar with the exception of one mismatch.

A

TGTAAGcNNNNtCtTACA
:: ::::::::::::::
WATCAANNNTTR

B

TGTAAGcNNNNtCtTACA
:::: ::::::::::::::
TNAGAAATTTCTNA

Chapter 2: Materials and Methods

2.1 Bacterial strains and plasmids

Strains used in this study are summarized in Table 2.1. All strains were stored in their corresponding culture media broth (stated in section 2.2) with 10% dimethyl sulfoxide (DMSO) at -80°C.

For all cloning procedures, *Escherichia coli* DH5 α (Table 2.1) was used as a host strain. For recombinant protein expression, *Escherichia coli* BL21 CodonPlus RILTM (Table 2.1) was used as a host strain and pET-16b (Novagen, Madison, WI) (Table 2.2) was used as the expression vector. All GFP assays were done in strains derived from *Legionella pneumophila* Lp02 (Table 2.1) with the promoterless GFP reporter plasmid pBH6119 (Hammer *et al.*, 1999) (Table 2.2). pETDuet-1 plasmid (Novagen, Madison, WI) (Table 2.2) containing a double multiple cloning site (MCS) was used to clone in *ihf α* and *ihf β* coding sequences to attain simultaneous expression. For allelic gene exchange to obtain marked deletion mutants, pRDX (Morash *et al.*, 2009) (Table 2.2) suicide vector was used for the implementation of an antibiotic cassette in place of the targeted gene. pBluescript SK+ (Stratagene) (Table 2.2) was used for the creation of the gene-replacement constructs.

Chemicals, media reagents, Hypure Hyclone molecular biology grade nuclease-free water, and antibiotics were procured from Sigma-Aldrich Canada (Oakville, Ontario) and GE Healthcare (Baie d'Urfe, Quebec), Fisher Scientific Canada (Ottawa, Ontario), and VWR International (Mississauga, Ontario). All restriction, modifying and PCR

Table 2.1: Catalogue of bacterial strains used in this study.

Strain	Genotype or Strain description	Source (Reference)
<i>Escherichia coli</i>		
BL21 (DE3) CodonPlus TM RIL	B F_ <i>ompT hsdS</i> (rB_ mB_) <i>dcm_ Tetr gal_</i> (DE3) <i>endA Hte _argU ileY leuW Camr</i>	Stratagene
BL21 IHF α	pET16b:: <i>IHFα</i> in BL21 (DE3) CodonPlus TM RIL; Cm ^R and Amp ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
BL21 IHF β	pET16b:: <i>IHFβ</i> in BL21 (DE3) CodonPlus TM RIL; Cm ^R and Amp ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
BL21 RpoS	pET16b:: <i>RpoS</i> in BL21 (DE3) CodonPlus TM RIL; Cm ^R and Amp ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
DH5 α	F' <i>endA1 hsdR17</i> (r _k - m _k -) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1</i> Δ (<i>lacZYA-argF</i>) <i>U169 deoR</i> (ϕ 80dlac Δ (<i>lacZ</i>)M15)	New England Biolabs
DH5 α pBH6119	Amp ^R ; Thymidine producing	M. Swanson (Hammer <i>et al.</i> , 2002)
DH5 α α 1	<i>E. coli</i> DH5 α pBH6119:: <i>ihfα</i> P1 – cloning	this study
DH5 α α 2	<i>E. coli</i> DH5 α pBH6119:: <i>ihfα</i> P2 – cloning	this study
DH5 α α 3	<i>E. coli</i> DH5 α pBH6119:: <i>ihfα</i> P3 – cloning	this study

DH5α α4	<i>E. coli</i> DH5α pBH6119:: <i>ihfα</i> P4 – cloning	this study
DH5α α5	<i>E. coli</i> DH5α pBH6119:: <i>ihfα</i> P5 – cloning	this study
DH5α α6	<i>E. coli</i> DH5α pBH6119:: <i>ihfα</i> P6 – cloning	this study
DH5α α7	<i>E. coli</i> DH5α pBH6119:: <i>ihfα</i> P7 – cloning	this study
DH5α α8	<i>E. coli</i> DH5α pBH6119:: <i>ihfα</i> P8 – cloning	this study
DH5α β1	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P1 – cloning	this study
DH5α β2	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P2 – cloning	this study
DH5α β3	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P3 – cloning	this study
DH5α β4	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P4 – cloning	this study
DH5α β5	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P5 – cloning	this study
DH5α β6	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P6 – cloning	this study
DH5α β7	<i>E. coli</i> DH5α pBH6119:: <i>ihfβ</i> P7 – cloning	this study
DH5α Y1	<i>E. coli</i> DH5α pBH6119:: <i>rsmY</i> P1 – cloning	this study
DH5α Y2	<i>E. coli</i> DH5α pBH6119:: <i>rsmY</i> P2 – cloning	this study
DH5α Y3	<i>E. coli</i> DH5α pBH6119:: <i>rsmY</i> P3 – cloning	this study

DH5α Z1	<i>E. coli</i> DH5α pBH6119:: <i>rsmZ</i> P1 – cloning	this study
DH5α Z2	<i>E. coli</i> DH5α pBH6119:: <i>rsmZ</i> P2 – cloning	this study
DH5α Z3	<i>E. coli</i> DH5α pBH6119:: <i>rsmZ</i> P1 – cloning	this study
DH5αλpir	F_-(<i>lacZYA-argF</i>)U169 <i>recA1 endA1</i> <i>hsdR1 supE44 thi-1 gyrA96 relA1 _::pir</i>	M. Swanson (Carlson <i>et al.</i> 2010)
DY330	W3110 Δ <i>lacU169 gal490 pgl</i> Δ8 λ[<i>P</i>]cI857 Δ(<i>cro-bioA</i>)	M. Swanson (Yu <i>et al.</i> 2000)
DY331	DY330 <i>recA</i>	M. Swanson (Yu <i>et al.</i> 2000)
<i>Legionella pneumophila</i>		
Lp02	Str ^R , Thy ⁻ , HsdR ⁻ derivative of Philadelphia-1 strain	M. Swanson (Berger and Isberg, 1993)
α1	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihfα</i> P1 – GFP assay	this study
α2	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihfα</i> P2 – GFP assay	this study
α3	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihfα</i> P3 – GFP assay	this study
α4	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihfα</i> P4 – GFP assay	this study
α5	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihfα</i> P5 – GFP assay	this study

$\alpha 6$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> α P6 – GFP assay	this study
$\alpha 7$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> α P7 – GFP assay	this study
$\alpha 8$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> α P8 – GFP assay	this study
$\beta 1$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P1 – GFP assay	this study
$\beta 2$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P2 – GFP assay	this study
$\beta 3$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P3 – GFP assay	this study
$\beta 4$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P4 – GFP assay	this study
$\beta 5$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P5 – GFP assay	this study
$\beta 6$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P6 – GFP assay	this study
$\beta 7$	<i>L. pneumophila</i> Lp02 pBH6119:: <i>ihf</i> β P7 – GFP assay	this study
Y1	<i>L. pneumophila</i> Lp02 pBH6119:: <i>rsmY</i> P1 – GFP assay	this study
Y2	<i>L. pneumophila</i> Lp02 pBH6119:: <i>rsmY</i> P2 – GFP assay	this study
Y3	<i>L. pneumophila</i> Lp02 pBH6119:: <i>rsmY</i> P3 – GFP assay	this study
Z1	<i>L. pneumophila</i> Lp02 pBH6119:: <i>rsmZ</i> P1 – GFP assay	this study
Z2	<i>L. pneumophila</i> Lp02 pBH6119:: <i>rsmZ</i> P2 – GFP assay	this study
Z3	<i>L. pneumophila</i> Lp02 pBH6119:: <i>rsmZ</i> P3 – GFP assay	this study

pBH6119-wt Lp02 $\Delta ihfaihf\beta$	<i>L. pneumophila</i> Lp02 pBH6119 – GFP assay, vector control <i>ihf\alpha::gent^R, ihf\beta::kan^R</i>	this study A.K. Brassinga (Morash <i>et al.</i> , 2009)
$\alpha 1$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P1 – GFP assay	this study
$\alpha 2$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P2 – GFP assay	this study
$\alpha 3$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P3 – GFP assay	this study
$\alpha 4$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P4 – GFP assay	this study
$\alpha 5$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P5 – GFP assay	this study
$\alpha 6$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P6 – GFP assay	this study
$\alpha 7$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P7 – GFP assay	this study
$\alpha 8$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\alpha</i> P8 – GFP assay	this study
$\beta 1$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\beta</i> P1 – GFP assay	this study
$\beta 2$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\beta</i> P2 – GFP assay	this study
$\beta 3$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\beta</i> P3 – GFP assay	this study
$\beta 4$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\beta</i> P4 – GFP assay	this study
$\beta 5$ - Δihf	Lp02 $\Delta ihfaihf\beta$ pBH6119:: <i>ihf\beta</i> P5 – GFP assay	this study

$\beta 6$ - Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>ihfβ</i> P6 – GFP assay	this study
$\beta 7$ - Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>ihfβ</i> P7 – GFP assay	this study
Y1- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>rsmY</i> P1 – GFP assay	this study
Y2- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>rsmY</i> P2 – GFP assay	this study
Y3- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>rsmY</i> P3 – GFP assay	this study
Z1- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>rsmZ</i> P1 – GFP assay	this study
Z2- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>rsmZ</i> P2 – GFP assay	this study
Z3- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119:: <i>rsmZ</i> P3 – GFP assay	this study
pBH6119- Δihf	Lp02 <i>$\Delta ihfaihf\beta$</i> pBH6119 – GFP assay, vector control	this study
Lp02 <i>$\Delta rpoS$</i>	<i>rpoS</i> ::Kan ^R	M. Swanson (Bachman and Swanson, 2004)
$\alpha 1$ - $\Delta rpoS$	Lp02 <i>$\Delta rpoS$</i> pBH6119:: <i>ihfα</i> P1 – GFP assay	this study
$\alpha 2$ - $\Delta rpoS$	Lp02 <i>$\Delta rpoS$</i> pBH6119:: <i>ihfα</i> P2 – GFP assay	this study
$\alpha 3$ - $\Delta rpoS$	Lp02 <i>$\Delta rpoS$</i> pBH6119:: <i>ihfα</i> P3 – GFP assay	this study
$\alpha 4$ - $\Delta rpoS$	Lp02 <i>$\Delta rpoS$</i> pBH6119:: <i>ihfα</i> P4 – GFP assay	this study
$\alpha 5$ - $\Delta rpoS$	Lp02 <i>$\Delta rpoS$</i> pBH6119:: <i>ihfα</i> P5 – GFP assay	this study

α 6- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> α P6 – GFP assay	this study
α 7- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> α P7 – GFP assay	this study
α 8- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> α P8 – GFP assay	this study
β 1- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P1 – GFP assay	this study
β 2- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P2 – GFP assay	this study
β 3- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P3 – GFP assay	this study
β 4- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P4 – GFP assay	this study
β 5- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P5 – GFP assay	this study
β 6- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P6 – GFP assay	this study
β 7- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119:: <i>ihf</i> β P7 – GFP assay	this study
pBH6119- Δ rpoS	Lp02 <i>ArpoS</i> pBH6119 – GFP assay, vector control	this study
Lp02 <i>ihfarpoS</i>	<i>ihf</i> α :: <i>gent</i> ^R , <i>rpoS</i> :: <i>kan</i> ^R	A.K. Brassinga
α 1- Δ rpoS <i>ihf</i> α	Lp02 <i>ihfarpoS</i> pBH6119:: <i>ihf</i> α P1 – GFP assay	this study
α 2- Δ rpoS <i>ihf</i> α	Lp02 <i>ihfarpoS</i> pBH6119:: <i>ihf</i> α P2 – GFP assay	this study
α 3- Δ rpoS <i>ihf</i> α	Lp02 <i>ihfarpoS</i> pBH6119:: <i>ihf</i> α P3 – GFP assay	this study

α 4- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfα</i> P4 – GFP assay	this study
α 5- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfα</i> P5 – GFP assay	this study
α 6- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfα</i> P6 – GFP assay	this study
α 7- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfα</i> P7 – GFP assay	this study
α 8- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfα</i> P8 – GFP assay	this study
β 1- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P1 – GFP assay	this study
β 2- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P2 – GFP assay	this study
β 3- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P3 – GFP assay	this study
β 4- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P4 – GFP assay	this study
β 5- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P5 – GFP assay	this study
β 6- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P6 – GFP assay	this study
β 7- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119:: <i>ihfβ</i> P7 – GFP assay	this study
pBH6119- Δ rpoSihfa	Lp02 <i>ΔihfarpoS</i> pBH6119 – GFP assay, vector control	this study
Lp02 <i>ΔletA</i>	<i>letA</i> ::kan ^R	M. Swanson (Hammer <i>et al.</i> 2002)
Y1- Δ letA	Lp02 <i>ΔletA</i> pBH6119:: <i>rsmY</i> P1 – GFP assay	this study

Y2- Δ letA	Lp02 Δ letA pBH6119:: <i>rsmY</i> P2 – GFP assay	this study
Y3- Δ letA	Lp02 Δ letA pBH6119:: <i>rsmY</i> P3 – GFP assay	this study
Z1- Δ letA	Lp02 Δ letA pBH6119:: <i>rsmZ</i> P1 – GFP assay	this study
Z2- Δ letA	Lp02 Δ letA pBH6119:: <i>rsmZ</i> P2 – GFP assay	this study
Z3- Δ letA	Lp02 Δ letA pBH6119:: <i>rsmZ</i> P3 – GFP assay	this study
pBH6119- Δ letA	pBH6119 – GFP assay, vector control	this study

Table 2.2: Catalogue of plasmids used in this study.

Plasmid	Description	Source (Reference)
pBH6119	RSF1010 ori, promoterless <i>gfpmut3 tdΔi</i> (Amp ^R)	M. Swanson
pBH α 1	pBH6119::ihf α P1 in Bam HI and Xba I	this study
pBH α 2	pBH6119::ihf α P2 in Bam HI and Xba I	this study
pBH α 3	pBH6119::ihf α P3 in Bam HI and XbaI	this study
pBH α 4	pBH6119::ihf α P4 in Bam HI and XbaI	this study
pBH α 5	pBH6119::ihf α P5 in Bam HI and XbaI	this study
pBH α 6	pBH6119::ihf α P6 in Bam HI and XbaI	this study
pBH α 7	pBH6119::ihf α P7 in Bam HI and XbaI	this study
pBH α 8	pBH6119::ihf α P8 in Bam HI and XbaI	this study
pBH β 1	pBH6119::ihf β P1 in Bam HI and XbaI	this study
pBH β 2	pBH6119::ihf β P2 in Bam HI and XbaI	this study
pBH β 3	pBH6119::ihf β P3 in Bam HI and XbaI	this study
pBH β 4	pBH6119::ihf β P4 in Bam HI and XbaI	this study
pBH β 5	pBH6119::ihf β P5 in Bam HI and XbaI	this study
pBH β 6	pBH6119::ihf β P6 in Bam HI and XbaI	this study
pBH β 7	pBH6119::ihf β P7 in Bam HI and XbaI	this study
pBHY1	pBH6119::rsmY P1 in Bam HI and XbaI	this study
pBHY2	pBH6119::rsmY P2 in Bam HI and XbaI	this study
pBHY3	pBH6119::rsmY P3 in Bam HI and XbaI	this study
pBHZ1	pBH6119::rsmZ P1 in Bam HI and XbaI	this study

pBHZ2	pBH6119::rsmZ P2 in Bam HI and XbaI	this study
pBHZ3	pBH6119::rsmZ P3 in Bam HI and XbaI	this study
pET16b	N-terminal 10-histidine-tagged fusion protein expression vector; Cm ^R , Amp ^R	Novagen
pET16b::IHF α	Expression vector; Cm ^R , Amp ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
pET16b::IHF β	Expression vector; Cm ^R , Amp ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
pET16b::RpoS	Expression vector; Cm ^R , Amp ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
pETDuet-1	Dual expression vector; Amp ^R	Novagen
pRDX (pKBXR)	Dual suicide vector pBOC20 with <i>Bacillus subtilis sacB</i> and <i>Helicobacter pylori rdxA</i> (nitroreducase) as counterselectable markers; Cm ^R	A.K. Brassinga (LeBlanc <i>et al.</i> , 2008)
pKBXR::ihf α	Dual suicide vector; Cm ^R , Gent ^R	A.K. Brassinga (Morash <i>et al.</i> , 2009)
pBluescript SK+	Cloning vector; Amp ^R	Stratagene
pKD3	Template plasmid; Cm ^R	M. Swanson (Bryan <i>et al.</i> 2011)

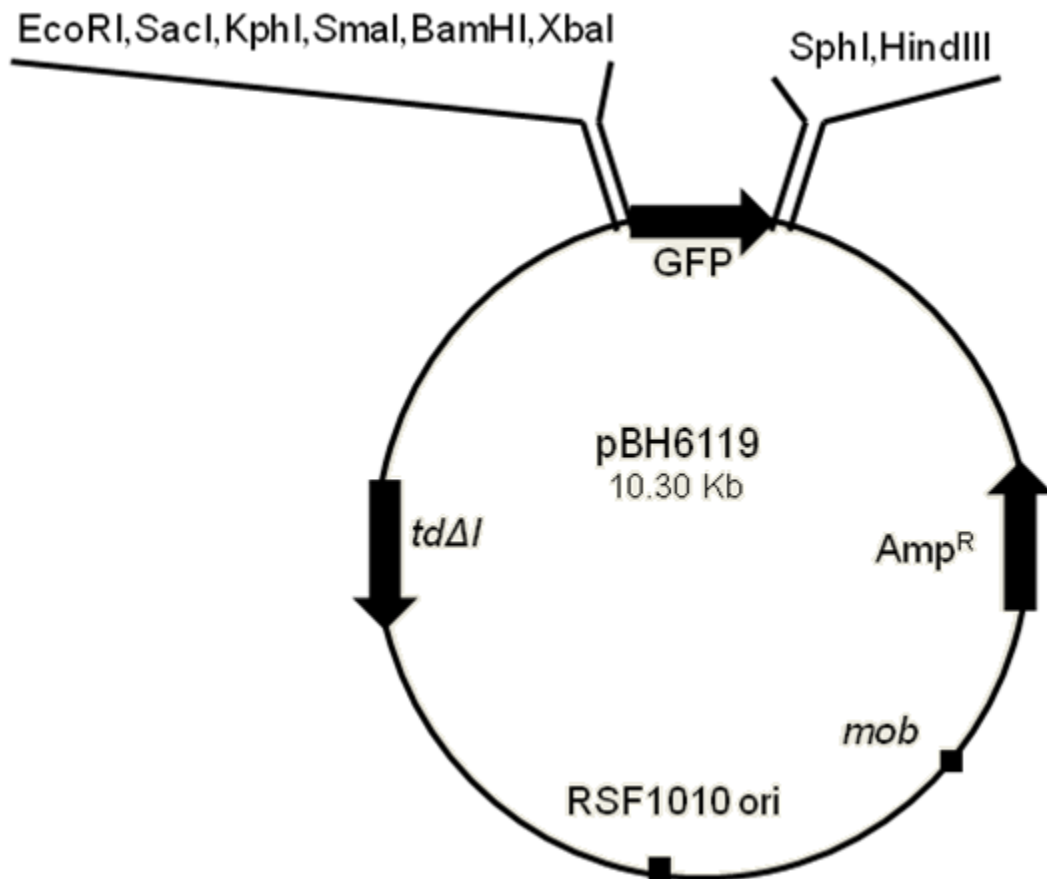


Figure 2.1: Genetic map of the promoterless GFP reporter vector pBH6119.

enzymes were obtained from New England Biolabs Canada (Toronto, Ontario). For sequencing the BigDye® Cycle Sequencing Kit v3.1 (Qiagen, Valencia, CA) was used.

2.2 Culture conditions:

2.2.1 *E. coli* culture conditions

The media used for all *E. coli* cultures was Luria-Burtani (LB) agar (per Litre: 10 g Tryptone, 5 g Yeast extract, 10 g NaCl, 15 g agar). The broth used was LB broth (same recipe minus the agar). Strains were struck out onto LB agar plates containing the appropriate antibiotics. Plates were incubated overnight at 37°C. A well isolated colony was selected and inoculated into a test tube containing 3 mL of LB broth and the appropriate antibiotics. Test tubes were incubated overnight with aeration at 37°C.

Where appropriate, antibiotics were added to the indicated final concentrations: ampicillin (100 µg/mL), kanamycin (40 µg/mL), gentamycin (20 µg/mL), and chloramphenicol (20 µg/mL). Isopropyl-β-D-galactoside (IPTG) was added to a final concentration of 1 mM where indicated.

2.2.2 *L. pneumophila* culture conditions

The medium used for all *L. pneumophila* cultures was Buffered Charcoal Yeast Extract (BCYE) (per litre: 10 g Bacto Yeast Extract, 1 g α-Ketoglutaric acid, 1 g ACES, 1.5 g Activated Charcoal, 15 g Agar). The broth used was BYE (10 g Bacto Yeast Extract, 1 g α-Ketoglutaric acid, 1 g ACES). For both the media and broth, the pH was adjusted to 6.6-6.7 via 6 M KOH and after being autoclaved. The medium was cooled to

55°C where the following supplements were added aseptically: 0.2 g L-Cysteine and 0.5 mL 25% Fe-pyrophosphate.

L-Cysteine was freshly prepared ~1 h prior to addition by measuring out 0.6 g and placed into a test-tube where 5 mL of dH₂O was added. 6M KOH was used to adjust the pH of the solution to 6.6 – 6.7. The solution was then topped up to 6mL and underwent sterilization via membrane filtration. 25% Fe-pyrophosphate was prepared by adding 10 g of Fe-pyrophosphate and bringing the volume up to 40 mL of dH₂O in a 50 mL falcon tube. The solution was vortexed, sterilized via 0.2 µm syringe membrane filtration, and stored at 4°C in 5 mL aliquots in sterile 15 mL conical tubes covered in aluminum foil.

Strains were stuck out onto BCYE plates, and placed in a 5% CO₂ humid 37°C incubator 48 – 72 h. Where appropriate, antibiotics and supplements were added to the indicated final concentrations: kanamycin (25 µg/mL), gentamycin (10 µg/mL), chloramphenicol (4 µg/mL), metronidazole (20 µg/mL) and thymidine (100 µg/mL).

2.3 Genome extraction

From a 48 – 72 h plate streaked with *L. pneumophila* Lp02, two loop fulls of Lp02 were collected and resuspended into an microfuge tube containing 440 µL of TE (pH 8.0), 50 µL of 10 mg/mL Proteinase K (in 50 mM Tris-HCl pH 8.0, 1 mM CaCl₂), and 10 µL of 10% SDS. The culture was then inverted for mixing and incubated at 37°C with gentle rocking for approximately 2 h, after which 50 µL of 10 M ammonium acetate was added. The lysate was extracted with an equal volume of (25:24:1) phenol/chloroform/isoamyl alcohol, vortexed, and spun in a microcentrifuge at 13,000

rpm for 10 min at room temperature (RT). The top aqueous layer was extracted with a volume of phenol/chloroform/isoamyl alcohol two more times before being placed in a sterile microcentrifuge tube with two volumes of ice-cold 100% ethanol and gently mixed by inversion. The sample was then spun at 13,000 rpm for 30 min at 4°C. The supernatant was removed and the pellet was washed with 70% ethanol and allowed to air dry. The final pellet was then resuspended in 100 µL of TE + RNase (100 µg/mL).

2.4 PCR

2.4.1 Cycling conditions

For gradient and colony PCR screening, Taq polymerase was used in 25µL reaction volumes as follows: 1 µL of *L. pneumophila* Lp02 genomic DNA as template for gradient PCR or 1µL of supernatant from colony inoculation in section 2.7 for colony PCR, 0.5 µL of dNTPs (10 mM), 2.5 µL of 10x of Thermo Pol buffer, 0.1 µM forward primer and 0.1 µM reverse primer (Table 2.3), 0.125 µL Taq polymerase units, and nuclease-free grade water. Cycling conditions were as follows: Initial denaturation at 94°C for 3 min, then denaturation at 94°C for 30 s, annealing temperature (Table 2.3) for 30 seconds, extension at 72° (for time see Table 2.3) for 35 cycles and final extension at 72° for 5 min. For gradient PCR, optimal temperatures for primer annealing were determined for each primer set by testing varying temperatures in the following range: 50°C, 51.5°C, 53.9°C, 57.5°C, 62.2°C, 66.0°C, 68.5°C, 70°C.

For amplification of Lp02 sequences for cloning purposes, high-fidelity Phusion™ Taq polymerase was used in 50 µL reaction volumes as follows: 1 µL of

Table 2.3: Description of amplification conditions constructs assembled in this study.

Amplicon Name	Primers(5' to 3' direction)	Annealing Temp	Extension Time (Taq/Phu)	Length
$\alpha 1$ (<i>infa</i> P1)	PF: GCGATAggatccGCCAGTCAGCTCAGATTGTGA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	70°C	30s/20s	469bp
$\alpha 2$ (<i>infa</i> P2)	PF: GCGATAggatccGATGCCTTTGATCTTGACCAGGA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	66°C	30s/20s	398bp
$\alpha 3$ (<i>infa</i> P3)	PF: GCGATAggatccGACTATTCCTCTGTATAAGCCTA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	66°C	30s/20s	333bp
$\alpha 4$ (<i>infa</i> P4)	PF: GCGATAggatccGTTGATAGGCAGATAAGTGCCA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	62.2°C	30s/20s	269bp
$\alpha 5$ (<i>infa</i> P5)	PF: GCGATAggatccGTAATCAGAAATACAGTTAAAGA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	53.9°C	30s/20s	233bp
$\alpha 6$ (<i>infa</i> P6)	PF: GCGATAggatccGACGTGTATATGGGTAAAGGTA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	62.2°C	30s/20s	182bp
$\alpha 7$ (<i>infa</i> P7)	PF: GCGATAggatccGACACTACAAGACGATACTCGA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	57.5°C	30s/20s	126bp
$\alpha 8$ (<i>infa</i> P8)	PF: GCGATAggatccGTGCTATAATCAAGAAACTGGA PR: GCGATAtctagaGCTTAGTGCGTTCACGATC	62.3°C	30s/20s	70bp

$\beta 1$ (<i>infβ</i> P1)	PF: GCGATAggatccCAAGTACCTTATTCGGTGCA PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	57.5°C	30s/20s	460bp
$\beta 2$ (<i>infβ</i> P2)	PF: GCGATAggatccCAACCGTCATTACTACTAGCA PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	57.5°C	30s/20s	394bp
$\beta 3$ (<i>infβ</i> P3)	PF: GCGATAggatccCGCATTTCATTCATAAGAAAGA PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	66°C	30s/20s	303bp
$\beta 4$ (<i>infβ</i> P4)	PF: GCGATAggatccGAACTATACTTATAAAAATCA PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	57.5°C	30s/20s	233bp
$\beta 5$ (<i>infβ</i> P5)	PF: GCGATAggatccGTAGCAGACATTAGGCCAGT PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	62.2°C	30s/20s	181bp
$\beta 6$ (<i>infβ</i> P6)	PF: GCGATAggatccGTATCGGCCACCCGTGTTTCA PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	57.5°C	30s/20s	136bp
$\beta 7$ (<i>infβ</i> P7)	PF: GCGATAggatccGAGGATGCAATCGGTCTAT PR: GCGATAtctagaTCAATGAGTTCGGATTTAATC	53.9°C	30s/20s	70bp
Y1 (<i>rsmY</i> P1)	PF: GCGATAggatccCTCGATGTATTTTCTGGTGG PR: GCGATAtctagaGGTCCCTTAGTTGACTTCCT	62.5°C	30s/20s	210bp
Y2 (<i>rsmY</i> P2)	PF: GCGATAggatccGGAGAGTAATGACGAAG PR: GCGATAtctagaGGTCCCTTAGTTGACTTCCT	62.5°C	30s/20s	146bp
Y3 (<i>rsmY</i> P3)	PF: GCGATAggatccCTCGATGACTTATACTGCCAAT PR: GCGATAtctagaGGTCCCTTAGTTGACTTCCT	62.5°C	30s/20s	82bp

Z1 (<i>rsmZ</i> P1)	PF: GCGATAggatccCCCCGCTACATTTTCATCGTA PR: GCGATAtctagaCAACTCAGAAATAGCCTTATA	66°C	30s/20s	210bp
Z2 (<i>rsmZ</i> P2)	PF: GCGATAggatccGACGTTATTTAAGCTGTAA PR: GCGATAtctagaCAACTCAGAAATAGCCTTATA	57.5°C	30s/20s	135bp
Z3 (<i>rsmZ</i> P3)	PF: GCGATAggatccCCTGACAATAATTCTTACA PR: GCGATAtctagaCAACTCAGAAATAGCCTTATA	57.5°C	30s/20s	73bp
MagA	PF: GCGATAggtaccGCTGTCAAAGAGGCAACA PR: GCGATAgcatgcATACCTCTCCTTATTTTTGGA	62.2°C	30s/20s	229bp
rsmY	PF: CTCGATGTATTTTCTGGTGG PR: GGTCCCTTAGTTGACTTCCT	53.9°C	30s/20s	210bp
rsmZ	PF: CCCCCTACATTTTCATCGTA PR: CAACTCAGAAATAGCCTTATA	53.9°C	30s/20s	210bp
Control	PF: CTCTACGCTAACGCACAAG PR: CAGACGGATTTTCCTGAGAGG	66.0°C	30s/20s	340bp
Ihfa(Int)	PF: GGCAGAAACGTTGTGTGACGA PR: GTAACAACCCTTCTGGCTTCCA	50.0°C	30s/-	216bp
Ihfa(Ext)	PF: GCGATAGAATTCCATCAGATTAATGAC PR: GCGATAGAGCTCAGATTGTGATTAACG	50.0°C	2.5min/-	2300bp
pDuet:: <i>Ihfa</i>	PF: GCGATAggatccAATGATCGTGAACGCACTAAGC PR: GCGATAgaatccCTACTTTCCTATTTTTTCAATTTTC	51.5°C	30s/20s	300bp

pDuet::Ihfb	PF: GCGATAcatatgATTAATCCGAACTCATTGAACAC PR: GCGATActcgagTTAATCTTTATCCAAAAGTGGGAAC	53.9°C	30s/20s	312bp
RpoS(FR)	PF: GCGATActcgagGCAGATGCTGGTGGTGGTAC PR: GCGATAg gatccGCAACCACTCCATGAGCGATA	50°C	3min/95s	3010bp
RpoS(Int)	PF: GAATCCTCTGAGCCAGATGATG PR: GCAAGAGTCGCATAATGGATTTC	62.2°C	30s/20s	239bp
Cm(FRT+RpoS)	PF: GTGCCAGTTAATCCATTGAATTATCTACAAAAA- GGTTGACATATAACTCATAGTTATAGCAACAAT- AGGCTGTGTAGGCTGGAGCTGCTTC PR: GTAATTAATTAATTTGGTTTCAGTTGGGTCAAA- TTTGACCCAACCTTGTGTGTAATTAATTAACTGG- TTAAACATATGAATATCCTCCTTAGTTCC	57.5°C	1min/30s	1024bp
LetA(FR)	PF: GCGATActgcagGAGATGAGTGAAGGAG PR: GCGATAg cggccgcGCATCCAATTCTTCATCTA	53.9°C	3min/95s	2537bp
LetA(Int)	PF: GATCTTGATCTAGTTGCAGATG PR: GCACCTAATTGCAATACTCGAG	62.2°C	30s/20s	218bp
Cm(FRT+LetA)	PF: GAGTGACTATGTATTGTAATGATTATCGAGTC- TTGTAAACATCGACCAACACAGTTTGTTCATT- GACTGATGTGTAGGCTGGAGCTGCTTC PR: GACTTGGGAGTTTCGTAAAAAATAAAGCGAG- TTCAGCGGAAAGCTGTAAGTCATTCATGAAAT- TAACATCATATGAATATCCTCCTTAGTTCC	57.5°C	1min/30s	1024bp
Sequencing(GFP)	PR: GTAAGTAGCATCACCTTCA			

1/10 dilution of (927.7 ng/ μ L) *L. pneumophila* Lp02 as template, 4 μ L of dNTPs (10 mM), 1.5 μ L of DMSO, 10 μ L of 5x PhusionTM HF buffer, 0.1 μ M forward primer and 0.1 μ M reverse primers (Table 2.3), 0.25 μ L PhusionTM polymerase, and nuclease-free water. Cycling conditions included: Initial denaturation at 98°C for 30 s then denaturation at 98°C for 30 s, annealing temperature (Table 2.3) for 30 s, extension at 72° for time (Table 2.3) for 35 cycles and final extension at 72° for 5 min.

2.4.2 Agarose gel electrophoresis

To visualize DNA, 5 μ L of PCR product was run on an 1xTAE (40 mM Tris acetate, 20 mM acetic acid, 1mM EDTA) agarose gel (1% for constructs \geq 1 kb or 2% for constructs <1 kb) supplemented with 0.5 mg/mL ethidium bromide via horizontal gel electrophoresis at 100V for 20 min. The DNA bands were visualized using an AlphaimagerTM 2200 equipped with an ethidium bromide filter.

2.5 Cloning of insert DNA into plasmid

All PhusionTM acquired PCR amplicons obtained for plasmid construction underwent a double digestion with selected restriction endonucleases (Table 2.4). Digestions were completed in 50 μ L reaction volumes which included: 10 μ L of PCR amplicon, 5 μ L of 10x BSA, 5 μ L of specified 10x buffer from New England Biolabs' double digest finder (http://www.neb.com/nebecomm/doubledigest_calculator.asp) (Table 2.4), 2 μ L of each restriction enzyme, and nuclease-free grade water. Digests were incubated at 37°C

Table 2.4: List of restriction enzymes used to digest various amplicons and vectors for plasmid construction in this study.

Amplicon or Vector	Restriction Enzymes (FP/RP)
$\alpha 1 - \alpha 8$	Bam HI/Xba I
$\beta 1 - \beta 7$	Bam HI/Xba I
Y1 – Y3	Bam HI/Xba I
Z1 – Z3	Bam HI/Xba I
pBH6119	Bam HI/Xba I
ihf α (for pDuet)	Bam HI/Eco RI
ihf β (for pDuet)	Nde I/Xho I
pDuet	Bam HI/Eco RI
pDuet::ihf α	Nde I/Xho I
RpoS (F1)	Xho I/Bam HI
pBS (for RpoS)	Xho I/Bam HI
LetA (F1)	Pst I/Not I
pBS (for LetA)	Pst I/Not I

for 2 h and products were purified following electrophoresis via a Qiagen gel extraction kit (Qiagen, Valencia, CA) in accordance to manufacturer's instructions.

All vectors obtained for the plasmid construction underwent a double digest with selected restriction endonucleases (Table 2.4). Digestions were completed in 70 μ L reaction volumes which included: 40 μ L of plasmid, 10 μ L of 10x BSA, 10 μ L of the specified 10x buffer from New England Biolabs' double digest finder (http://www.neb.com/nebecomm/doubledigest_calculator.asp) (Table 2.4), 3 μ L of each restriction digest enzyme, and nuclease-free water. The digests were incubated at 37°C for 1 h, at this time 7 μ L of calf intestinal alkaline phosphatase (CIP) was added and the digest was left to incubate at 37°C for another hour. The entire plasmid reaction was subjected to agarose gel electrophoresis (section 2.4.2) and run for 30 min. The section of gel with the band of appropriate size was extracted with a clean razor blade. Both the digested insert and gel extracted digest plasmid were then purified via a Qiagen gel extraction kit as the protocol stated.

Digested amplicons and vectors were ligated using T4 DNA ligase in 10 μ L reaction volumes which included: 7 μ L of amplicon, 1 μ L of vector, 1 μ L of T4 DNA ligase, and 1 μ L of 10x T4 DNA ligase buffer. The reaction was incubated at 15°C for 24 hours. Ligations were then transformed into rubidium chloride competent *E. coli* DH5 α cells.

2.6 Constructed plasmid transformation or electroporation into *E. coli* cells

2.6.1 Preparation of rubidium chloride chemically competent *E. coli* DH5 α cells

E. coli DH5 α was struck out on LB agar plates and incubated overnight at 37°C. One colony was selected and inoculated into a test tube containing 3 mL of LB broth and incubated overnight at 37°C. The overnight culture was subcultured into 500 mL of LB broth and incubated at 37°C. Once an OD₆₀₀ of ~0.5 was reached the culture was chilled on ice for 5 min. At this point the culture was then placed into centrifuge bottles where it was spun at 3700 rpm for 20 min at 4°C. The supernatant was then poured off. The cells were resuspended in 200 mL of TFB I (30 mM Potassium acetate, 100 mM RbCl₂, 10 mM CaCl₂, 50 mM MnCl₂, 0.0225% glycerol in dH₂O, pH to 5.8 with 0.2 M acetic acid) and chilled on ice for 5 min. The cells were then spun for 15 min at 3700 rpm at 4°C. The supernatant was poured off. The cells were resuspended in 20 mL of sterile TFB II (10 mM MOPS, 75 mM CaCl₂, 0.0563% glycerol in dH₂O, pH to 6.5 with 1 M KOH, sterilized via membrane filtration) and chilled on ice for 15 min. 200 μ L aliquots of the cells were added to microfuge tubes, flash frozen in a dry-ice/ethanol bath and stored at -80°C.

2.6.2 Transformation of ligations into rubidium chloride chemically competent *E. coli* DH5 α cells

Chemically competent *E. coli* cells were thawed on ice. The 10 μ L ligation reaction and 100 μ L of competent cells were mixed together in a microfuge tube and chilled on ice for 1 h. The mixture was then heat shocked at 37°C for 90 s and resuspended in 500 μ L of LB. The resuspension was then incubated at 37°C while shaking for 1 h. The tube was then vortexed and 100 μ L aliquots were spread plated onto LB (containing appropriate antibiotics) and incubated overnight at 37°C.

2.6.3 Preparation of electrocompetent *E. coli* DY330 and DY331 cells

Each strain was struck out on LB and grown overnight at 30°C. Well isolated colonies were inoculated into 3 mL of LB and grown overnight at 30°C. Cells were then subcultured at 1:50 fold dilution in two flasks of LB (1 flask-induced, 1 flask-uninduced) and incubated shaking at 30°C until the OD₆₀₀ reached 0.4 – 0.5. The flask containing the culture to be induced was then incubated at 42°C for 15 min with shaking, while the culture to be uninduced was left to incubate at 30°C. Both cultures were immediately chilled in an ice-water slurry for 5 min. Cultures were transferred to 50mL conical tubes and spun at 4600 rpm for 7 min at 4°C. After decanting, the cultures were then washed with 50mL of ice-cold sterile dH₂O and spun at 4600 rpm for 7 min at 4°C. The supernatant was removed and the cells were resuspended in 1 mL of ice-cold sterile dH₂O and each transferred to a microfuge tube and spun for 1 min 13,000 rpm at 4°C. The supernatant was removed and the cultures were then washed again with 1 mL of dH₂O and resuspended in a final volume 250 µL of a 10% glycerol solution. Electroporation of electrocompetent cells were as stated in section 2.15 below.

2.7 PCR colony confirmation of ligated plasmid construct in *E. coli*

Up to 50 colonies were randomly selected and subjected to a colony PCR. Colonies were picked, streaked on a master LB plate supplemented with appropriate antibiotics, and resuspended into a PCR tube containing 50 µL of nuclease-free water at a quantity of 5 colonies per tube. The PCR tubes containing the inoculated cells were subjected to lysis at 95°C for 10 min in a thermocycler. The tubes were then centrifuged

at 13,000 rpm and 1 μ L of the supernatant containing the plasmid was subjected to PCR using Taq polymerase following the protocol (as stated in section 2.4.1) with specified primers (Table 2.3) to determine if desired insert was present within the plasmid. From a tube that gave a positive result, the corresponding five colonies on the master plate were subjected to single-colony PCR with Taq polymerase. The colony that yielded a positive result was then inoculated into 3 mL of LB broth containing the appropriate antibiotics for an overnight culture. The entire culture was then subjected to plasmid DNA extraction via QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA).

2.8 Sequencing

Amount of DNA was determined via measurement with a Nanodrop 2000c spectrophotometer (Thermo Scientific, Ottawa, Ontario) at a wavelength of 260 nm.

Constructs were prepared via PCR amplification of 20 μ L reaction volumes including: 2 μ L of 2.5x reaction premix, 2 μ L of Big Dye Buffer, 1.53 pmol/ μ L of (Sequencing(GFP)) primer, 150-300ng of DNA template and nuclease-free water. Cycling conditions included: Initial denaturation at 96°C for 1 min, then 96°C for 10 s, 50°C for 5 s, 60°C for 4 min, for 25 cycles.

All constructs were sequenced using the BigDye® Cycle Sequencing Kit v3.1 (Qiagen, Valencia, CA) protocol. Purification of each sample included adding 5 μ L of 125 mM EDTA and 66 μ L of 95% EtOH. The sample was mixed by inversion and incubated at RT for 15 min. The sample was then centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was removed and 60 μ L of 70% EtOH was added. The last two

steps were then repeated twice with final step being the removal of the supernatant and air drying of the pellet. The sample was then resuspended in 20 μ L of formamide and heated at 94 $^{\circ}$ C for 5 min where it was then loaded into the Applied Biosystems 3130 Sequencer.

2.9 Constructed plasmid electroporation into *L. pneumophila* cells

2.9.1 Preparation of electrocompetent *L. pneumophila* cells

L. pneumophila strains were struck out onto BCYE supplemented with appropriate additives and antibiotics and incubated at 37 $^{\circ}$ C for 48 h. The bacteria were then harvested from the plate with a sterile disposable loop and heavily streaked out on a fresh BCYE plate and incubated at 37 $^{\circ}$ C for 24 h. The entire plate culture was harvested with a sterile disposable loop and resuspended into 40mL of cold sterile water and spun at 3700 rpm at 4 $^{\circ}$ C for 15 min. The supernatant was removed and the pellet was resuspended in 40 mL of cold sterile water and spun at 3700 rpm at 4 $^{\circ}$ C for 15 min, and the washing repeated three more times. After the last spin the pellet was resuspended into 2 mL of cold sterile 15% glycerol. 100 μ L aliquots were placed into microfuge tubes, flash frozen in dry ice/ethanol bath and stored at -80 $^{\circ}$ C.

2.9.2 Electroporation of constructed plasmids into *L. pneumophila* cells

The electrocompetent cells were thawed on ice. 5 μ L of desired plasmid and 90 μ L of electrocompetent bacteria were mixed together. This mixture was then added to a 1 mm-gap BioRadTM electroporation cuvette and pulsed at a manual setting of 2.1 kV.

The electroporated cells were recovered with 1 mL of BYE and incubated on a shaker at 37°C for 2 h. The culture was then plated onto the appropriate selective BCYE media and incubated for 48 – 72 h.

2.10 Fluorometer assays

L. pneumophila strains containing GFP reporter plasmids were subjected to GFP assays which measured the intensity of the fluorescence of liquid bacterial cultures at normalized optical density and the results were expressed in relative fluorescence units.

Initial GFP assays were done by growing strains on BCYE 37°C for 48 h after which approximately 4 colonies were inoculated into 3 mL of BYE. The OD₆₀₀ was measured, the cells were subcultured into 50 mL of BYE at a calculated OD₆₀₀ of 0.005 and grown overnight shaking at 37°C such that the starting OD₆₀₀ for measurements would be at midlog phase, between 0.4 and 0.6 (approximately 16 h post inoculation). Every two hours starting at time zero (when midlog phase was reached) for 8 hours the optical density of each culture was measured. In addition a calculated sample of 0.1 OD₆₀₀ was taken from each culture where it was spun down and the supernatant was resuspended in 2mL of dH₂O. A fluorescence reading of the sample was then read by a BioRadTM fluorometer equipped with a 488Ex/510Em nm filter set. This initial GFP assay was completed once for all wild-type and *Δihfaihfβ* strains containing the $\alpha 1 - \alpha 8$ and $\beta 1 - \beta 7$ constructs ($\alpha 1 - \alpha 8$, $\beta 1 - \beta 7$, $\alpha 1 - \Delta ihf - \alpha 8 - \Delta ihf$, and $\beta 1 - \Delta ihf - \beta 7 - \Delta ihf$) and their negative controls (pBH6119-wt and pBH6119- Δihf) (Table 2.1).

All subsequent GFP assays were completed at least three times in the following manner. *L. pneumophila* strains harbouring GFP reporter plasmids were grown on BCYE for 48 h. Approximately $\frac{3}{4}$ of a disposable loop full of each culture was resuspended in 3 mL of BYE where the optical density was measured and subcultured into 10mL of BYE at an OD₆₀₀ of 0.2. 150 μ L of each culture was placed into one well within a 96 well black microclear plate (Greiner Bio-One). GFP (Ex 485/20, E@m 528/20) and OD₆₀₀ readings were taken every hour within a SynergyTM 2 (Gen 5 program) for 24 h (kinetic assay) where the plates were incubated shaking (at the setting speed fast) at 37°C. The results were calculated by dividing GFP by OD and normalizing the values by subtracting the negative controls (pBH6119-wt, pBH6119- Δ ihf, pBH6119- Δ rpoS, pBH6119- Δ rpoSihf α , pBH6119- Δ letA).

2.11 Protein purification

2.11.1 Growth and protein induction

Strains: *E. coli* BL21 CodonPlus pET16b::IHF α , *E. coli* BL21 CodonPlus pET16b::IHF β , and *E. coli* BL21 CodonPlus pET16b::RpoS, were struck out onto LB agar containing ampicillin and chloramphenicol and incubated overnight at 37°C. Approximately $\frac{1}{2}$ of a disposable loop full of bacteria was inoculated into 30 mL of LB containing ampicillin and incubated on a shaker overnight at 37°C. The entire culture was subcultured into 1 L of LB broth containing ampicillin and incubated on a shaker at 37°C until an OD₆₀₀ of \sim 0.5 was reached. After a 250 μ L pre-induction sample was taken, the culture was induced with 1 mM IPTG on a shaker at 37°C for 1 h. A 250 μ L post-

induction sample was taken, and the culture was then spun at 6,000 rpm for 30 min at 4°C. The supernatant was removed and the pellet was resuspended in 40mL of 0.05M Tris (pH 8). The culture was then spun at 3,800 rpm for 20 min at 4°C. The supernatant was removed. The pellet was stored at -80°C until processed.

2.11.2 Preparation of beads and French press

Approximately 1 mL of well shaken Qiagen Ni-NTA beads was added to a 15 mL conical tube and left for ~1 h at 4°C to settle. The supernatant was discarded and the beads were resuspended in 10 mL of sterile water. The solution was left for ~2 h at 4°C to settle. The supernatant was then removed and the beads were resuspended in 10mL of Binding buffer (5mM Imidazole, 0.5 M NaCl, 0.02 M Tris pH 8).

The cell pellet was then resuspended in 40 mL of Binding buffer plus 200 µL each of a premixed solution of Protease inhibitor and EDTA (Fisher). The cells were lysed via a French Press (20K cell, American Instrument Company, Silver Spring, Md.) and a 250 µL post-lysis sample was taken. The beads were then added to the lysate and mixed at 4°C on the Orbitron Rotator II (Mandel Scientific, Guelph, Ontario) for 1 h to allow the protein to bind to the beads.

2.11.3 Gravity column and dialysis

The protein bead mixture was added to a gravitational column (GE Healthcare, Baie d'Urfe, Quebec) and a 250µL sample of the flow though was collected. The protein bead mixture was then subjected to 3 separate washes (0.5M NaCl, 0.02M Tris

pH 8) with increasing amounts of imidazole (wash 1: 0, wash 2: 10 mM, wash 3: 25 mM). A sample of the flow though was collected from wash 2. The final step involved collecting 1.5 mL aliquots of the flow through from the Elution buffer (0.25 M NaCl, 0.05 Tris pH8, 0.5 mL of concentrated HCl, 250 mM Imidazole, up to 100 mL H₂O). 15 μ L of the collected aliquots were loaded into a 12% SDS gel (2 mL Resolving gel buffer [1.5 M Tris, 0.4% SDS, pH 8.8], 12% polyacrylamide, 50 μ L 10% APS, 12 μ L Temed [Biorad, Mississauga, Ontario], up to 8 mL dH₂O) and ran at 120V for 1 h and 15 min in 1x Running Buffer (0.025 M Tris, 0.192 M glycine, 0.1% SDS). The gel was then stained with Coomassie Brilliant Blue (BioRad) and destained with Destain Solution #1 (50% Ethanol, 10% Acetic acid) for 2 h and Destain Solution #2 (5% Ethanol, 7% Acetic acid) for 1 h to visualize protein bands to determine which aliquots contained protein. In addition, 10 μ L of Bradford Protein Assay Dye Reagent (Bio-Rad) and 10 μ L of eluted protein were added to 80 μ L of water to verify which aliquots contained protein through visualization. The sample which contained protein were then pooled and placed into dialysis tubing and a 250 μ L pre-dialysis sample was taken. The pooled eluted protein was subject to dialysis with Buffer 1 (0.02 M Tris pH 8, 0.3 M KCl, 77 mg DTT, 0.4 mM EDTA, 10% glycerol) for 4 – 24 h and repeated with new Buffer 1 for an additional 4 – 24 h.

2.11.4 HiTrapTM Heparin HP column and concentration

HiTrapTM Heparin HP column (GE Healthcare, Baie d'Urfe, Quebec) was equilibrated with 10 volumes of Buffer A (40 mM Hepes pH 7.5, 200 mM NaCl, 3 mM β -mercaptoethanol). The protein sample was then loaded onto the column and the

column washed with 5 volumes of Buffer A. 5 volumes of Buffer A plus 0.5 mM DTT was then added to the column. Next Buffer A and Buffer B (40 mM Hepes pH 7.5, 1.5 M NaCl, 3 mM β -mercaptoethanol) were added together to give washes of increasing NaCl concentrations beginning with 200 mM NaCl increasing in 100 mM increments to 1.5 M NaCl. All washes contained 0.5 mM DTT. Flow through was taken at each point starting from adding the protein sample to the final wash of 1.5 M NaCl. Samples of the flow through were then checked for the presence of protein via: SDS-PAGE and Bradford analyses. The samples which contained protein were pooled together and concentrated in storage buffer (20 mM Tris pH 7.9, 200 mM KCl, 0.2 mM EDTA, 30% glycerol) via centrifugation in a Amicon® Ultra 10K Centrifugal filter units (Milipore, Long Beach, California). Samples were spun at 8,500 rpm at 4°C until the final volume reached approximately 500-750 μ L. Samples were stored at -20°C.

2.11.5 Western blot analysis of eluted recombinant protein

To verify the elution of His-tagged protein, 25 μ L of protein sample was added to a PCR tube and mixed with 22.5 μ L of 2x loading buffer (2 mL 50% glycerol, 2 mL 10% SDS, 60 mM Tris pH 6.8, 0.5 mL of 0.1% Bromophenol Blue, up to 10 mL H₂O) and 2.5 μ L of β -mercaptoethanol and heated at 95°C for 5 min. 15 μ L of sample was loaded into a 12% SDS gel and run at 120V for 1 h and 15 min in 1x Running Buffer. Samples were then transferred from gel to nitrocellulose membrane in 1x Transfer buffer (0.025 M Tris, 0.192 M glycine, 0.19% ethanol) and run at 100 V for 2 h in a gel transfer tank placed in an ice bath. The nitrocellulose containing the transferred protein was rinsed with dH₂O, stained with Ponceau red (0.01% Ponceau Red, 0.1% acetic acid) for 10 min, washed

with water to visualize the bands, and destained with 1xPBS (0.116 M NaCl, 2 mM KCl, 1.4 mM KH_2PO_4 , 1.4 mM Na_2HPO_4) for 10 min. The membrane was placed in blocking solution (100mg Skim milk powder, 100mg BSA, 10 mL 1xTTBS [0.02 M Tris pH 7.5, 0.5mM NaCl, 0.05% Tween 20]) on a shaker for 1 h at RT. The membrane was rinsed twice with 1xTTBS for 5 min each. Primary antibody [1:3,000 Anti-His Antibody is an IgG₂ subclass of monoclonal antibody produced against mouse GST-(histidine)₆-tagged protein (GE Healthcare, Baie d'Urfe, Quebec), 100 mg BSA, 10 mL 1xTTBS] was then added to membrane and incubated on shaker for 1 h at RT. After removal of the primary antibody solution, the membrane was rinsed three times with 1x Tris-NaCl (0.02 Tris pH 9.5, 0.05 M NaCl) solution for 10 min each, and placed in a Secondary antibody [1:30,000 of α -mouse IgG alkaline phosphatase, developed in Goat (Sigma-Aldrich Canada, Oakville, Ontario), 10mL Tris-NaCl] solution and incubated for 1 h at RT. After removal of the secondary antibody solution, the membrane was washed twice with Tris-NaCl solution for 10 min each after which AP buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 0.05 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) was added to the membrane and incubated on a shaker for 10 min at room temperature. The AP buffer was discarded and 3mL of BCIP/NBT (Amresco®, Solon, Ohio) solution was added to the membrane, and once bands were satisfactorily visualized, the colourimetric reaction was stopped with 1xPBS.

2.11.6 Determination of the concentration of protein and DNA in samples

Protein concentration was determined via a Bio-Rad protein assay. The protein concentration was determined by plotting it on a standard curve of increasing Bovine BGG50 concentrations. 200 μL of Bio-Rad protein reagent was added to increasing

amounts of BGG50 and brought up to 1 mL with dH₂O to create a curve with BGG50 concentrations at set points of 0, 2.5, 5, 10, 20 µg/µL. The protein sample was diluted by one tenth by adding 10 µL of protein sample to 90 µL of protein dilution buffer (20 mM Tris pH 8, 10 mM MgCl₂, 100 µM KCl, 10% glycerol 0.05 mg/mL BSA). The sample was further diluted by 1/500 by adding 20 µL of the 1/10 dilution to 780 µL of dH₂O. A₂₈₀ readings of the protein were compared against the standard curve to determine protein concentration.

A Nanodrop 2000c spectrophotometer (Thermo Scientific, Ottawa, Ontario) was used to determine percent of DNA within the protein samples by comparing the ratio of A₂₆₀ (DNA) to A₂₈₀ (protein).

2.12 Electrophoretic mobility shift assay

The probe mixture was made with a total volume of 50 µL containing: (10 ng or 250 ng) desired PCR amplicon, 22 µL Binding Buffer (0.2 M Tris pH 8.0, 0.2 M KCl, 0.5 M MgCl₂, 50 µL 100x BSA), 44 µL of 50% glycerol. Each 20 µL reaction consisted of 10µL of probe mixture, 0, 1, 3, or 5 µL of recombinant protein in increasing amounts and 10, 9, 7, and 5 µL of nuclease-free water, respectively. The reactions were left to incubate for 30 min at RT to allow for protein/DNA complex formation. For loading on to a 5% 0.5X TBE gel, 2 µL of 10x Loading buffer (600 µL of 50% glycerol, 9.4 mL 5x TBE [500 mL solution containing 27 g Tris, 13.75 g Boric Acid, 0.01 M EDTA pH 8], 0.1 g bromophenol blue) was added to each reaction. To prepare the 5% 0.5X TBE gel, (1 mL 5x TBE, 5% polyacrylamide, 62.5 µL 10%APS, 12 µL TEMED (Bio-Rad,

Mississauga, Ontario), up to 10 mL with dH₂O), the nonpolymerized gel mixture was degassed for 15 min in order to increase the polymerization time and to decrease the background noise before the TEMED was added. The gel was run at 100 V for the desired amount of time. The gel was then stained with a 1:10,000 dilution of SYBR[®] Green for 10 min. Visualization of bands was done by AlphaImager[™] 2200 or BioRad[™] VersaDoc 4000.

2.13 Double knockout strategy (*ΔihfaletA*)

Escherichia coli DH5α pKBXR::ihfα-gent was struck out onto LB supplemented with gentamycin and incubating it overnight at 37°C. Since the strain contains a low copy number plasmid, two overnight cultures were created by inoculating a well isolated colony into 3mL of LB and incubated overnight at 37°C with aeration. Both overnight cultures were combined and subjected to plasmid DNA extraction via QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The plasmid was then electroporated into *L. pneumophila* Lp02 *ΔletA* and plated onto BCYE containing the appropriate antibiotics and additives and incubated for 48 h at 37°C. Colonies were patched onto BCYE (supplemented with gentamycin, kanamycin, thymidine) and BCYE (supplemented with metronidazole and thymidine). Colonies that were resistant to gentamycin, kanamycin and metronidazole were subjected to genomic isolation and PCR verification.

2.14 Triple knockout strategy (*ΔihfaihfbprpS* & *ΔihfaihfbletA*)

All primers and PCR conditions are indicated in Table 2 for this section. The targeted gene to be mutated plus 500 bp before the start codon and 500 bp after the stop codon was PCR amplified using primers RpoS(FR) for RpoS KO and LetA(FR) for LetA KO (Table 2.3) with PhusionTM polymerase (see section 2.4), and cloned into restriction sites in pBluescript SK+ (pBS) (Table 2.2). A chloramphenicol cassette plus FRT sites was PCR amplified from pKD3 where the primers [Cm(FRT+RpoS) for RpoS KO and Cm(FRT+LetA) for LetA KO] contained approximately 60 bp of the beginning and end of the targeted gene to be mutated (CmFRTgene). To promote insertion of the antibiotic cassette between the two flanking regions, 3 μ L of pBS containing gene amplicon and 7 μ L of amplified CmFRTgene were mixed and electroporated into 50 μ L of both induced and uninduced *E. coli* recombineering strains DY331 and DY330 (Table 2.1). The electroporation was done in a 1mm-gap electroporation cuvette at 1.8 kV; the cells recovered in 500 μ L of LB and incubated with aeration at 30°C for 3 – 12 h, after which 100 μ L aliquots were plated onto LB plus chloramphenicol and incubated for 24-48 h at 30°C. Colonies that grew from the induced cells were checked with internal primers specific to gene of interest [RpoS(Int) for RpoS KO and LetA(Int) for LetA KO] for the absence of the gene and therefore confirm the presence of the antibiotic cassette alone. Once the desired construct was attained the plasmid was isolated and transformed into *E. coli* DH5 α to prepare DNA to recheck the construct with the internal primers [RpoS(Int) and LetA(Int)]. Once the construct was reconfirmed then the plasmid was electroporated into *L. pneumophila* Lp02 and recombination was checked via PCR with the internal primers [RpoS(Int) and LetA(Int)] and sequenced (as per protocol in section 2.8).

Chapter 3: Results

3.1 Bioinformatic pattern searches

3.1.1 Computational bioinformatic pattern search for IHF binding sites in the upstream promoter regions of *ihfα* and *ihfβ* in *L. pneumophila*

The *E. coli* IHF is a heterodimeric protein that consists of an alpha (encoded by *himA*) and a beta subunit (encoded by *himD*) (Aviv *et al.*, 1994). In *E. coli*, IHF (EcIHF) recognizes a 13-bp consensus sequence WATCAANNNTTR (W = A/T, R = A/G), and when bound covers a region of approximately 35 bp (Rice *et al.*, 1996). Expression of IHF in *E. coli* is negatively autoregulated; one IHF binding site and two IHF binding sites are located in the promoter regions upstream of *himA* and *himD* genes, respectively (Aviv *et al.*, 1994). In *L. pneumophila*, genes homologous to *himA* and *himD* were identified and determined to encode IHF, *ihfα* and *ihfβ*, respectively (Morash *et al.*, 2009). Comparative sequence analysis using Blast_p revealed *himA* (*E. coli*) to be 71% identical to *ihfα* (*L. pneumophila*) and *himD* (*E. coli*) to be 62% identical to *ihfβ* (*L. pneumophila*) (<http://blast.ncbi.nlm.nih.gov>). To ascertain if the IHF in *Legionella* (LpIHF) is also autoregulated, a bioinformatic search via the pattern search option on the Legiolist website (genolist.pasteur.fr/LegioList/), was performed using the *E. coli* consensus IHF binding sequence on the *ihfα* and *ihfβ* promoter regions to identify the presence of possible sites. Although exact matches were not found, the search pattern parameters were expanded to allow up to 3 mismatches to the *E. coli* consensus sequence in which four and two putative sites were identified in the *ihfα* and *ihfβ* promoter regions, respectively (Figure 3.1). The identified sites when compared with the *E. coli* consensus

Figure 3.1: Identification of putative IHF consensus sequences within the promoter regions of *ihfα* and *ihfβ*. In orange font are the locations of the putative *E. coli*, IHF 13-bp consensus sequence WATCAANNNTTR (W = A/T, R = A/G). Underlined are the locations of the sequences related to the *E. coli* RpoS consensus sequence. The starts of the *ihfα* and *ihfβ* open reading frame are represented in green and blue font, respectively. Sequences from genolist.pasteur.fr/LegioList/.

ihf α :

CGCTTACCCCCGGCCAGTCAGCTCAGATTGTGATTAACGGCAAGCATTTCAGGCTGGATC
GGTGTGCTGCATCCTCGCTTGTCCGATGCCTTTGATCTTGACCAGGATGTGGTTTTGT
TGAGTTGAATCTTGAATCATTAAATCAATCCGACTATTCTCTGTATAAGCCTATTTCCA
AAATATCCACAATAAGGCGCGATTTGTCTTTCTGGTTGATAGGCAGATAAGTGCCATG
CAGATTGAGCGAGTAAATCAGAAATACAGTTAAAGAGGATTGGTTAAAATCGTTTGACGT
CTTTGACGTGTATATGGGTAAAGGTATTCAGAAGATAAAAAAGTATTGCTGTGGCTA
TGACACTACAAGACGATACTCGAACTTTAGTTGATGCCGAGATCAATTTAACAATTAGT
GCTATAATCAAGAACTGGAAAATGAATTTTCAATCCTTTTGAGGGAATGATCGTGAAC
GCACCTAAGCAAAGCAATAATGGCAGAAACGTT . . .

ihf β :

GGCGACCTTAATATTTATTAATTCAAGTACCTTATTCGGTGCAACCTGGGAAGCCCTTA
ACACCCGTCTCAATAGCCTGCCACAACCTTTCAACCGTCATTACTACTAGCAAAACCTTA
TCATCCAGTCGTTTTAAATTAGTTACTCGCGCTAAAATACAAATGAGTTGGGGTGTAGT
TTTCGCATTCATTCATAAGAAAGAAAATAATTTTTACTAACCAGCTTGAAAACATTGA
ATAATTTTTTACCCGAACTATACTTATAAAAATCAAAATGGGAAATTGTCAGCGCCTGATG
TGTCTTGGTAGCAGACATTAGGCCAGTTGCTCAGCTCGTGGAGTTTATTTTAGTATCGG
CCACCCGTGTTTTCAAGATACAAAGCGAATAGAAACATAGGCAACCAAGCTAAAATAATA
GAGGATGCAATCGGTCTATTAAATACGCGACAAAACGAGGTGAGTATATGATTAAATCC
GAACTCATTGAACACATCGCTGCTCGAATGACGCATCT . . .

site revealed 1 through 3 mismatches (Figure 3.2). When the six identified IHF sites are compared to one another, commonality among the group is a reserved WATC region (Figure 3.3) and the mismatches from the *E. coli* site when compared amongst the identified sites appear to be random in localization.

3.1.2 Computational bioinformatic pattern search for RpoS binding sites in the upstream promoter regions of *ihfα* and *ihfβ* in *L. pneumophila*

In *E. coli*, the stationary sigma factor RpoS plays a role in the regulation of IHF expression as shown by reduced *himA* and *himD* promoter activities in an *rpoS* mutant strain (Aviv *et al.*, 1994). Identification of possible RpoS binding sites indicate one location upstream each of *ihfα* and *ihfβ* (Figure 3.1).

3.2 Creation of *ihfα* and *ihfβ* truncated promoter plasmid constructs

To determine whether the putatively identified sites located within the *ihfα* and *ihfβ* promoter regions are involved in expression of LpIHF (i.e. autoregulation) and if RpoS plays a role in LpIHF expression, the functionality of the sites in binding LpIHF were assessed *in vivo* by green fluorescent protein (GFP) reporter assays and *in vitro* by electrophoretic mobility shift assays (EMSA). Thus, truncated promoter regions of the *ihfα* and *ihfβ* promoter regions were PCR-amplified for ligation into the promoterless GFP reporter plasmid pBH6119 and for employment in EMSAs.

Forward primers were designed to create PCR amplicons of decreasing fragment size from the promoter region upstream of each gene with one reverse primer anchored

Figure 3.2: Putative LpIHF binding sites. Comparison of partially matched IHF binding sites identified within the *ihf α* and *ihf β* promoter regions with the *E. coli* consensus sequence WATCAANNNTTR (W = A/T, R = A/G). Colons represent similarity between sequences.

Ihfa site one:

AATCAATCCGACT
::::::::::::
WATCAANNNTTR

Ihfa site two:

TATCCACAAATAA
:::: : :::: :
WATCAANNNTTR

Ihfa site three:

AATCAGAAATACA
:::: : ::: :
WATCAANNNTTR

Ihfa site four:

AATCAAGAACTG
:::::::::: ::
WATCAANNNTTR

Ihfb site one:

TATCATCCAGTCG
:::: : :::: :
WATCAANNNTTR

Ihfb site two:

AATCAAATGGGAA
:::::::::: :
WATCAANNNTTR

Figure 3.3: Comparison of partially matched IHF binding sites identified within the *ihf α* and *ihf β* promoter regions. Colons represent the commonality among the group, the reserved ATC region.

Ihfa site one:

AATCAATCCGACT

Ihfa site two:

:::

TATCCACAAATAA

Ihfa site three:

:::

AATCAGAAATACA

Ihfa site four:

:::

AATCAAGAACTG

Ihfβ site one:

:::

TATCATCCAGTCG

Ihfβ site two:

:::

AATCAAATGGGAA

just within the beginning of the gene coding sequence. Primers $\alpha 1$ (*ihfa* P1) through $\alpha 8$ (*ihfa* P8) were used to create eight truncated constructs of the *ihfa* promoter region, and primers $\beta 1$ (*ihfb* P1) through $\beta 7$ (*ihfb* P7) were used to create seven truncated constructs of *ihfb* promoter region (Figure 3.4) (see materials and methods; Table 2.3). Restriction sites for BamHI and XbaI were engineered into the 5' and 3' ends of the amplicon fragment to facilitate directional cloning into pBH6119 (Figure 3.5 and Figure 3.6).

For each primer set a gradient PCR was performed using the Taq polymerase enzyme to determine the optimal annealing temperature for implementation with PhusionTM polymerase to obtain the final product (see materials and methods). Subsequent agarose gel electrophoresis analysis of the product along with the appropriate negative control (i.e. no inclusion of DNA in the PCR reaction) confirmed specificity of the amplification as DNA bands were not visualized in the negative control lane (see materials and methods). The amplicon $\beta 3$ is shown in Figure 3.7 as a representation of a gradient PCR trial. Based on brightness and clarity of the band, the optimal annealing temperature was concluded to be 66.0°C. Figure 3.8 depicts a PhusionTM PCR of $\beta 3$. The approximate band size of 303 bp is confirmed and based on the negative control being blank verifies that this is a sample which does not contain contamination.

After purification with the Qiagen Gel Extraction kit, desired PCR products representing the truncated promoter regions of *ihfa* and *ihfb*, as well as mini-prepped vector pBH6119 were subjected to double restriction endonuclease digestion (see material and methods; Table 2.4). The digested pBH6119 vector was also treated with calf-alkaline phosphatase to prevent re-ligation of the vector ends (Table 2.2), and subjected to agarose gel electrophoresis for linearized band extraction. Both digested

Figure 3.4: Promoter regions of *ihf α* and *ihf β* . In bold with directional arrows are the forward primer binding regions $\alpha 1 - \alpha 8$ and $\beta 1 - \beta 7$. The numbers identify the position to which the start of each region begins relative to the start codon. In orange font is the IHF 13-bp consensus sequence WATCAANNNTTR (W = A/T, R = A/G). Underlined are the locations of the RpoS consensus sequence. The starts of the *ihf α* and *ihf β* gene sequences are represented in green and blue font, respectively.

ihfa:

(-448) α_1 →
CGCTTCACCCCGGCCAGTCAGCTCAGATTGTGATTAACGGCAAGCATTTCAGGCTGGATCGGTGTG
(-377) α_2 →
CTGCATCCTCGCTTGTCGGATGCCTTTGATCTTGACCAGGATGTGGTTTTGTTTGAGTTGAATCT
(-312) α_3 →
TGAATCATT**AATCAATCCGACT**ATTCCTCTGTATAAGCCTATTTCCAAAT**TATCCACAAATAAGGC**
(-248) α_4 → (-211) α_5 →
GCGATTTGTCTTTCTG**GTTGATAGGCAGATAAGTGCCA**TGCAGATTGAGCGAGT**AATCAGAAAT**
(-160) α_6 →
ACAGTTAAAGAAGGATTGGTTAAATCGTTTGACGCTTT**GACGTGTATATGGGTAAAGGTA**TTCC
(-105) α_7 →
AGAAGATAAAAAAGTATTGCTGTGGCTAT**GACACTACAAGACGATACTCGA**ACTTTAGTTGATG
(-49) α_8 →
CCGAGATCAATTTAACAATTA**GTGCTATAATCAAGAACTGGA**AAATGAATTTTCAATCCTTTTG
PR (+21) ←
AGGGAAT**GATCGTGAACGCCTAAGCAAAGCAATAATGGCAGAAACGTT**...

ihf β :

(-473) β_1 →
GGCGACCTTAATATTTATTAATT**CAAGTACCTTATTCGGTGCA**ACCTGGGAAGCCCTTAACACCC
(-371) β_2 →
GTCTCAATAGCCTGCCACAACCTTT**CAACCGTCATTACTACTAGCA**AAACCT**TATCATCCAGTCGT**
(-280) β_3 →
TTTAAATTAGTTACTCGCGCTAAAATACAAATGAGTTGGGGTGTAGTTTT**CGCATTCATTCATAA**
(-210) β_4 →
GAAAGAAACTAATTTTTACTAACCAGCTTGAAAACATTGAATAATTTTTTACCC**GAACTATACT**
(-158) β_5 →
TATAA**AATCAAATGGGAA**ATTGTCAGCGCCTGATGTGTCTTG**GTAGCAGACATTAGGCCAGT**TGC
(-113) β_6 →
TCAGCTCGTGGAGTTTATTTT**GTATCGGCCACCCGTGTTTCA**AGATACAAAGCGAATAGAAACA
(-47) β_7 →
TAGGCAACCAAGCTAAAATAAT**GAGGATGCAATCGGTCTAT**TAAATACGCGACAAAACGAGGTG
PR (+23) ←
AGTATAT**GATTAAATCCGA**ACT**CATTGA**ACACATCGCTGCTCGAATGACGCATCT...

Figure 3.5: Ligated *ihf* α constructs within the promoterless GFP vector pBH6119.

Purple arrows 1 through 4 identify the putative IHF locations with each construct. The red arrow identifies the putative RpoS location.

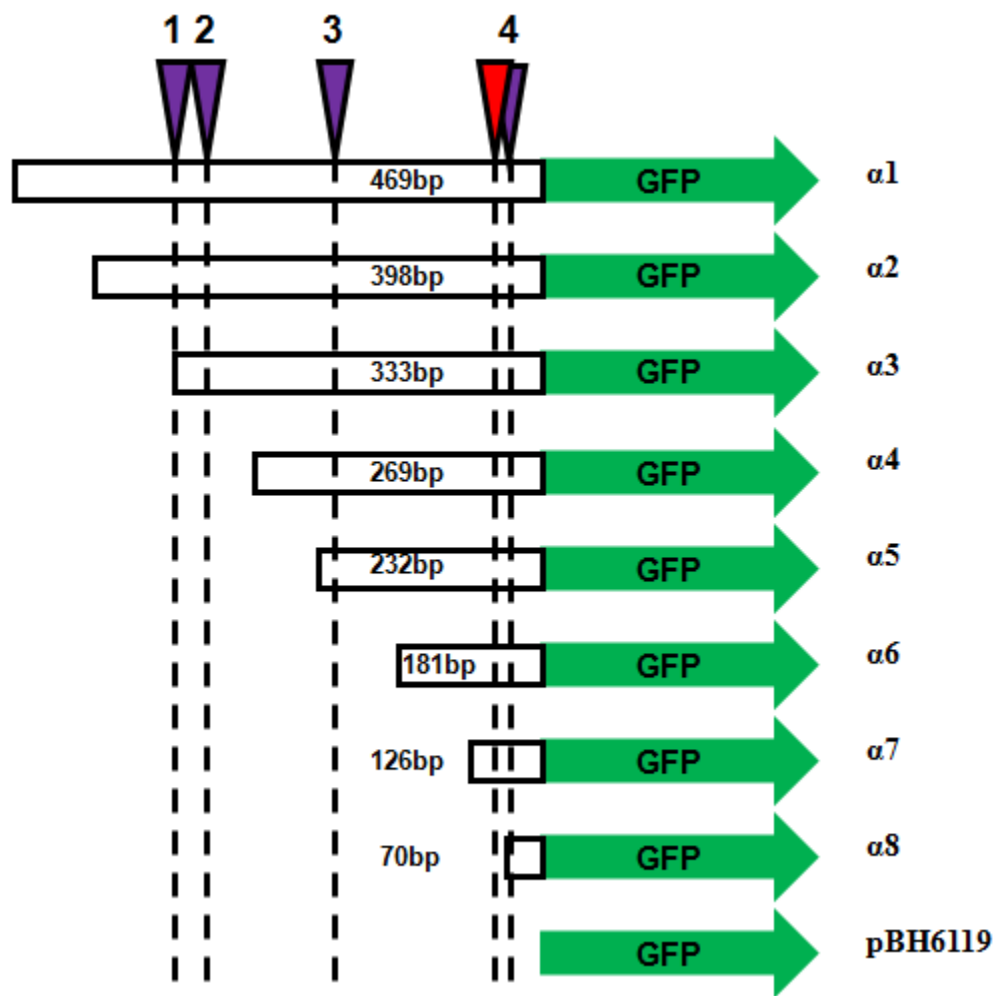


Figure 3.6: Ligated *ihf* β constructs within the promoterless GFP vector pBH6119.

Purple arrows 1 and 2 identify the putative IHF locations with each construct. The red arrow identifies the putative RpoS location.

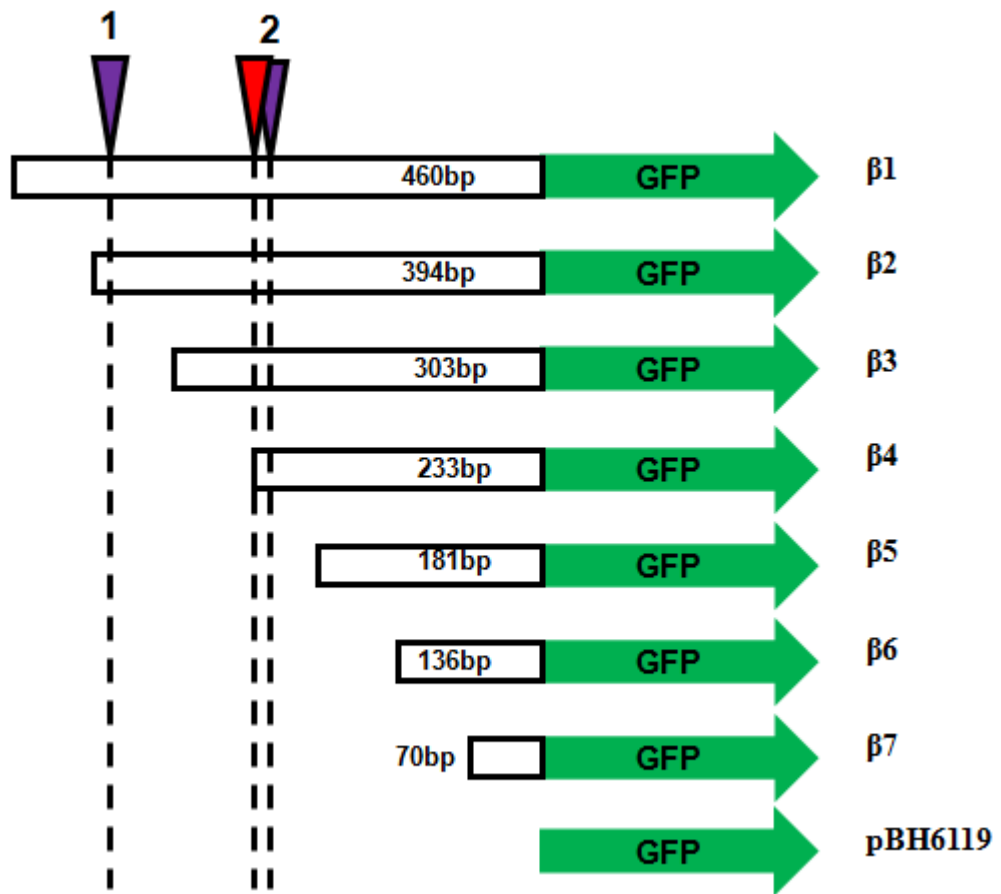


Figure 3.7: Gradient PCR of $\beta 3$. 5 μ L of the PCR reaction loaded into a 2% agarose gel and run against a 100-bp ladder. Letters A through H refer to specific temperature (A: 70°C, B: 68.5°C, C: 66.0°C, D: 62.2°C, E: 57.5°C, F: 53.9°C, G: 51.5°C, H: 50°C. The optimal annealing temperature was concluded to be 66.0°C. (Product size for $\beta 3$ is 303 bp).

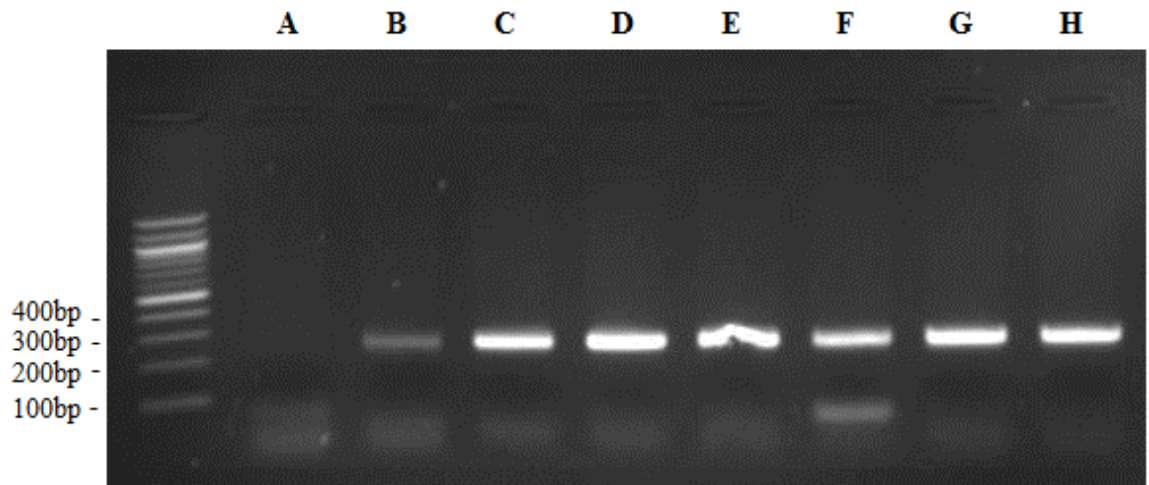
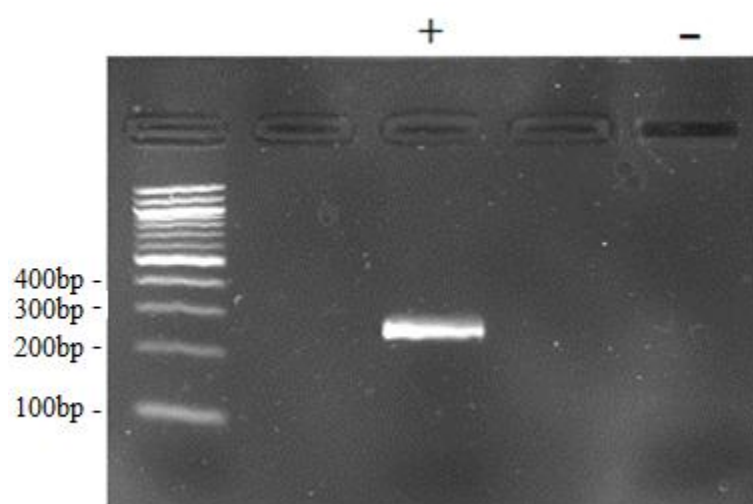


Figure 3.8: β 3 PhusionTM PCR. 5 μ L of the PCR reaction loaded into a 2% agarose gel and run against a 100-bp ladder. Symbols + and – refer to PCR product and negative control, respectively. (Product size for β 3 is 303 bp).



PCR products and vector pBH6119 were purified using the Qiagen Gel Extraction kit (see materials and methods). Each PCR product was then ligated into pBH6119 separately in order to create plasmid constructs pBH α 1, pBH α 2, pBH α 3, pBH α 4, pBH α 5, pBH α 6, pBH α 7, pBH α 8, pBH β 1, pBH β 2, pBH β 3, pBH β 4, pBH β 5, pBH β 6, and pBH β 7.

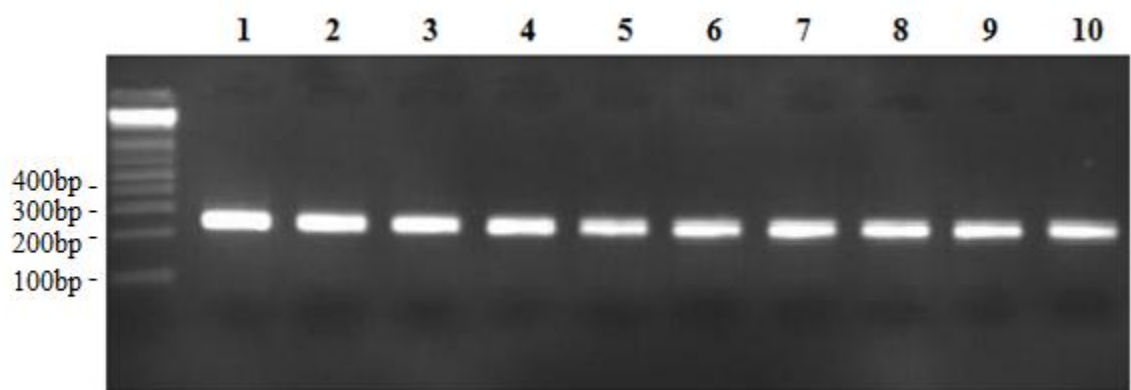
The resulting plasmids (Table 2.2) were transformed into competent *E.coli* DH5 α and were confirmed via pooled colony and single colony PCR screening (see materials and methods). The amplicon β 3 is shown in Figure 3.9 as a representation of a visualized single colony PCR on an agarose gel (where each lane represents an individual colony). Based on the presence of a band at the expected amplicon size 303 bp, it was observed that all 10 colonies screened contain pBH6119::ihf β P3. Therefore colony 1 was chosen to freeze and store for future use (see materials and methods).

All of the PCR confirmed plasmid constructs were then subjected to sequencing to verify the desired truncated promoter sequence (see materials and methods). The GFP primer Seq (GFP), which anneals from within the GFP coding sequence on pBH6119 (Table 2.3) was used to sequence the constructs.

3.3 Expression of GFP based on the regulation of the truncated promoter constructs

To determine if the putative IHF binding site(s) are functional in binding LpIHF, if LpRpoS plays a functional role in the expression of LpIHF and if together LpIHF and LpRpoS are the only contributing regulators to LpIHF expression, fluorescence levels of GFP expressed from truncated promoter plasmid constructs thereby correlating with promoter activity were monitored over time in Lp02 wild-type, Lp02 Δ ihfaihf β , Lp02

Figure 3.9: Individual colony PCR of $\beta 3$. 5 μ L of the PCR reaction loaded into a 2% agarose gel and run against a 100-bp ladder. Numbers refer to individual colonies numbers determined by the grid location of the struck out colonies on the master LB plate. (Product size for $\beta 3$ is 303 bp).



ΔrpoS, and Lp02 *ΔrpoSihfa* strains. Plasmid constructs pBH α 1, pBH α 2, pBH α 3, pBH α 4, pBH α 5, pBH α 6, pBH α 7, and pBH α 8 (Table 2.2) representing the *ihfa* promoter region were electroporated into wild-type Lp02 creating strains α 1 – α 8, into Lp02 *Δihfaihfb* creating strains α 1- Δ ihf – α 8- Δ ihf, into Lp02 *ΔrpoS* creating strains α 1- Δ rpoS – α 8- Δ rpoS, and into Lp02 *ΔrpoSihfa* creating strains α 1- Δ rpoSihfa – α 8- Δ rpoSihfa (Table 2.1). Plasmid constructs pBH β 1, pBH β 2, pBH β 3, pBH β 4, pBH β 5, pBH β 6, and pBH β 7 (Table 2.2) were electroporated into wild-type Lp02 creating strains β 1 – β 7, into Lp02 *ihfaihfb* creating strains β 1- Δ ihf – β 7- Δ ihf, into Lp02 *ΔrpoS* creating strains β 1- Δ rpoS – β 7- Δ rpoS, and into Lp02 *ΔrpoSihfa* creating strains β 1- Δ rpoSihfa – β 7- Δ rpoSihfa (Table 2.1). As Lp02 is auxotrophic for thymidine and pBH6119 carries the gene encoding thymidylate synthetase, verification of plasmid uptake was confirmed via selection on BCYE plates lacking thymidine.

3.3.1 Expression profiles of *ihfa* and *ihfb* in Lp02 wild-type and *Δihfaihfb*

An initial set of assays was completed for α 1 – α 8, β 1 – β 7, α 1- Δ ihf – α 8- Δ ihf, β 1- Δ ihf – β 7- Δ ihf, pBH6119-wt and pBH6119- Δ ihf (see materials and methods). In the wild-type Lp02 strain, promoter activities as indicated by normalized (to OD) fluorescence levels were similar in level for seven (α 1 – α 7) truncated *ihfa* promoter constructs (Figure 3.10). For the eighth promoter construct, a loss of promoter activity as indicated by decreased fluorescence was evident when the *ihfa* promoter was truncated to a region of 70bp (α 8) suggesting a functional role of one or a combination of the three putative IHF sites located upstream of the 70 bp (α 8) region (Figure 3.4). The 70 bp (α 8)

truncated *ihf α* promoter region still exhibited fluorescence above background which may be attributed to inadequate binding of IHF to a putative site located just inside of the 5' end of the 70 bp (α 8) region (Figure 3.4). In regards to the *ihf β* promoter region, promoter activity levels via normalized fluorescence levels for three (β 1 – β 3) were overall similar to one another (despite the abnormal reading at the 2 h timepoint for β 3) with a drastic drop to background level as depicted by the vector control for the remaining four promoter constructs (Fig. 3.10). In the mutant Lp02 Δ *ihf α ihf β* strain background, the promoter activities of the truncated *ihf α* and *ihf β* promoter constructs were remarkably similar (Figures 3.11 and 3.13) when compared to those observed in the wild-type Lp02 background (Figures 3.10 and 3.12). These surprisingly similar results in wild-type and mutant backgrounds may have stemmed from the parameters of the fluorescence assay conducted which are as follows: 1) the number of transformant strains assessed for fluorescence levels is restricted to four or five at one time; 2) the period of time necessary to prepare the samples for the single-unit fluorometer instrument which is approximately 20 min from the time of sampling; 3) the variability in optical densities and resultant fluorescence levels in samples between strains and sampling timepoints; and 4) the manual nature of the single-unit fluorometer restricts the number of hours in the testing period of time. Thus, these factors may have contributed to the inaccuracies associated with the *ihf α* and *ihf β* expression profiles and therefore, an automated microplate reader equipped with a heater and shaker was opted to be employed in the subsequent fluorescent assays.

Figure 3.10: Fluorescence detected from *L. pneumophila* Lp02 containing vector control pBH6119 and *P_{ihf α}* promoter DNA-GFP constructs. Readings were taken every two hours for an eight hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data is representative of one experiment.

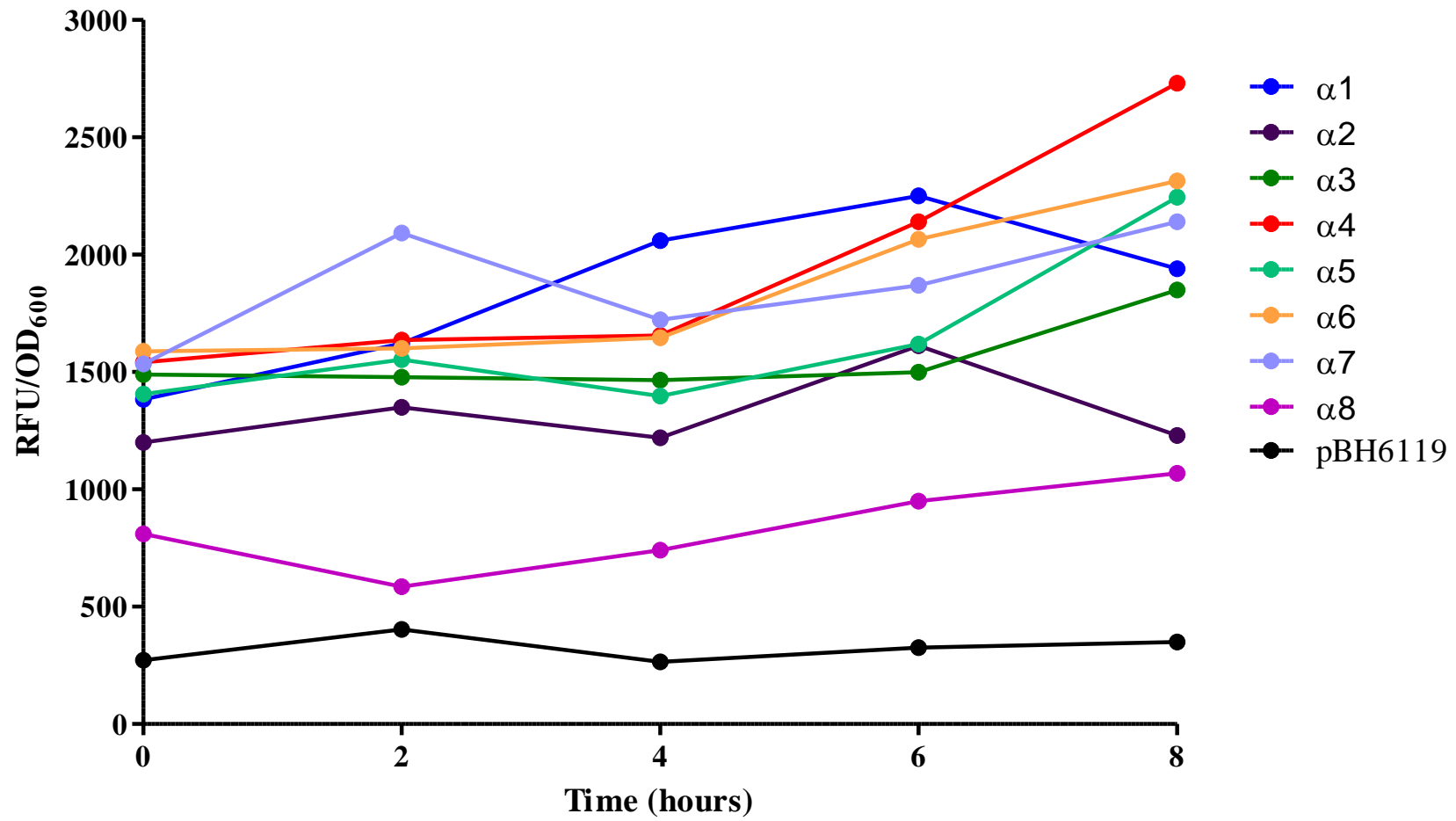


Figure 3.11: Fluorescence detected from *L. pneumophila* Lp02 $\Delta ihfaihf\beta$ containing vector control pBH6119 and $P_{ihf\alpha}$ promoter DNA-GFP constructs. Readings were taken every two hours for an eight hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data is representative of one experiment.

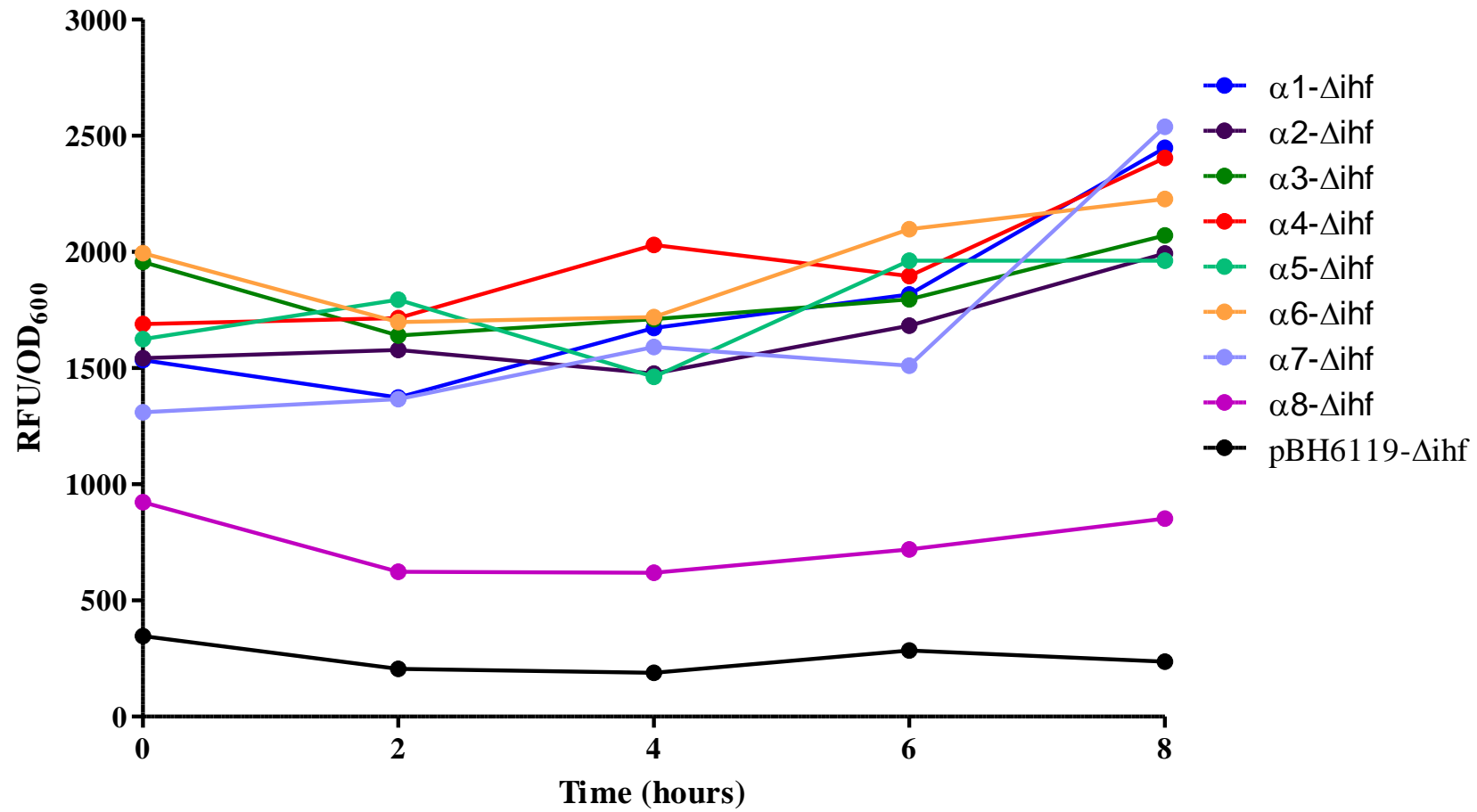


Figure 3.12: Fluorescence detected from *L. pneumophila* Lp02 containing vector control pBH6119 and $P_{ihf\beta}$ promoter DNA-GFP constructs. Readings were taken every two hours for an eight hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data is representative of one experiment.

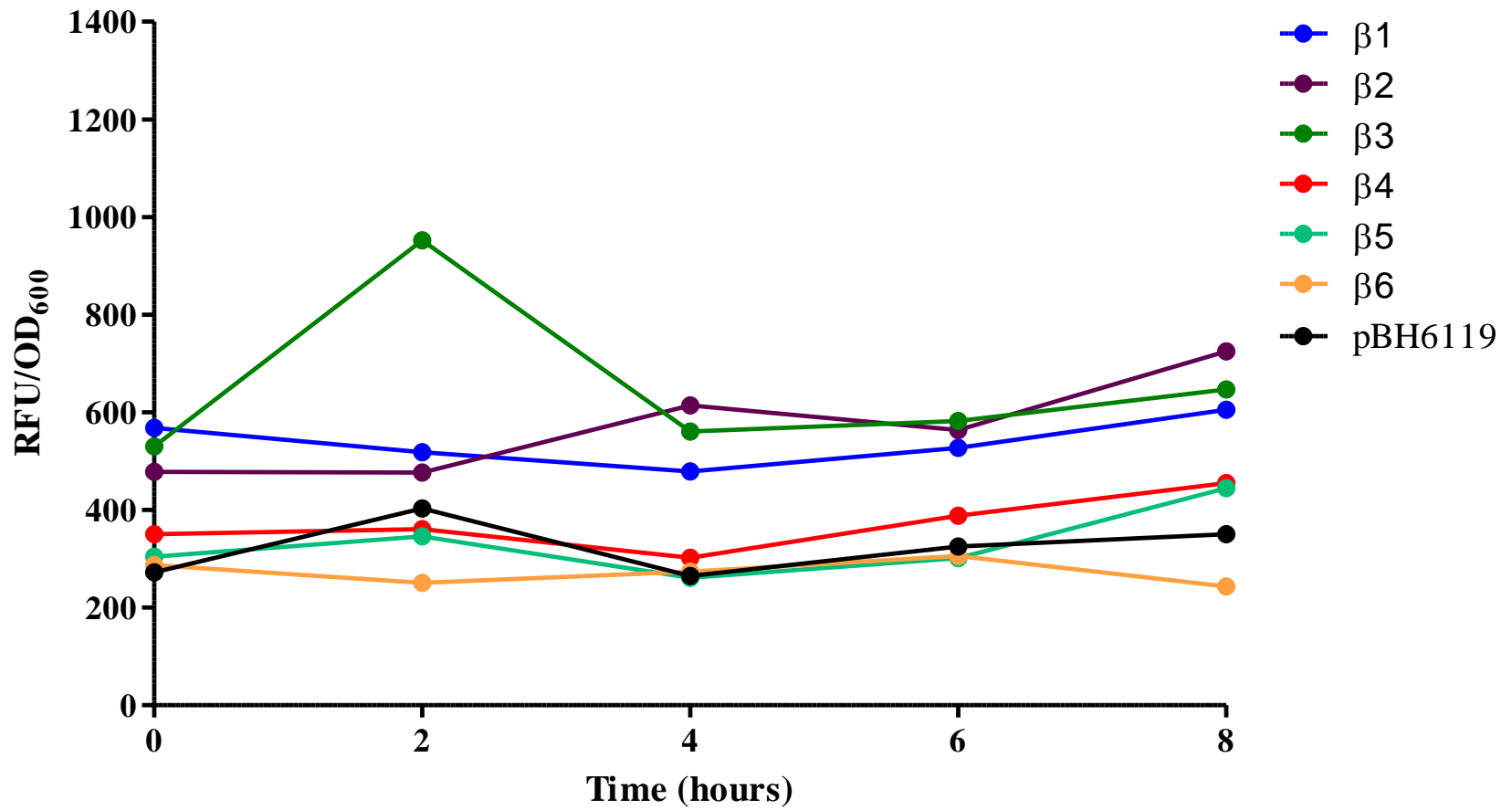
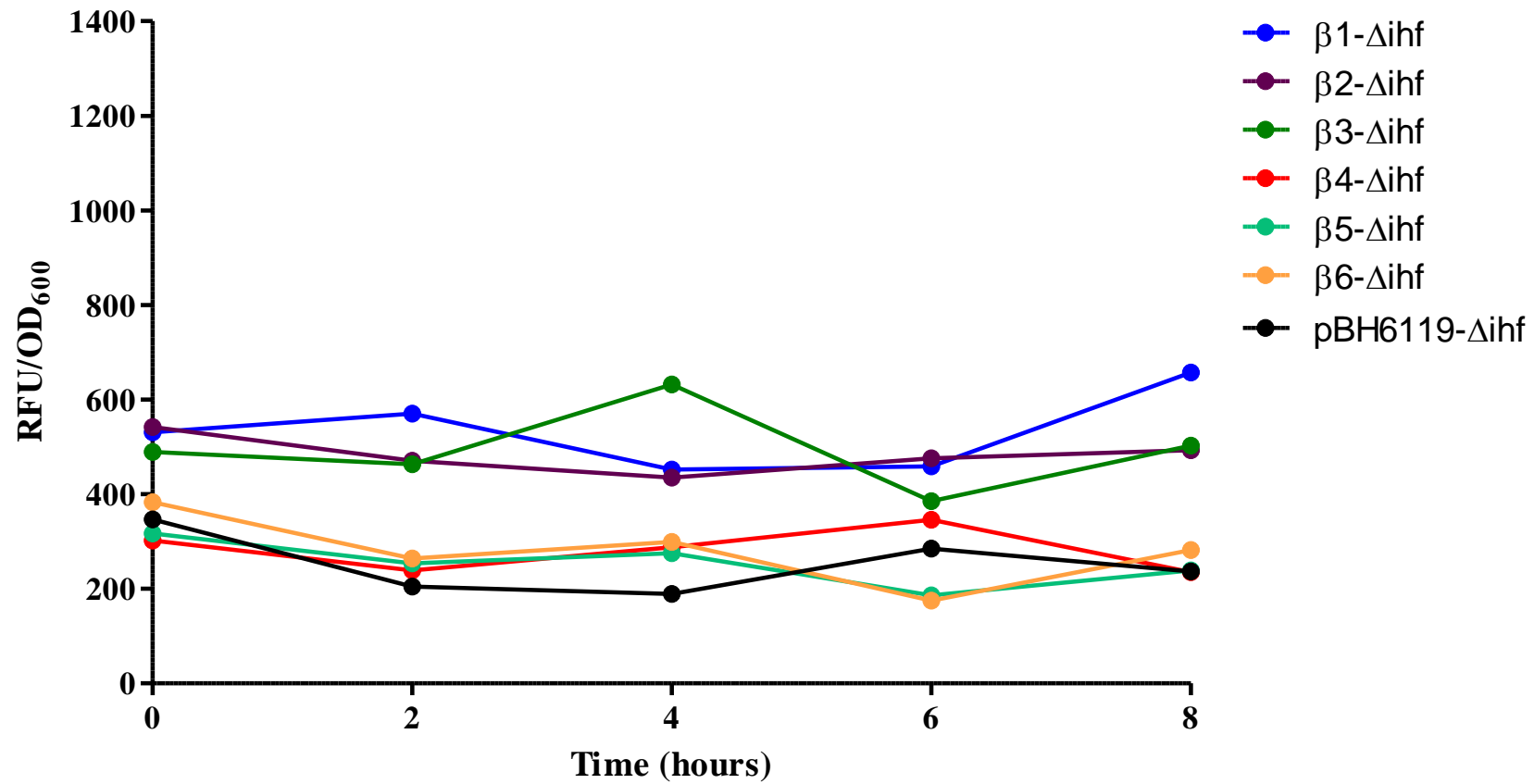


Figure 3.13: Fluorescence detected from *L. pneumophila* Lp02 $\Delta ihfaihf\beta$ containing vector control pBH6119 and $P_{ihf\beta}$ promoter DNA-GFP constructs. Readings were taken every two hours for an eight hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data is representative of one experiment.



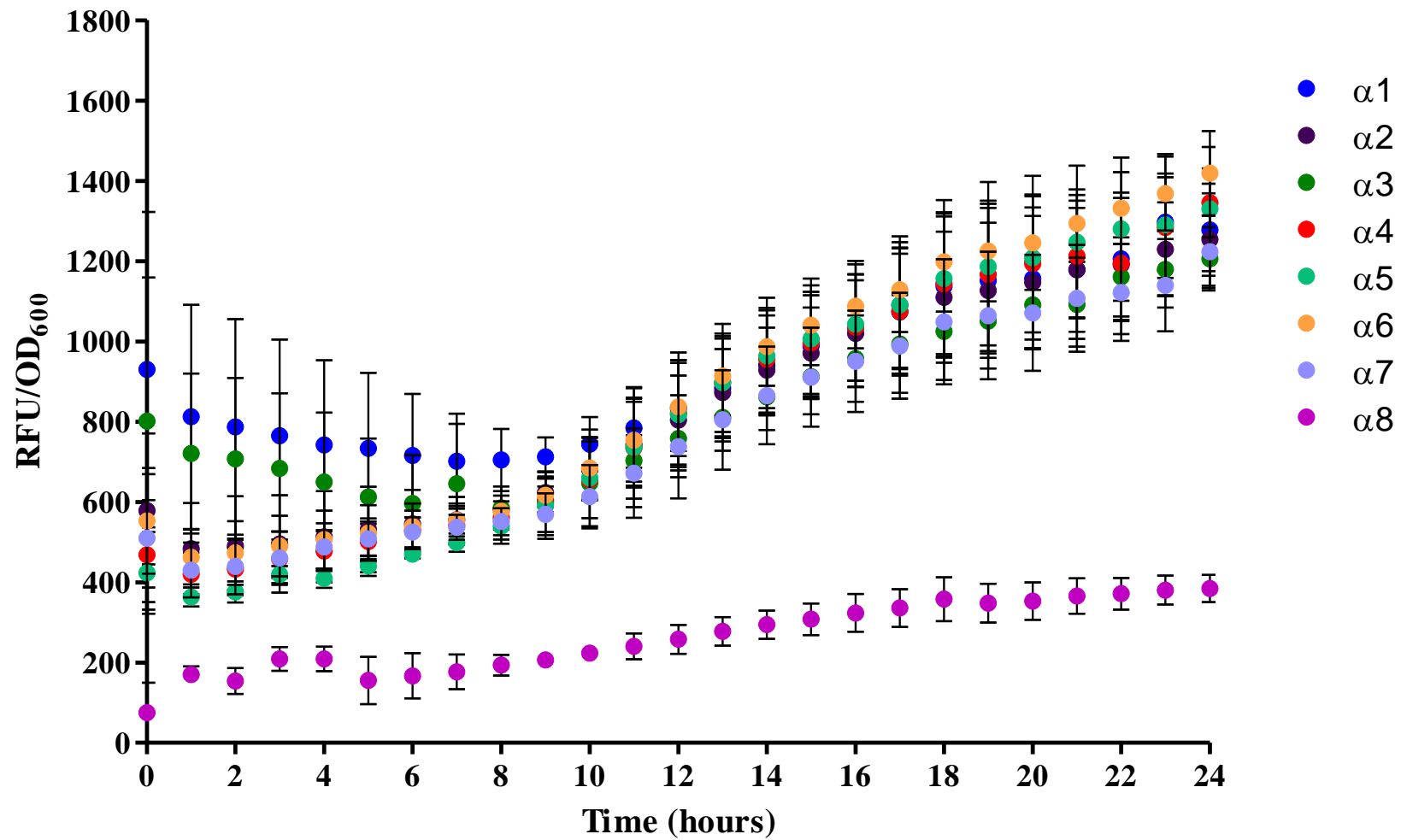
3.3.2 Microplate expression profiles of *ihfa* and *ihfβ* in Lp02 wild-type, $\Delta ihfaihf\beta$, $\Delta rpoS$ and $\Delta rpoSihfa$

In general, growth curves generated from the 96-well microplate fluorometer assays conducted over a 24 h period with sampling performed on an hourly basis revealed that the exponential and post-exponential growth phases initiated at hours 9 and 18, respectively (data not shown). To assess the expression profiles of the truncated *ihfa* promoter constructs plate-grown strains (wt, $\Delta ihfaihf\beta$, $\Delta rpoS$, $\Delta rpoSihfa$) were normalized to OD₆₀₀ 0.2 in BYE broth and aliquoted in 150 μ l into a 96-well format microplate specialized for fluorometry applications (see materials and methods). Optical density and fluorescence data were normalized to the vector control, and expressed as a ratio of relative fluorescence units (RFU) over optical density.

3.3.2.1 Microplate expression profile of *ihfa* in Lp02 wild-type

The expression profiles of constructs $\alpha 1$ through $\alpha 7$ are comparable although $\alpha 1$ and $\alpha 3$ appeared to have higher levels in comparison to the levels attained by the cells expressing the other constructs ($\alpha 2$, $\alpha 4 - \alpha 7$) (Figure 3.14). However, this difference falls within the range of the standard deviation of error (represented by error bars) and therefore is not considered to be a significant difference. In comparison to the other *ihfa* promoter constructs, a much lower expression level was observed for the $\alpha 8$ promoter construct. These results indicate that the regulatory sites responsible for the transcriptional activation of the *ihfa* gene appears to be restricted to the region between -448bp and -49bp of the upstream promoter region with perhaps one additional site (or basal site)

Figure 3.14: Fluorescence detected from *L. pneumophila* Lp02 containing P_{ihfa} promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.



bordering -49 bp as fluorescence was observed with the $\alpha 8$ promoter construct (Figures 3.5).

3.3.2.2 Microplate expression profile of *ihfA* in Lp02 $\Delta ihfA ihfB$

The expression profiles of $\alpha 1$ - Δihf through $\alpha 7$ - Δihf (Figure 3.15) are comparable to those observed with $\alpha 1$ through $\alpha 7$ (Fig. 3.14). A small difference is observed between the two profiles of approximately 200 RFU/OD_{600S} but this difference lies within the standard error and therefore may not be significant. However, the expression levels between the two strains $\alpha 8$ and $\alpha 8$ - Δihf are somewhat interesting. From hours 0 to 9, $\alpha 8$ - Δihf differs from $\alpha 8$ as it is observed to have a level of expression comparable to $\alpha 1$ - Δihf – $\alpha 7$ - Δihf and $\alpha 8$ expression is much lower than $\alpha 1$ – $\alpha 7$. From hours 9 through 24 the $\alpha 8$ - Δihf and $\alpha 8$ levels are comparable. These data suggest the possibility of LpIHF negative feedback where high enough concentrations of LpIHF may bind and inhibit LpIHF expression with the possible binding site located within the -49 bp to 0 bp region.

3.3.2.3 Microplate expression profile of of *ihfA* in Lp02 $\Delta rpoS$

Comparison of the expression profiles of $\alpha 1$ – $\alpha 8$ (Figure 3.14) with $\alpha 1$ - $\Delta rpoS$ – $\alpha 8$ - $\Delta rpoS$ (Figure 3.16) indicates that the $\alpha 1$ - $\Delta rpoS$ – $\alpha 7$ - $\Delta rpoS$ overall have significantly lower levels of GFP expression, with the exception of overlap seen during exponential phase. These results suggest that RpoS may act as a positive transcriptional activator of *ihfA*. No observable difference was observed between $\alpha 8$ and $\alpha 8$ - $\Delta rpoS$.

Figure 3.15: Fluorescence detected from *L. pneumophila* Lp02 $\Delta ihfAihfB$ containing *P_{ihfA}* promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.

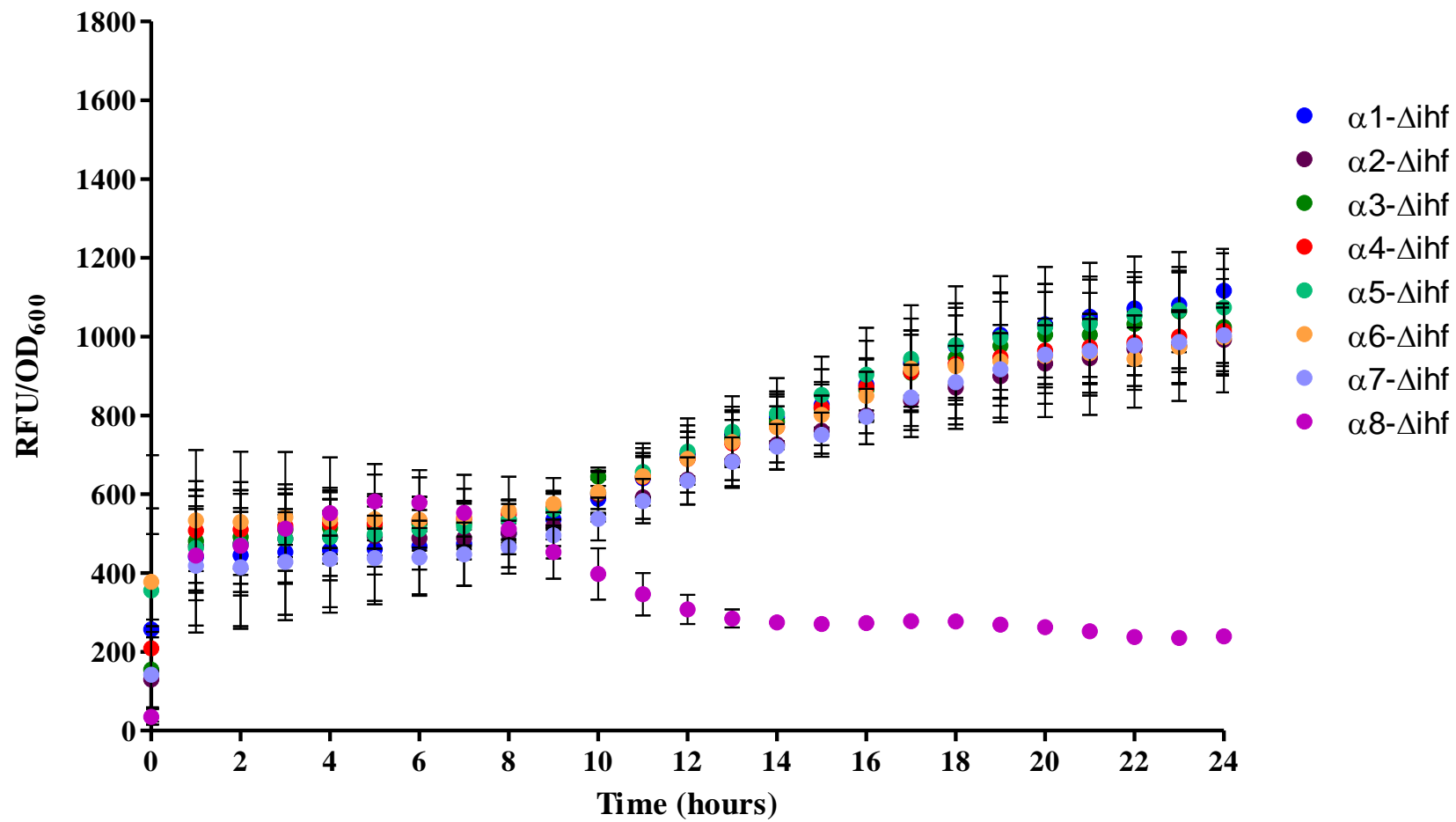
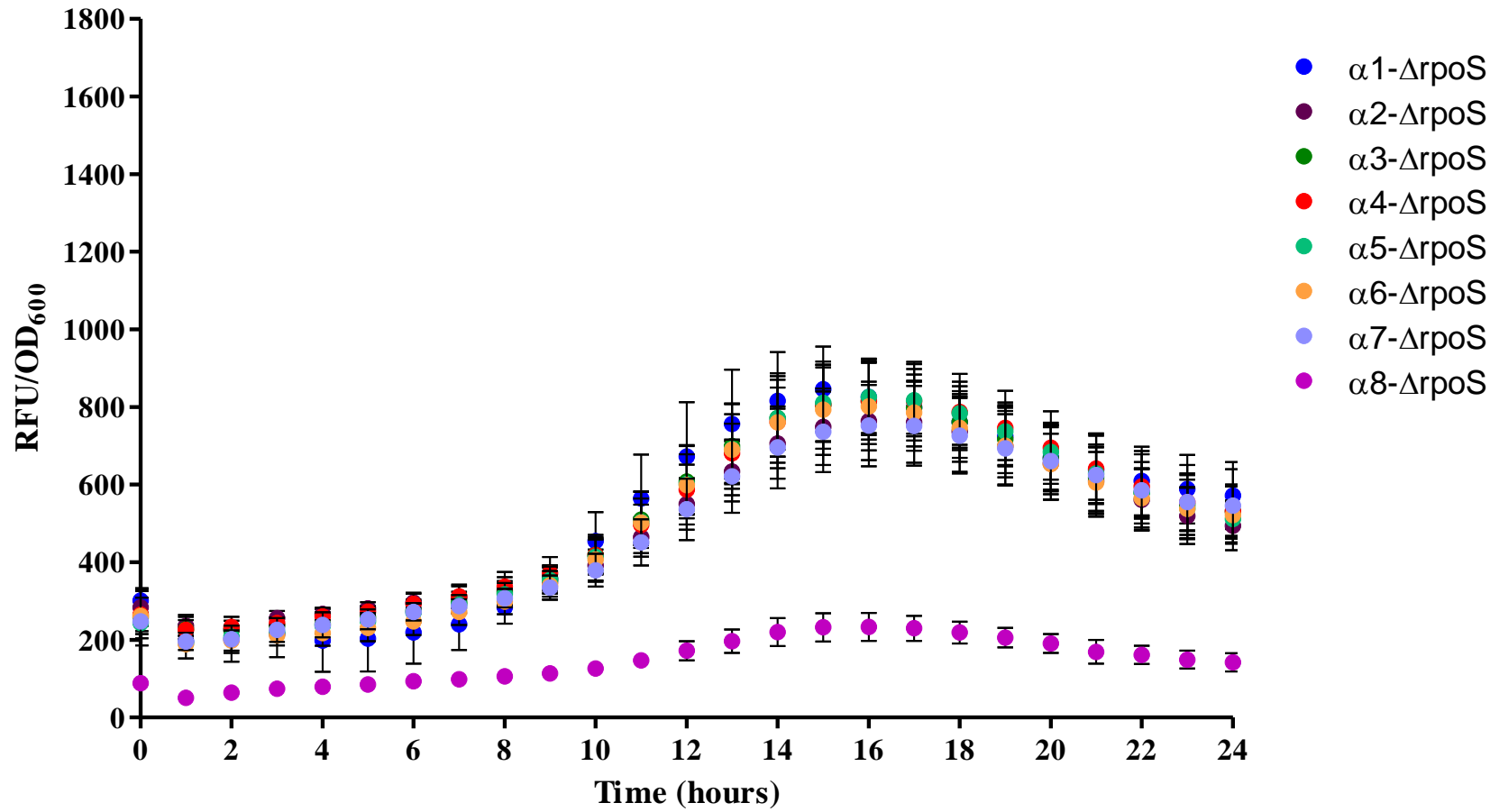


Figure 3.16: Fluorescence detected from *L. pneumophila* Lp02 $\Delta rpoS$ containing *P_{ihf α}* promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.



3.3.2.4 Microplate expression profile of *ihf α* in Lp02 Δ rpoSihf α

In order to ascertain whether LpRpoS and LpIHF are the sole regulatory factors responsible for the regulation of *ihf α* expression, a triple gene deletion in Lp02 (Δ *ihf α ihf β rpoS*) would need to be created. However, due to a limited number of antibiotic cassettes available, the double gene deletion Lp02 Δ *ihf α rpoS* was used on the assumption that Ihf β does not act as a homodimer on the regulation of *ihf α* . This assumption is based on studies which revealed that Ihf β homodimers did not bind with great affinity to the *magA* promoter region as experimentally determined by Morash *et al.* (2009). Thus on this basis, the double gene deletion was used in place of a triple gene knockout to promoter activities of *ihf α* and *ihf β* in the absence of functional RpoS and IHF. The expression profiles of α 1- Δ rpoS – α 8- Δ rpoSihf α (Figure 3.17) appear to be significantly lower than α 1 – α 8 (Figure 3.14). In addition, when comparing the expression profiles of α 1- Δ rpoSihf α – α 8- Δ rpoSihf α with α 1- Δ rpoS – α 8- Δ rpoS (figure 3.16), at first glance the overall shape of the curves differs somewhat; however there is no significant difference between the two. Based on the assumption that Ihf β homodimers do not actively functionally bind to the promoter region and regulate expression of *ihf α* , these results suggest that the overlap observed between α 1 – α 7 and α 1- Δ rpoS – α 7- Δ rpoS during exponential phase cannot be attributed to LpIHF.

3.3.2.5 Microplate expression profile of *ihf β* in Lp02 wild-type

The expression profiles for β 1 through β 7 indicate some variation in fluorescence between the truncated promoter constructs (Figure 3.18). Fluorescence was observed for constructs β 1 – β 3 commencing during exponential phase (10 h) with a gradual

Figure 3.17: Fluorescence detected from *L. pneumophila* Lp02 Δ rhoSihf α containing *P*_{ihf α} promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.

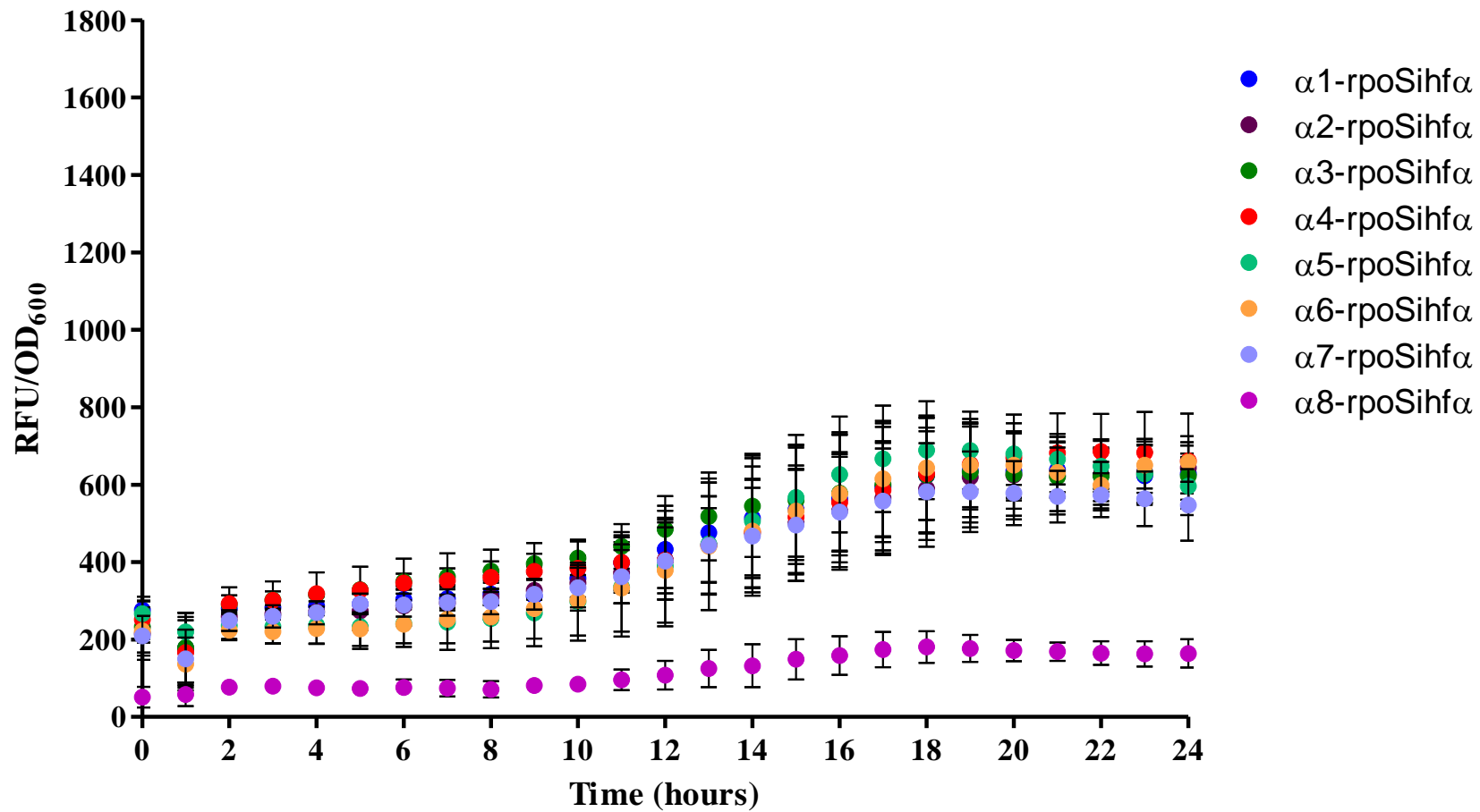
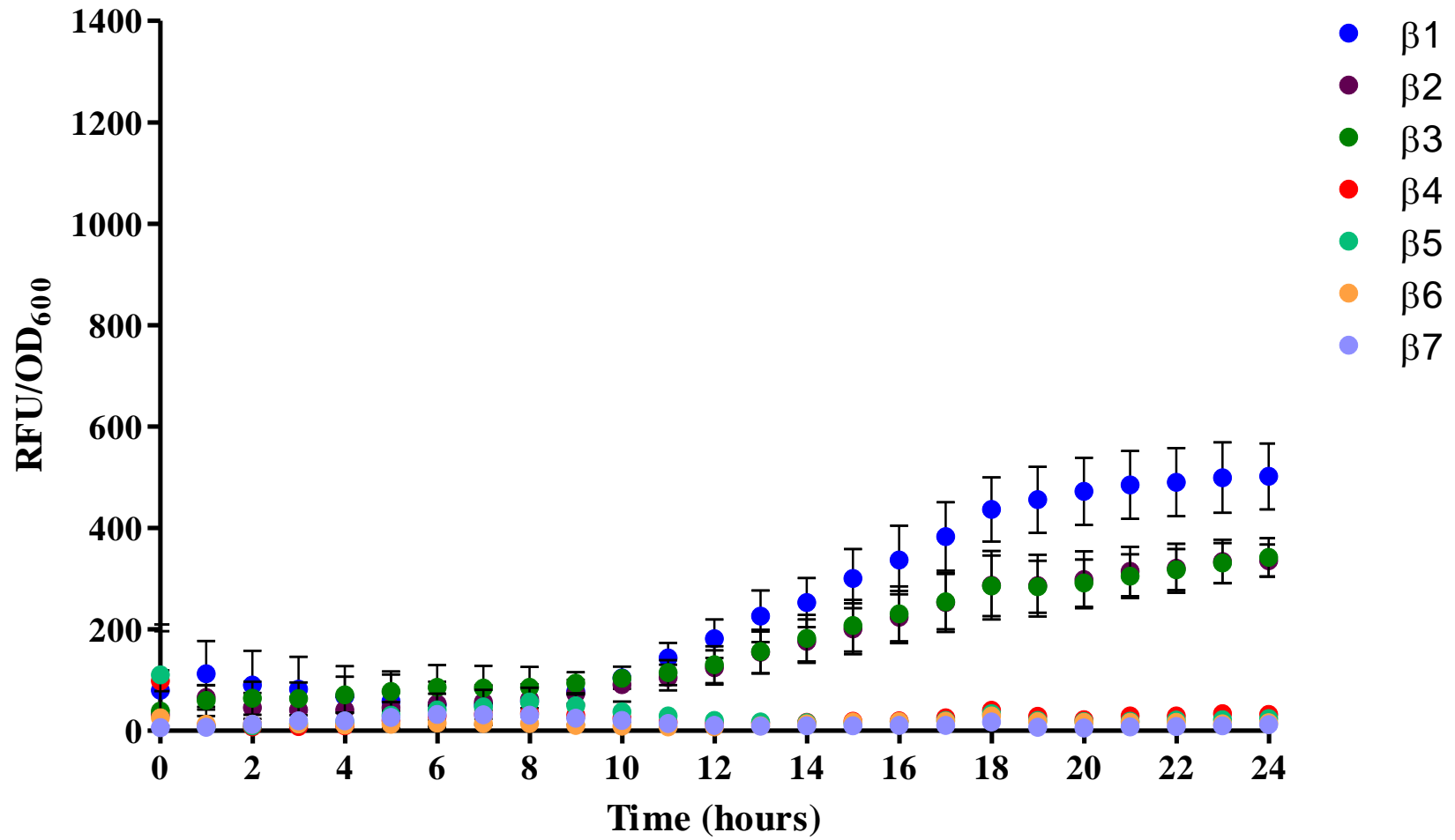


Figure 3.18: Fluorescence detected from *L. pneumophila* Lp02 containing P_{ihff} promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.

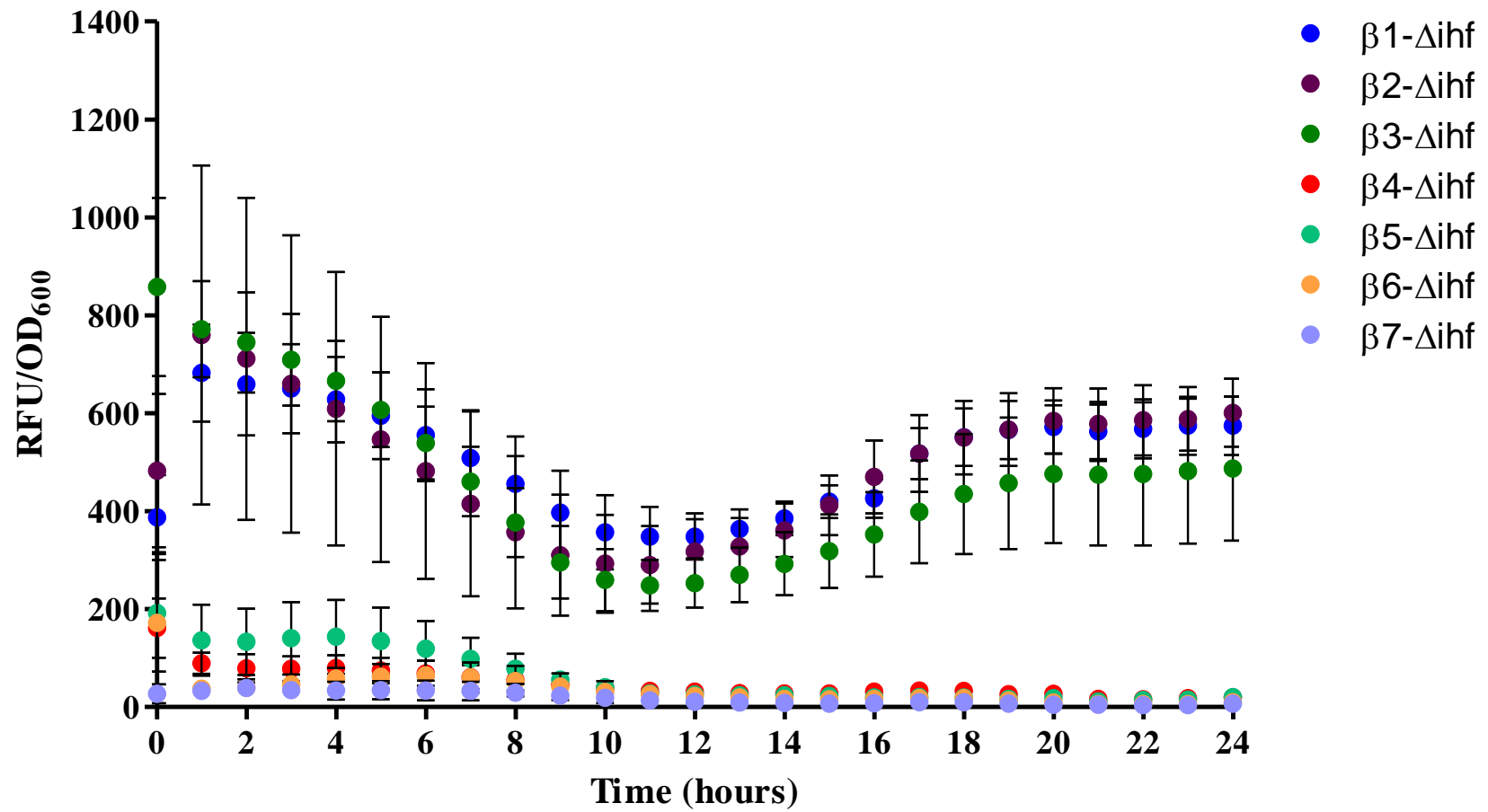


incremental increase through the transition into post-exponential phase; construct $\beta 1$ appears to produce the highest level of fluorescence with slightly reduced fluorescence levels observed from constructs $\beta 2$ and $\beta 3$. Constructs $\beta 4 - \beta 7$ exhibited minimal to no fluorescence. Thus, it would appear that regulatory site(s) that have a functional role in activating transcription of *ihf β* are restricted to the promoter region between -437bp and -210bp as suggested by the $\beta 1 - \beta 3$ constructs (Figure 3.4).

3.3.2.6 Microplate expression profile of *ihf β* in Lp02 $\Delta ihfaihf\beta$

Similarly to the fluorescence levels of cells with constructs $\beta 4 - \beta 7$ (Figure 3.18), minimal to no fluorescence was observed for constructs $\beta 4-\Delta ihf - \beta 7-\Delta ihf$ (Figure 3.19). However, the expression profiles of $\beta 1-\Delta ihf - \beta 3-\Delta ihf$ (Figure 3.19) were quite different from those attained for $\beta 1 - \beta 3$ (Figure 3.18). Specifically, high levels of fluorescence were initially observed with gradual decrease in fluorescence for these constructs in the time period correlating to the lag growth phase (hours 0-8) in which there is an absence of cellular LpIHF, which is otherwise prevalent in post-exponential and stationary phase in the Lp02 wild-type strain. This suggests that LpIHF may act as a negative transcriptional regulator of *ihf β* . Upon transition into exponential and subsequently post-exponential growth phases, the fluorescence levels increased once again to levels mimicking the levels observed with in $\beta 1 - \beta 3$ cells albeit a bit higher than $\beta 1-\Delta ihf - \beta 3-\Delta ihf$. The vast standard deviation range (denoted by error bars) of normalized fluorescence readings during lag growth phase may be due to the fact that stationary phase bacteria are harvested from culture plates after a 3 - 4 day incubation leading to variability in regulatory protein levels (Figure 3.19). Regardless, regulatory sites

Figure 3.19: Fluorescence detected from *L. pneumophila* Lp02 $\Delta ihfAihfB$ containing P_{ihfB} promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.



responsible for the transcription of *ihfβ* appear to be restricted to the promoter region (-437 bp to -210 bp) as defined by truncated promoter constructs β1 – β3.

3.3.2.7 Microplate expression profile of *ihfβ* in Lp02 *ArpoS*

The normalized fluorescence levels of β1-Δ*rpoS* – β3-Δ*rpoS* (Figure 3.20) were drastically reduced in comparison to levels attained during the post-exponential and exponential growth phases with β1 – β3 (Figure 3.18) and β1-Δ*ihf* – β3-Δ*ihf* (Figure 3.19), respectively, suggesting that LpRpoS may act as a positive transcriptional activator of *ihfβ*. No fluorescence was observed for β4-Δ*rpoS* – β7-Δ*rpoS*. The very low fluorescence levels were observed for β1-Δ*rpoS* – β3-Δ*rpoS* during exponential and post-exponential growth phases (hours 11 – 24) suggesting the involvement of perhaps another minor regulatory factor in the transcriptional activation of *ihfβ* (Figure 3.20).

3.3.2.8 Microplate expression profile of *ihfβ* in Lp02 *ArpoSihfa*

The expression profiles of the *ihfβ* promoter constructs in the Lp02 *ΔrpoSihfa* mutant strain background (in which cellular RpoS is absent and IHF is presumably non-functional due to the absence of the α subunit) are similar to those observed for the constructs in the Lp02 *ΔrpoS* mutant strain background (Figure 3.21). Minimal expression of β1-Δ*rpoSihfa* – β3-Δ*rpoSihfa* constructs in the Lp02 *ΔrpoSihfa* mutant strain background is noted suggesting the involvement of another minor regulatory factor in the transcriptional activation of *ihfβ*.

Figure 3.20: Fluorescence detected from *L. pneumophila* Lp02 *ArpoS* containing *P_{ihfβ}* promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.

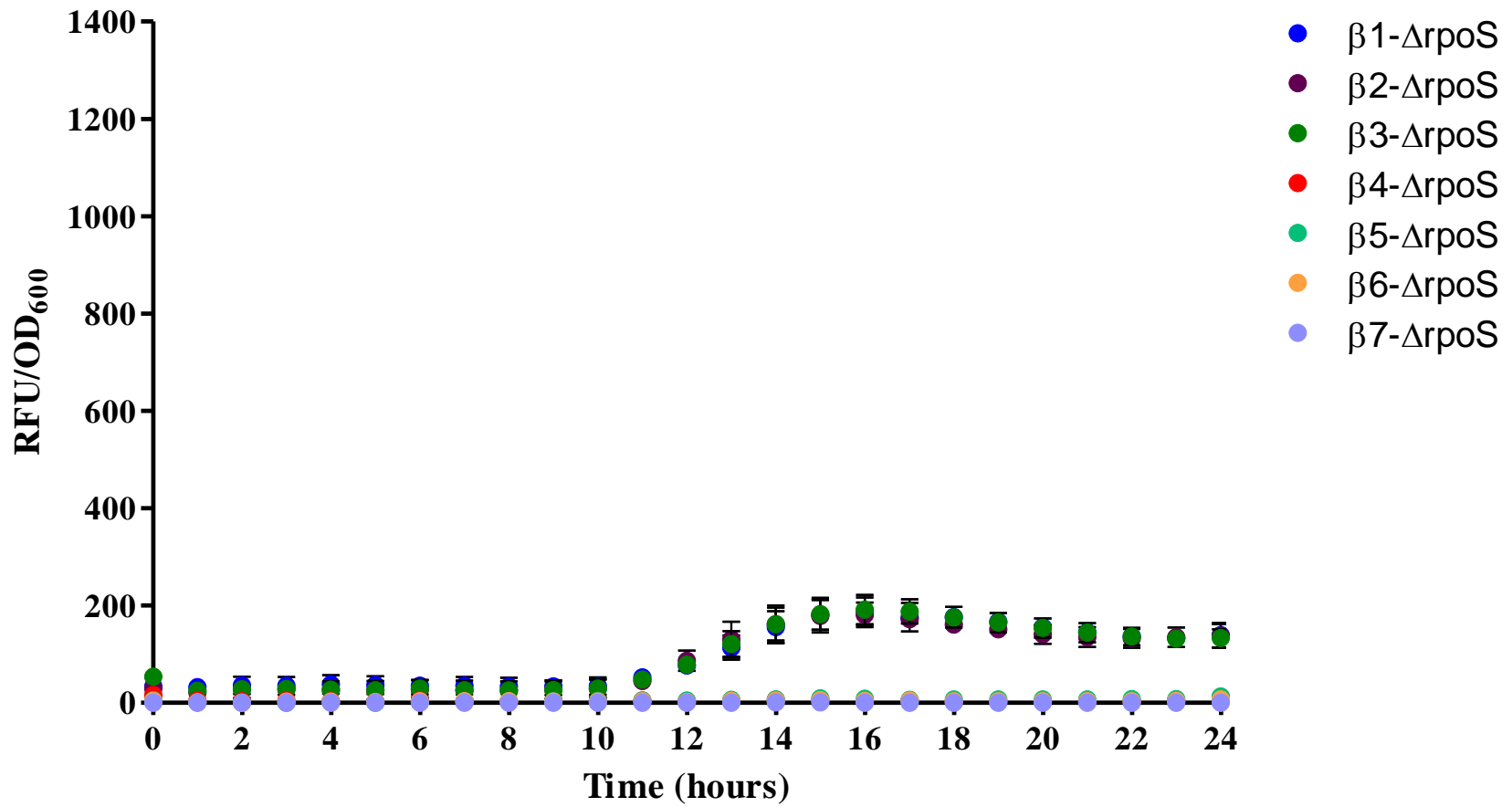
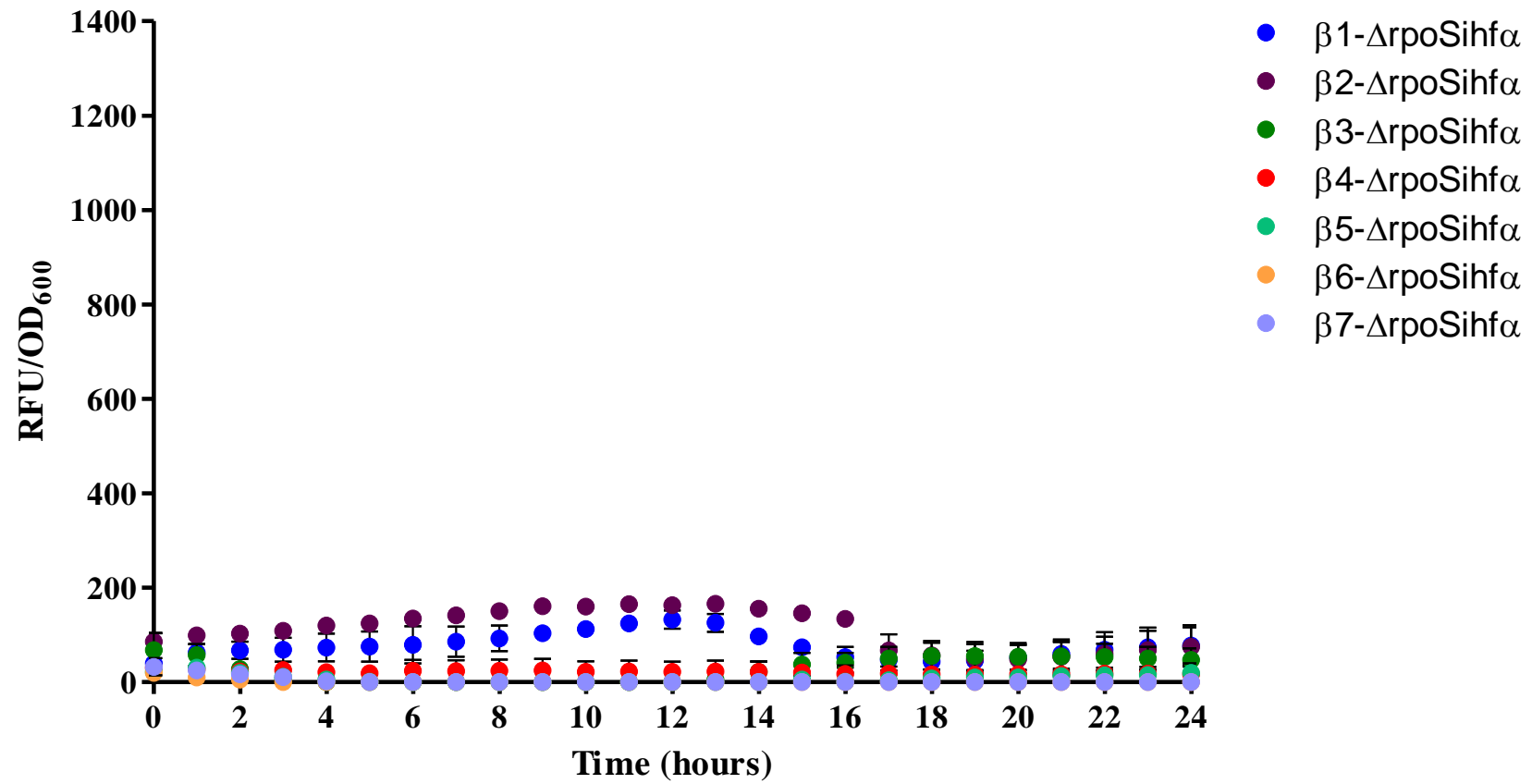


Figure 3.21: Fluorescence detected from *L. pneumophila* Lp02 Δ rhoSihfa containing *P_{ihf β}* promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.



3.4. Protein Purification of LpIHF and LpRpoS

To verify whether the putatively identified IHF binding sites located in the promoter regions upstream of the *ihf α* and *ihf β* are functional in binding LpIHF, recombinant LpIHF was expressed and purified for subsequent implementation in electrophoretic mobility shift assays (EMSA) with the truncated promoter constructs. As LpIHF is a heterodimeric protein, the His₁₀-tagged α and β subunits were individually expressed and purified separately on nickel-charged gravity columns, and then combined in a 1:1 ratio during dialysis prior to concentration (see materials and methods). The purity of the recombinant LpIHF protein was assessed via Ponceau red staining of the nitrocellulose blot; however, a considerable number of non-specific bands was observed in the background although immunoblotting with monoclonal anti-His₆-tag antibody detected the band correlating to recombinant LpIHF (Figure 3.22). To improve the purity of the recombinant LpIHF protein preparation, a protease inhibitor cocktail was included in the cell lysis mixture and the HiTrapTM Heparin HP column was implemented as an additional purification step (see materials and methods) and ultimately improved the protein purity (Fig. 3.23). Likewise, to assess if the putatively identified RpoS sites located in the promoter regions upstream of *ihf α* and *ihf β* is functional as well in binding LpRpoS protein, recombinant His₁₀-tagged LpRpoS protein was expressed and purified using the newly modified purification protocol for subsequent employment in EMSAs (Fig. 3.24). Thus, the modifications to the purification protocol greatly improved the purity of the recombinant LpIHF protein and therefore potentially reduced the risk of non-specific binding in subsequent EMSAs.

Figure 3.22: Recombinant LpIHF after purification, dialysis and concentration without implementation of the HiTrapTM Heparin HP column in the purification protocol. (A) Approximately 15.25 μg of LpIHF was separated through a 12% polyacrylamide SDS-PAGE gel, blotted to nitrocellulose membrane and stained with Ponceau Red. Note the number of non-specific protein bands in the background. (B) Monoclonal His₆-tag antibody immunoblot after Ponceau Red destaining. Arrows denote recombinant LpIHF (~12.5 kDa per subunit).

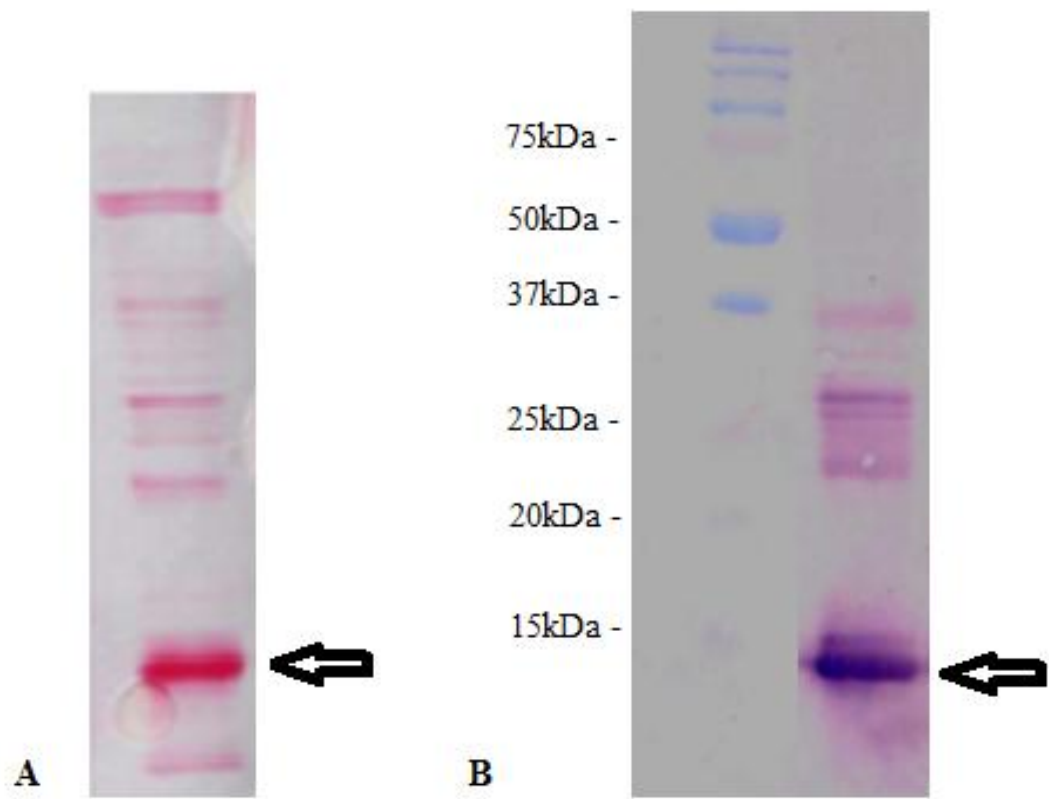


Figure 3.23: Recombinant LpIHF after purification, dialysis and concentration with implementation of the HiTrapTM Heparin HP column in the purification protocol. (A) Approximately 1.26 μg of LpIHF was separated through a 12% polyacrylamide SDS-PAGE, blotted to nitrocellulose membrane and stained with Ponceau Red. Note the number of non-specific protein bands in the background. (B) Monoclonal His₆-tag antibody immunoblot after Ponceau Red destaining. Arrows denote recombinant LpIHF (~12.5 kDa per subunit).

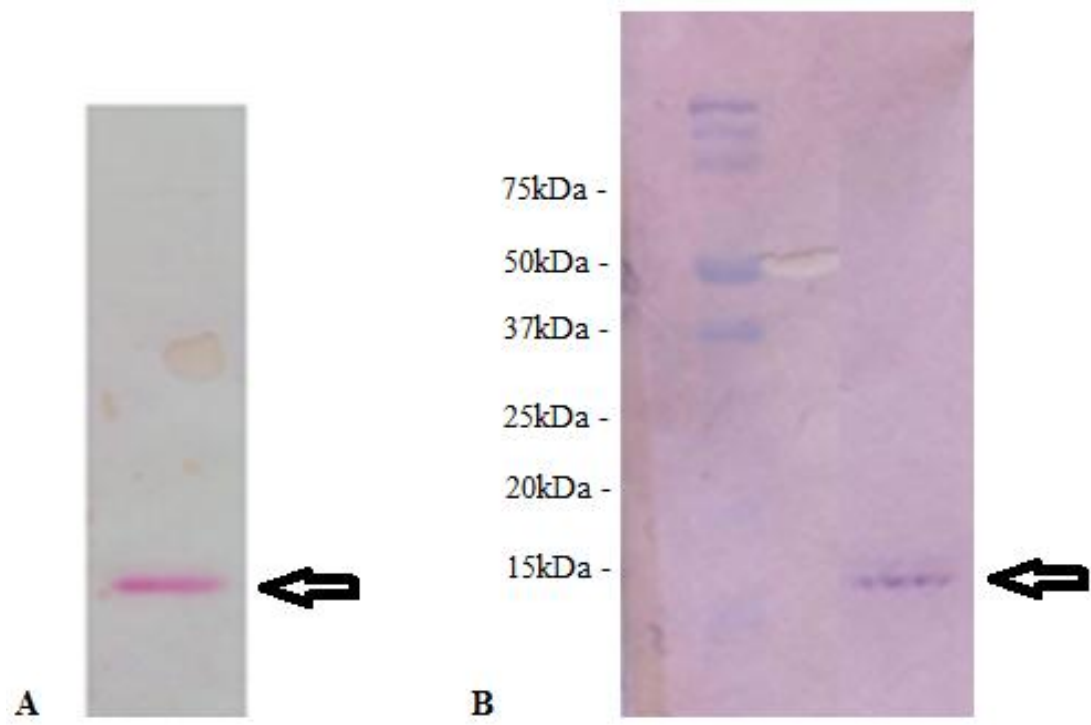
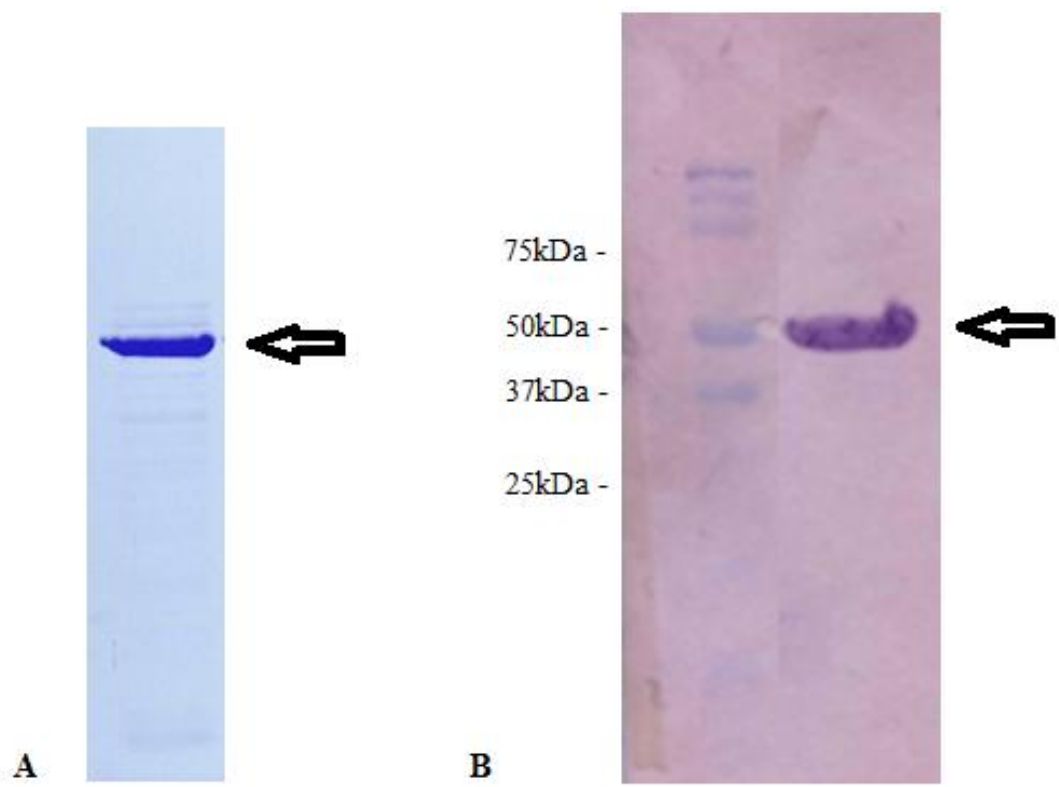


Figure 3.24: Recombinant LpRpoS after purification and concentration. (A) Approximately 5.57 μg was separated through a 12% SDS-PAGE and stained with Coomassie Blue. (B) Monoclonal His₆-tag antibody immunoblot after blotting onto nitrocellulose membrane. Arrows denote location of His₁₀-tagged LpRpoS (42.8 kDa).



3.5 Electrophoretic mobility shift assays (EMSA)

To assess whether LpIHF bound the putatively identified IHF sites in the promoter regions upstream of *ihfα* and *ihfβ*, purified recombinant heterodimeric LpIHF protein was employed in EMSAs. The promoter region of *magA*, a known gene to be regulated by LpIHF (Morash *et al.*, 2009), was used as a positive control and the internal segment of gene *lpg2112* (macrophage induced major protein) was used as the negative control. The PCR-amplified upstream promoter region of *magA*, using primer pair MagA, along with the internal segment of *lpg2112* (negative control), using primer pair Control, were incubated with purified recombinant LpIHF, run on non-denaturing polyacrylamide gel and stained with SYBR[®] Green nucleic acid dye to detect band shifts indicative of LpIHF binding to DNA (Table 2.3) (see materials and methods) (Figure 3.25 and Figure 3.26). The EMSAs on the *magA* promoter and internal *lpg2112* fragments were initially done using recombinant LpIHF protein purified prior to the modifications of the purification protocol (henceforth referred to as pre-HiTrap[™] LpIHF). Band shifts were observed at 18.3 μM of LpIHF for the positive control *magA* promoter region, but not for the negative control internal *lpg2121* fragment, indicating that pre-HiTrap[™] LpIHF was active functionally in specifically recognizing and binding the identified IHF site within the upstream promoter region of *magA*.

3.5.1 Binding of Recombinant pre-HiTrap[™] LpIHF protein with upstream promoter regions of *ihfα* and *ihfβ*

To determine if the putative IHF sites located within the upstream promoter regions of *ihfα* and *ihfβ* were functional in binding LpIHF, truncated promoter regions

Figure 3.25: Binding of recombinant LpIHF with upstream promoter region of *magA*. Approximately 250 ng of PCR-amplified *magA* promoter region was incubated with incremental amounts of pre-HiTrapTM LpIHF protein (0 μ M, 6.1 μ M, 18.3 μ M, 30.5 μ M), run on 0.5X TBE 6% polyacrylamide gel, and stained with SYBR[®] Green nucleic acid dye.

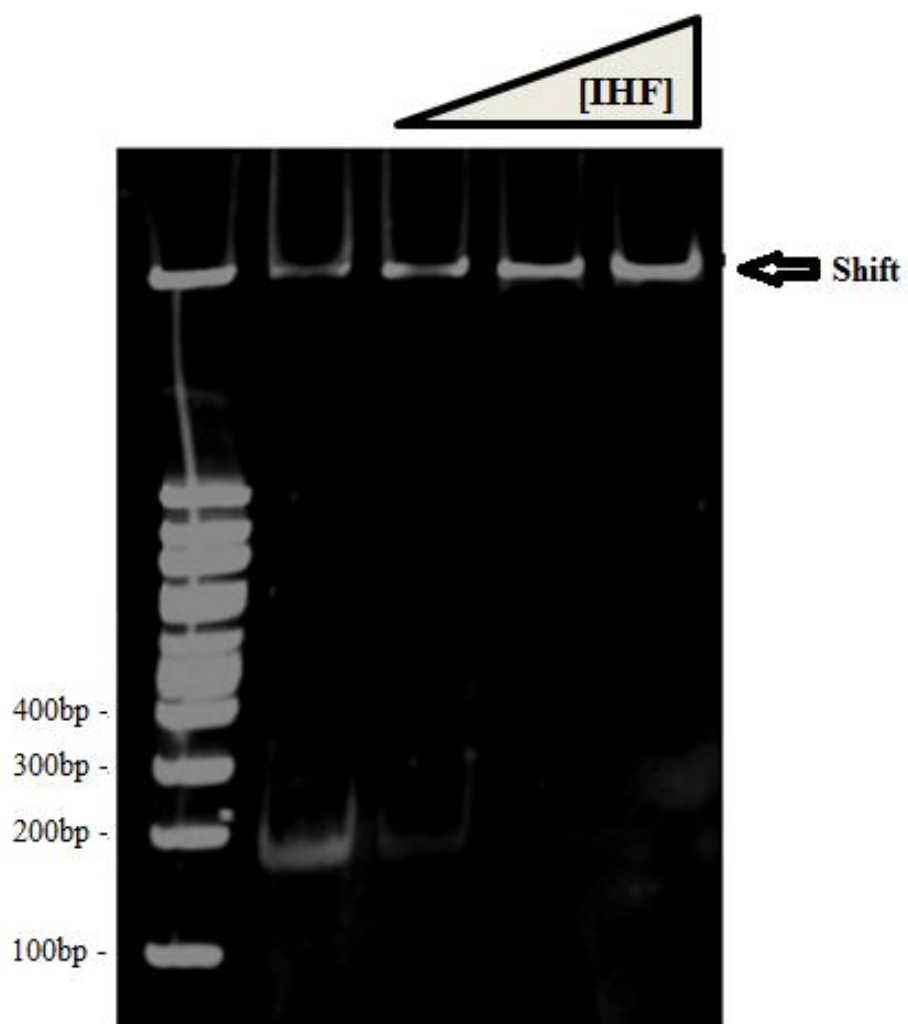
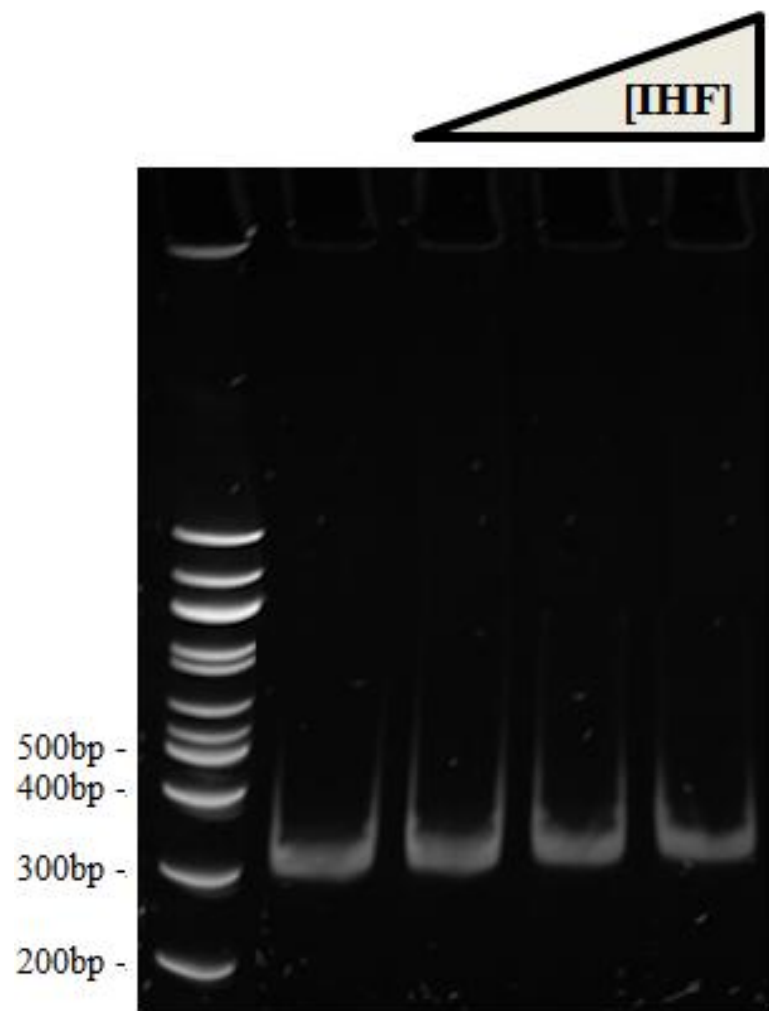


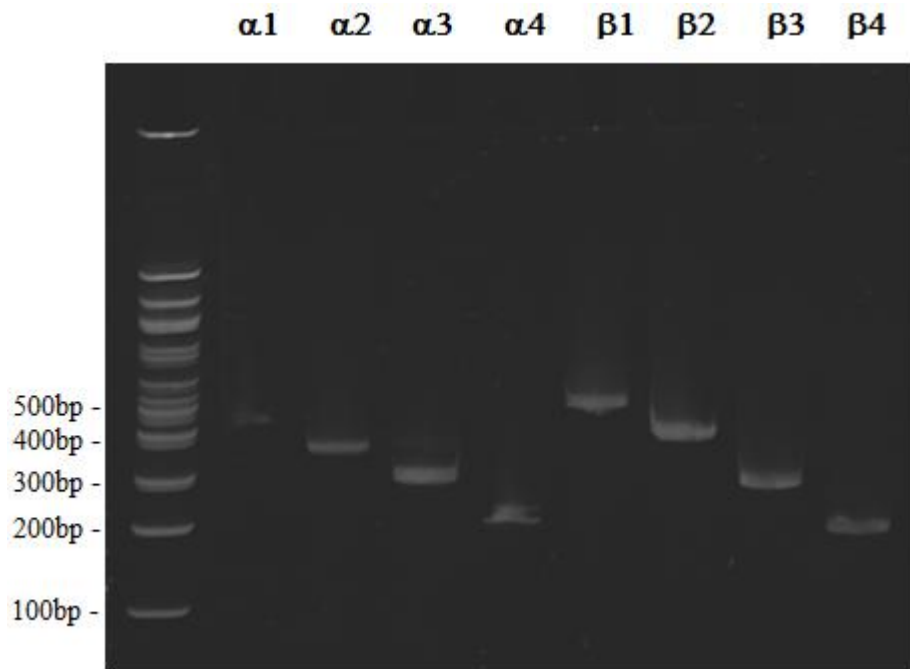
Figure 3.26: Lack of binding of recombinant LpIHF with internal segment of lpg2112. Approximately 250 ng of PCR-amplified lpg2112 internal segment was incubated with incremental amounts of pre-HiTrapTM LpIHF protein (0 μ M, 6.1 μ M, 18.3 μ M, 30.5 μ M), run on 0.5X TBE 6% polyacrylamide gel, and stained with SYBR[®] Green nucleic acid dye.



were employed in EMSAs with a specific concentration (18.3 μM) of pre-HiTrapTM LpIHF to achieve binding as empirically determined in Figure 3.25. The migration band pattern of the free (i.e. absence of pre-HiTrapTM LpIHF) truncated promoter regions (Figs. 3.27 A and 3.28 A) were compared to the migration band pattern of truncated promoter regions incubated with of pre-HiTrapTM LpIHF (Figs. 3.27 B and 3.28 B). Full band shifts were observed for the truncated promoter regions $\alpha 1 - \alpha 4$ and $\beta 1 - \beta 3$, whereas shifting of only a subpopulation of the $\beta 4$ fragment was observed. With the remaining truncated promoter regions, there was ambiguity in the interpretation of the EMSAs due to excessive “streaking” pattern observed with the DNA fragments. It appeared that there were shifts of only a subpopulation for promoter regions $\alpha 5 - \alpha 7$ with no band shifts observed for $\alpha 8$, and $\beta 5 - \beta 7$. The resultant band shifts of the truncated promoter region fragments seem to indicate that pre-HiTrapTM LpIHF was fully functional in binding the *ihf α* promoter region fragments that included sites #1 through #3, whereas site #4 does not appear to bind pre-HiTrapTM LpIHF as no shift was observed suggesting the necessity of site #3 for binding with pre-HiTrapTM LpIHF (Figure 3.5). However, shifting of only a subpopulation of $\alpha 6$ and $\alpha 7$ and the fact that site #3 is not present in either of these constructs suggests the possibility of #4 being a necessary binding site. Regarding the *ihf β* promoter region, full band shifts of the truncated promoter region fragments included both sites #1 and #2 (Figure 3.6). Once site #2 was eliminated band shifting was lost completely suggesting the necessity of site #2, and possibly site #1, for binding with pre-HiTrapTM LpIHF.

Figure 3.27: Binding of recombinant LpIHF to truncated *ihf* α and *ihf* β promoter regions (α 1 – α 4 & β 1 – β 4). Truncated promoter region fragments (250 ng) in the absence (A) and presence of (B) 18.3 μ M pre-HiTrapTM LpIHF protein were run on non-denaturing 0.5X TBE 5% polyacrylamide and stained with SYBR[®] Green nucleic acid dye.

A



B

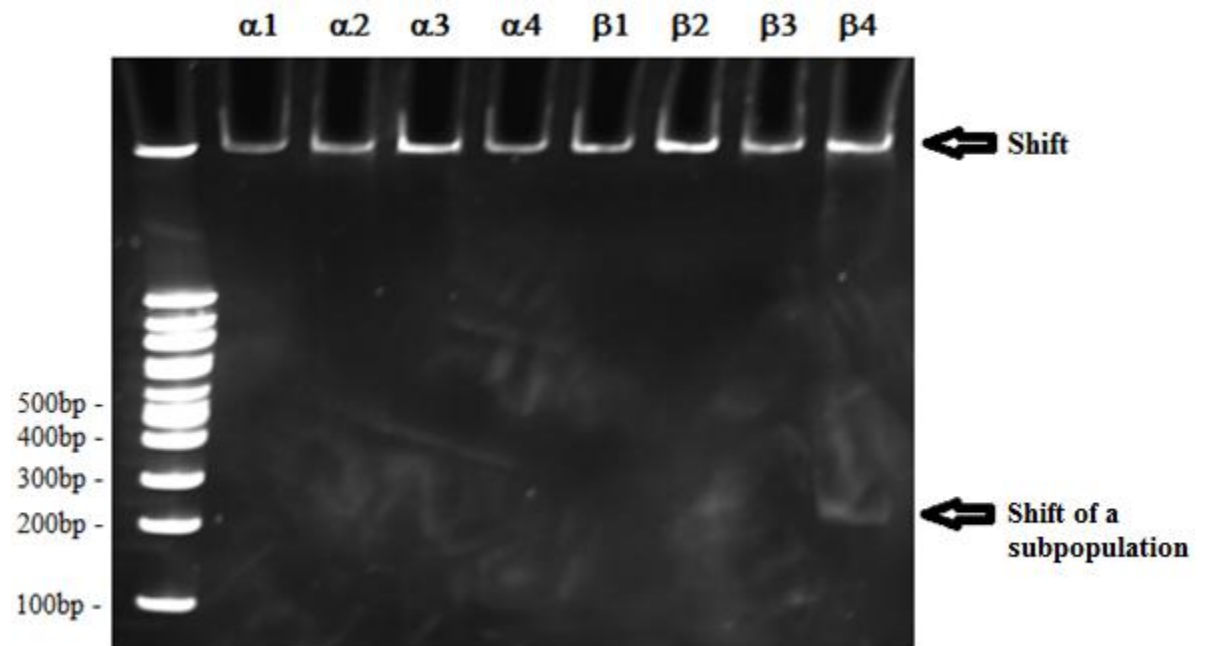
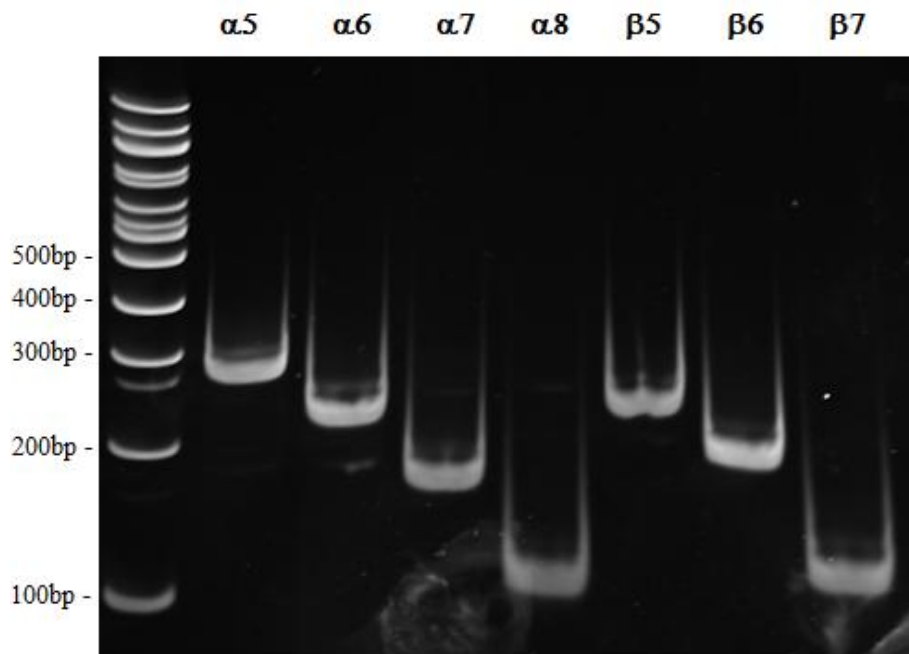
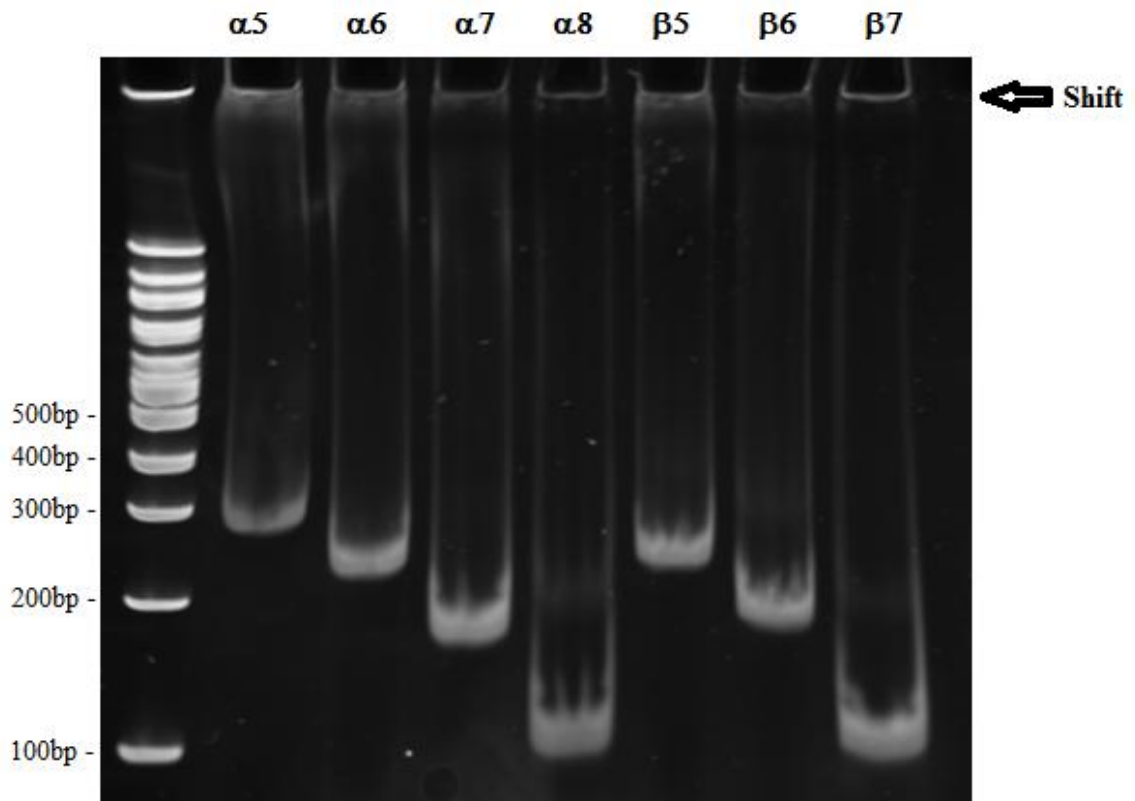


Figure 3.28: Binding of recombinant LpIHF to truncated *ihf* α and *ihf* β promoter regions (α 5 – α 8 & β 5 – β 7). Truncated promoter region fragments (250 ng) in the absence (A) and presence of (B) 18.3 μ M pre-HiTrapTM LpIHF protein were run on non-denaturing 0.5X TBE 5% polyacrylamide and stained with SYBR[®] Green nucleic acid dye.

A



B



3.5.2 Binding of Recombinant post-HiTrapTM LpIHF protein with upstream promoter regions of *ihfα* and *ihfβ*

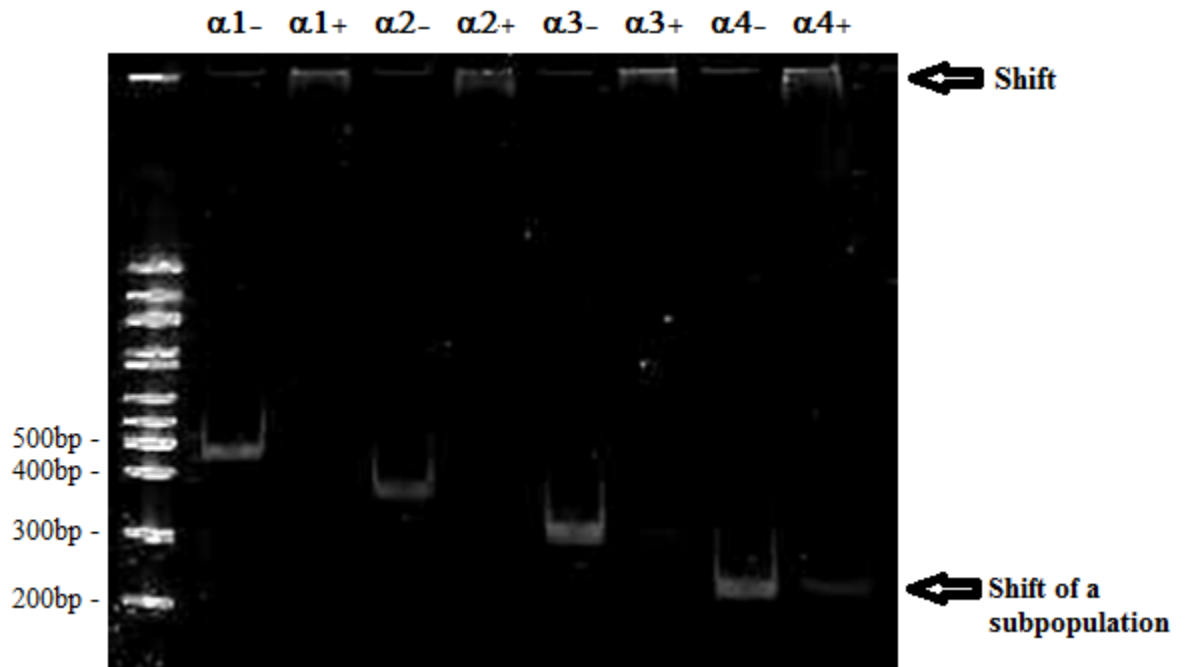
Although band shifts were observed with some of the truncated *ihfα* and *ihfβ* promoter region fragments indicating the specificity of pre-HiTrapTM LpIHF of recognizing and binding the putative IHF sites as supported by the positive (*mgaA* promoter region) and negative (internal fragment of lp2112) controls employed (Figures 3.25 and 3.26), there was some concern with the purity of the pre-HiTrapTM LpIHF protein. Though immunoblotting with monoclonal His₆ antibody indicated the presence of His₁₀-tagged α and β subunits of the heterodimeric LpIHF protein, SDS-PAGE analysis revealed considerable background protein contamination (Figure 3.22). In addition, there was consistent retention of full band shifts in the wells of EMSA gels (Figures 3.27 and 3.28) indicating possible non-specific binding and/or protein aggregation. In addition there was ambiguity in the interpretation of the resultant shifting due to excessive streaking of the promoter region fragments in question. Thus, measures were employed that included protease inhibitor in the cell lysis mixture and further purification of the recombinant protein using the HiTrapTM Heparin HP column that greatly improved the purity of the recombinant protein (Figure 3.23, see materials and methods) henceforth referred to as post-HiTrapTM LpIHF.

EMSAs were conducted to determine if post-HiTrapTM LpIHF (Figures 3.29 and 3.30) would improve and/or alter the band shifting pattern previously observed with EMSAs of truncated *ihfα* and *ihfβ* promoter region fragments with pre-HiTrapTM LpIHF (Figures 3.27 and 3.28). Similarly to the results achieved with pre-HiTrapTM LpIHF, full band shifts were observed for $\alpha 1 - \alpha 3$ and shifting of only a subpopulation were observed

Figure 3.29: Binding of recombinant LpIHF to truncated *ihfa* promoter region.

Truncated promoter region fragments (10 ng) in the absence (-) and presence of (+) 2.52 μ M post-HiTrapTM LpIHF protein were run on non-denaturing 0.5X TBE 5% polyacrylamide and stained with SYBR[®] Green nucleic acid dye.

A



B

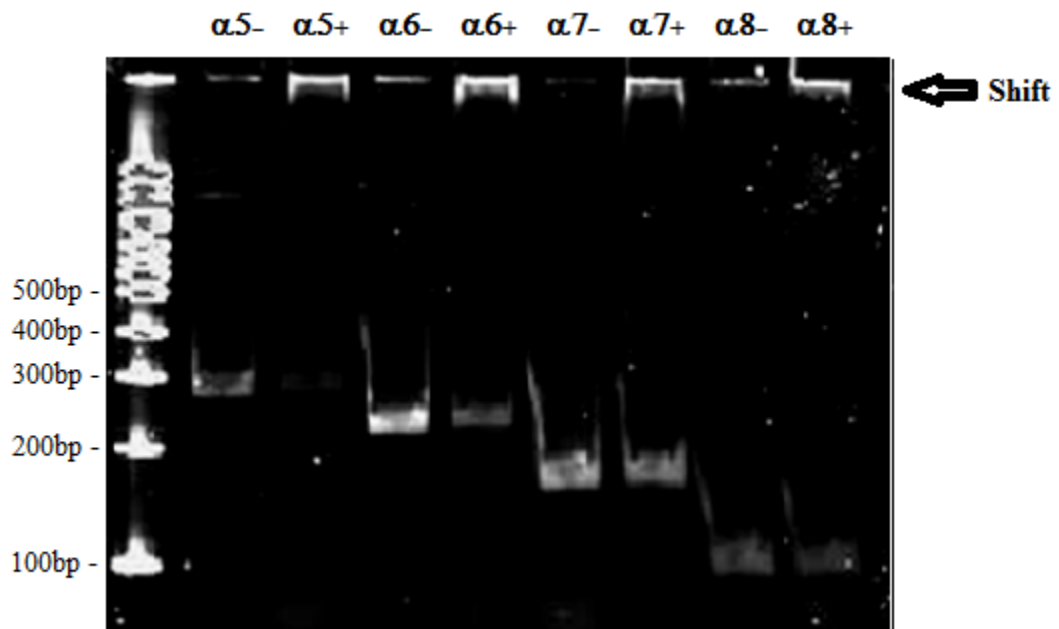
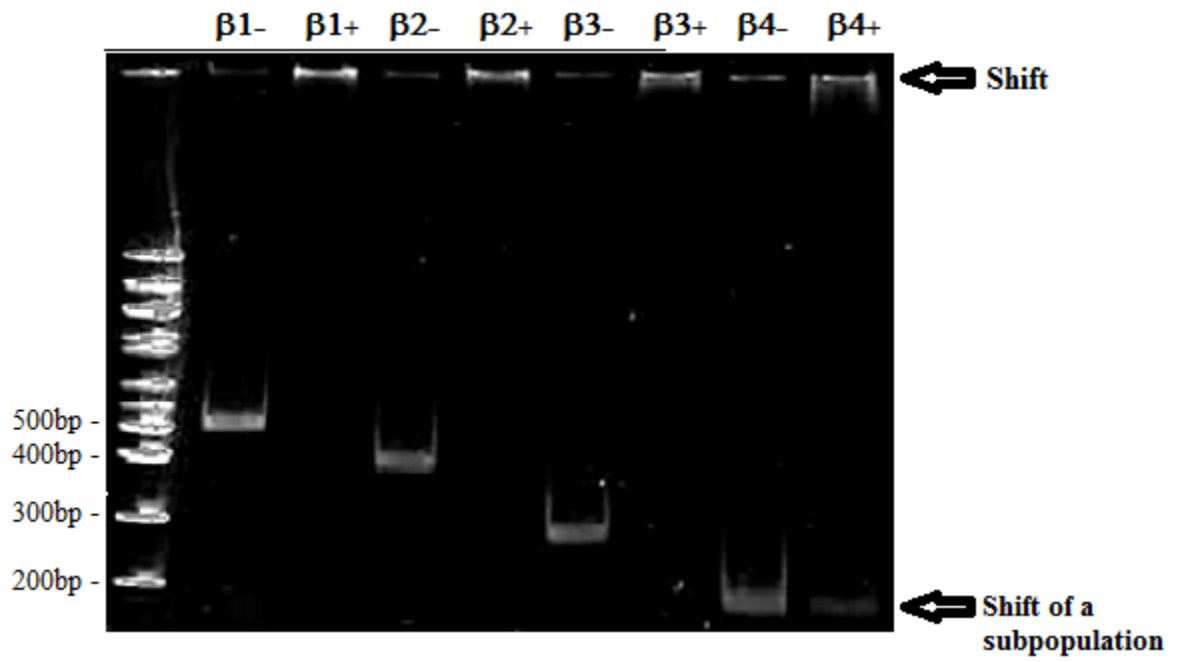


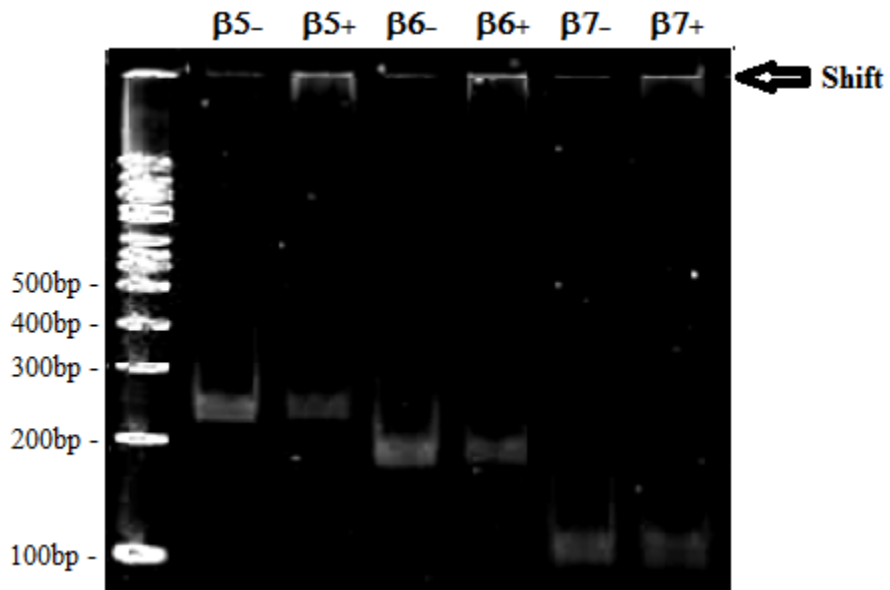
Figure 3.30: Binding of recombinant LpIHF to truncated *ihf* β promoter region.

Truncated promoter region fragments (10 ng) in the absence (-) and presence of (+) 2.52 μ M post-HiTrapTM LpIHF protein were run on non-denaturing 0.5X TBE 5% polyacrylamide and stained with SYBR[®] Green nucleic acid dye.

A



B



for $\alpha 5 - \alpha 7$. However, shifting of only a subpopulation were observed for $\alpha 4$ and $\alpha 8$ with post-HiTrapTM LpIHF whereas a full band shift and no band shift were observed previously with pre-HiTrapTM LpIHF, respectively. Regarding the *ihf β* promoter region, full band shifts and shifting of only a subpopulation were observed for $\beta 1 - \beta 3$ and $\beta 4$, respectively, similar to results achieved with pre-HiTrapTM LpIHF. However, shifting of only a subpopulation were observed for the remaining fragments $\beta 5 - \beta 7$ whereas no band shifts were observed for these fragments previously with pre-HiTrapTM LpIHF. Thus, the band shift pattern of the truncated *ihf α* and *ihf β* promoter region fragments with post-HiTrapTM LpIHF has greatly improved over that obtained with pre-HiTrapTM LpIHF. Inference of the results concerning the *ihf α* promoter region suggest that now site #4 is necessary for binding and that perhaps site #2 may be involved in binding IHF as shifting of only a subpopulation was observed in the absence of site #2 (Figure 3.5). Regarding the *ihf β* promoter region, as observed previously with pre-HiTrapTM LpIHF site #2 appears to be involved in binding LpIHF as shifting of only a subpopulation was observed with the exclusion of site #2; however, shifting of only a subpopulation were observed with $\beta 5 - \beta 7$ suggesting the presence of an additional site that escaped detection by the initial bioinformatic analysis, perhaps due a high degree of mismatch to the *E. coli* IHF consensus sequence, that may manifest weak affinity binding for post-HiTrapTM LpIHF (Figure 3.6).

3.6 RsmY and RsmZ

The two-component signal transduction system LetA/LetS is involved in the differentiation of *L. pneumophila* from vegetative replicative form to cyst-like form (Hammer *et al.*, 2002). Recently, the response regulator LetA was shown to regulate the expression of the non-coding RNAs RsmY and RsmZ which in turn sequester CsrA abolishing its post-transcriptional repressive activities on targeted genes associated with differentiation (Sahr *et al.*, 2009). Interestingly, the 14-bp LetA consensus binding site sequence is remarkably similar to the 13-bp *E. coli* IHF consensus binding site sequence such that there are only three mismatches noted between the two sequences (Figure 3.31). To investigate whether the LetA consensus binding site sequence is similarly recognized by LpIHF thereby contributing to the expression of *rsmY* and *rsmZ*, truncated promoter region constructs of the promoter regions upstream of *rsmY* and *rsmZ* were created via PCR amplification and directionally cloned into the promoter-less GFP reporter plasmid similar to the strategy employed for *ihf α* and *ihf β* promoter regions (see section 3.2 and see materials and methods). Primers pairs Y1 (*rsmY* P1) through Y3 (*rsmY* P3) were used for the amplification of the truncated *rsmY* promoter constructs, and primers Z1 (*rsmZ* P1) through Z3 (*rsmZ* P3) were used for the amplification of the truncated *rsmZ* promoter constructs creating three constructs each for *rsmY* (constructs Y1 – Y3) and *rsmZ* (constructs Z1 – Z3).

3.6.1 Microplate expression profiles of *rsmY* and *rsmZ* in Lp02 wild-type, Δ *ihf α ihf β* , Δ *letA*

To assess the expression profiles of *rsmY* and *rsmZ* in the presence and absence of

Figure 3.31: Binding site comparisons. Comparison of the *E. coli* IHF consensus sequence [WATCAANNNTTR (W = A/T, R = A/G and N = G/A/T/C) and the *L. pneumophila* LetA consensus sequence (TNAGAAATTTCTNA). (A) and (B) are equally strong, alternative alignments, where both sets of alignments contain 10 identified matches (indicated by colons).

A

W A T C A A N N N N T T R
: : : : : : : : : :
T N A G A A A T T T C T N A

B

W A T C A A N N N N T T R
: : : : : : : : : :
T N A G A A A T T T C T N A

LpIHF and LetA separately, truncated *rsmY* and *rsmZ* promoter plasmid constructs pBHY1, pBHY2, pBHY3, pBHZ1, pBHZ2, pBHZ3 (Table 2.2) were electroporated into wild-type Lp02 creating strains Y1 – Y3, and into Lp02 $\Delta ihfaihf\beta$ creating strains Y1- Δihf – Y3- Δihf and Lp02 $\Delta letA$ creating strains Y1- $\Delta letA$ – Y3- $\Delta letA$. These cells were subjected to microplate fluorometer assays. The resulting growth curves were very similar to those generated by strains harboring the *ihf α* and *ihf β* truncated promoter GFP plasmid constructs in that exponential and post-exponential phases commenced at 9 and 18 hours, respectively. In the wild-type Lp02 strain background, high expression was observed with the Y1 promoter construct during lag phase and it steadily decreased to low levels during the transition to exponential phase, increasing to higher levels during the transition to post-exponential phase (Figure 3.32). Expression levels for Y2 and Y3 promoter constructs were observed to be minimal to none. Interestingly, minimal to no expression was observed for Y1- Δihf – Y3- Δihf and Y1- $\Delta letA$ – Y3- $\Delta letA$ (Figure 3.32). As expression occurred only with the Y1 construct in the wild-type strain background, it would appear that both LetA and LpIHF are required for *rsmY* expression and that the sites for these regulatory proteins may be restricted to the region bordered by the Y1 and Y2 primers as expression was lost when this region was excluded from the Y2 and Y3 promoter constructs.

Regarding the expression of *rsmZ* in the wild-type strain background, levels were initially low for the Z1 promoter construct during lag phase and steadily increased to higher levels throughout the transitions to exponential and post-exponential growth phases (Figure 3.33). The promoter construct Z2 mimicked the trend displayed by Z1 albeit at much lower levels and the expression was completely abolished with the Z3

Figure 3.32: Fluorescence detected from *L. pneumophila* Lp02 wild-type, *Δihfaihfβ*, and *ΔletA* mutant strains containing P_{rsmY} promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.

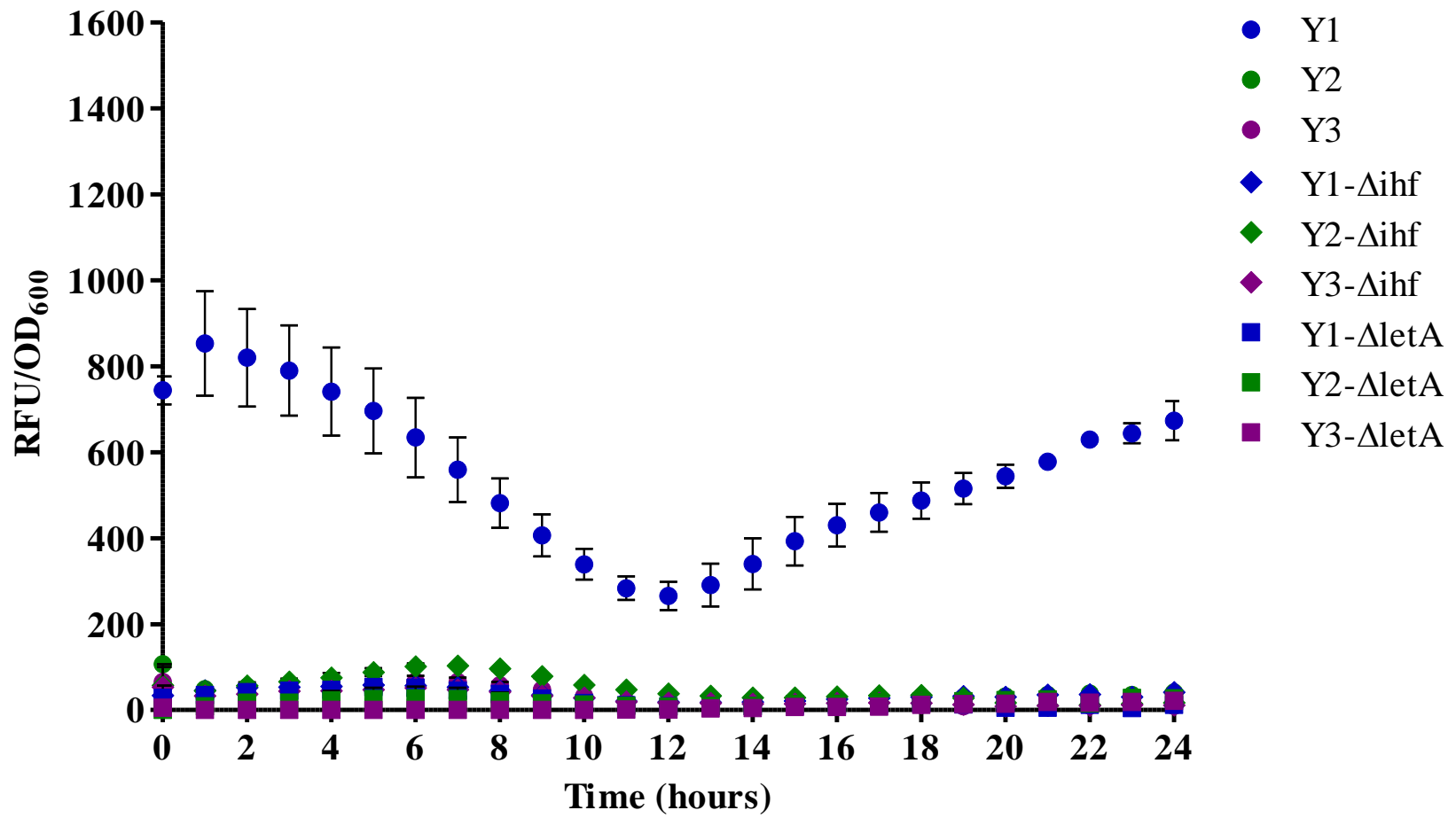
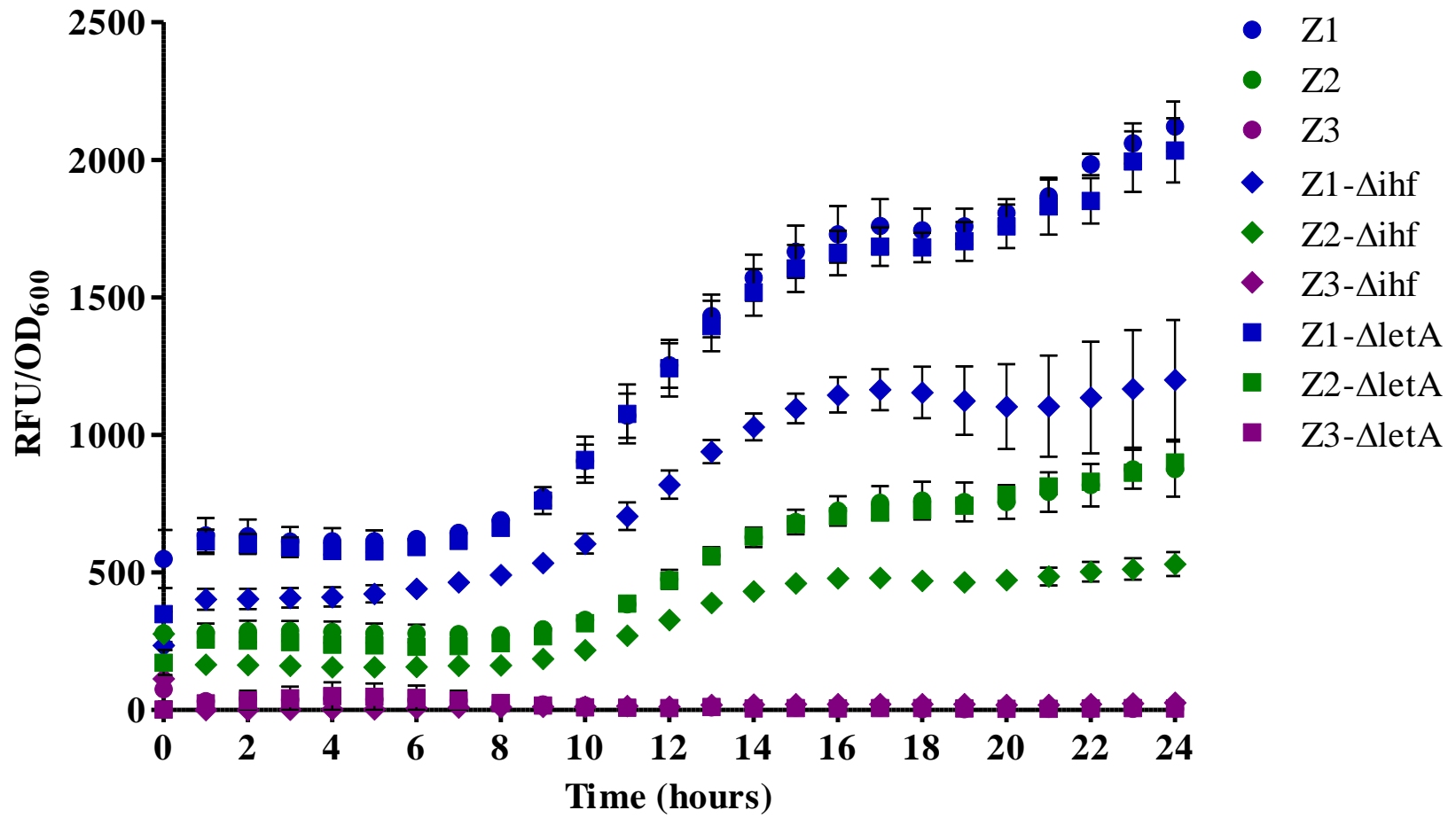


Figure 3.33: Fluorescence detected from *L. pneumophila* Lp02 wild-type, $\Delta ihfaihf\beta$, and $\Delta letA$ mutant strains containing P_{rsmZ} promoter DNA-GFP constructs. Readings were taken on an hourly basis for a 24 hour period of growth. Data are presented as normalized units of expression (RFU/OD₆₀₀). Data points are the average of three independent experiments.



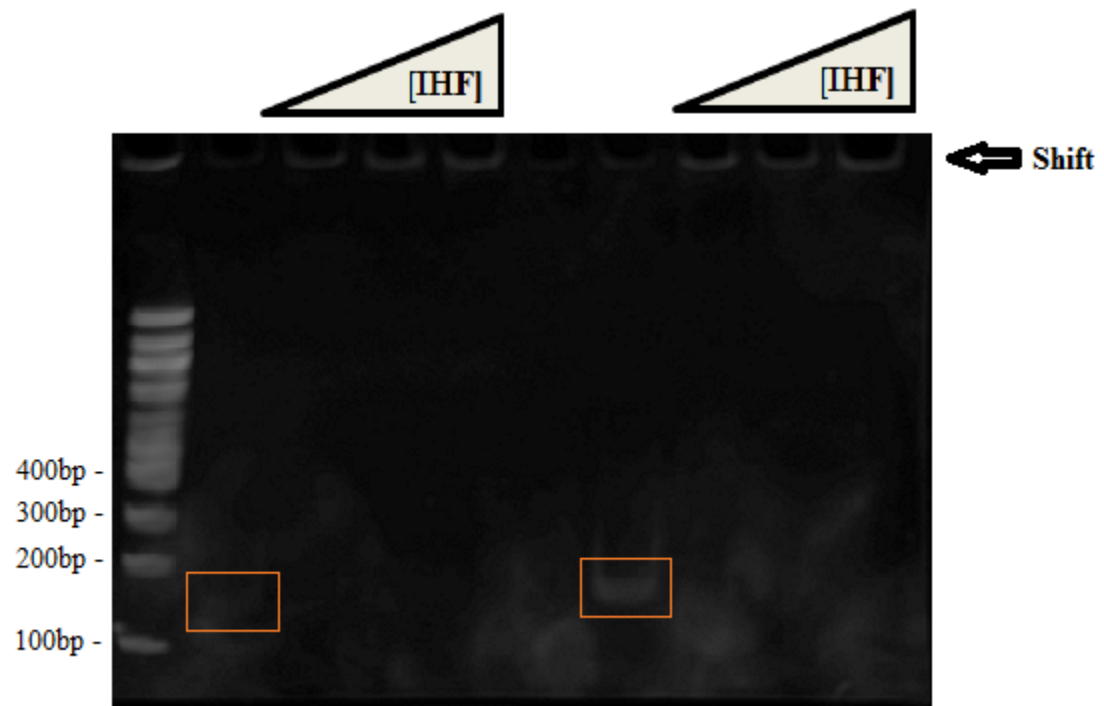
promoter construct. In the Lp02 $\Delta ihfaihf\beta$ mutant strain background, Z1- Δihf and Z2- Δihf followed the trends displayed by the Z1 and Z2 promoter constructs in the wild-type strain background, respectively, though their expression was reduced by approximately 30% of the wild-type expression, and expression was abolished with the Z3 promoter construct. The expression of Z1- $\Delta letA$, Z2- $\Delta letA$ and Z3- $\Delta letA$ surprisingly were similar to the levels of the same three promoter constructs in the wild-type background (Z1, Z2 and Z3). The expression profiles suggest that LetA may not be required for the expression of *rsmZ* as the fluorescence levels of the three promoter constructs were similar in both the Lp02 wild-type and Lp02 $\Delta letA$ mutant strain backgrounds. However, LpIHF seems to be required for optimal expression of *rsmZ* as the expression level for Z1- Δihf dropped approximately 30% in comparison the levels achieved by Z1 in the wild-type strain and the levels for Z2- Δihf also dropped approximately 30% in comparison the levels achieved by Z2 and abolishment of expression when only the Z3- Δihf segment remains. This indicates the site for binding LpIHF appears to be located within the region defined by the Z2 and Z3 primers. In addition the similar trends observed between the three constructs where all Z2 constructs (wt, - Δihf , and - $\Delta letA$) have 50% lower expression levels than all Z1 constructs (wt, - Δihf , and - $\Delta letA$) respectively, indicates the possibility of an additional transcriptional activator.

3.6.2 EMSA of LpIHF on *rsmY* and *rsmZ* promoter regions

To assess whether LpIHF bound to the promoter regions upstream of *rsmY* and *rsmZ*, the upstream promoter regions of *rsmY* and *rsmZ* were PCR amplified using *rsmY* and *rsmZ* primers (Table 2.3, see materials and methods) and employed in EMSAs using

recombinant pre-HiTrapTM LpIHF protein. Band shifts were observed at 6.1 μ M for both *rsmY* and *rsmZ* promoter regions (Figure 3.34). This suggests that LpIHF can form complexes with both *rsmY* and *rsmZ* promoter regions.

Figure 3.34: Binding of recombinant LpIHF with upstream promoter regions of (A) *rsmY* and (B) *rsmZ*. Approximately 100 ng of *rsmY* and 200 ng of *rsmZ* PCR-amplified promoter region were incubated with incremental amounts of pre-HiTrap™ LpIHF protein (0 μM, 6.1 μM, 18.3 μM, 30.5 μM), run on 0.5X TBE 6% polyacrylamide gel, and stained with SYBR® Green nucleic acid dye. Both *rsmY* (210 bp) and *rsmZ* (210 bp) bands representing DNA alone are encased in orange boxes to identify their position.



Chapter 4: Discussion

Originally identified as an architectural protein involved in the supercoiling and compaction of DNA in *E. coli*, IHF also functions in bending DNA promoting, by itself or in cohort with other regulatory factors, transcriptional activation or repression of genes (Goosen and van de Putte, 1995). The role of IHF as a transcriptional regulator appears to be widespread and IHF is conserved in function as homologs of IHF in a number of closely- and distantly-related bacteria have been determined to be involved in the regulation of gene expression (Pérez-Rueda *et al.*, 2009). Recently, IHF has gained increased recognition for the importance of its role in the regulation of virulence gene expression in a number of bacterial pathogens such as *Shigella flexneri*, *Neisseria gonorrhoeae* and *Salmonella enterica* serovar Typhimurium (Dorman *et al.*, 2001; Fyfe and Davies, 1998; Mangan *et al.*, 2006). In some cases, IHF is an integral part of the global regulatory cascade that controls the temporal expression of genes associated with a developmental cell cycle program as exemplified by the aquatic bacterium *Caulobacter crescentus* and the obligate intracellular pathogen *Chlamydia trachomatis* (Gober and Shapiro, 1990; Zhong *et al.*, 2001). In *L. pneumophila*, post-exponentially expressed LpIHF is required for the complete differentiation of the CLF form and full virulence in the natural amoebic host *Acanthamoeba castellanii* and in the HeLa mammalian cell model (Morash *et al.*, 2009). However, regulation of LpIHF is poorly understood. Thus, this study was undertaken to define the regulation of the heterodimeric LpIHF and characterize its cognate binding site sequences upstream of targeted genes associated with CLF differentiation.

4.1 Regulation of *ihfA* expression by LpIHF and LpRpoS

In *E. coli*, heterodimeric IHF expression is upregulated upon entry into postexponential or stationary phase (Morash *et al.*, 2009). Regulation of alpha subunit (encoded by *himA*) was modulated by IHF and the stationary sigma factor σ^{38} (RpoS) such that transcription of *himA* was found to be negatively and positively regulated by IHF and RpoS, respectively (Aviv *et al.*, 1994). In this study with *L. pneumophila*, microplate fluorometer assays indicate that expression of *ihfA* was upregulated upon transition from exponential to post-exponential phase and this upregulation was maintained in the absence of cellular LpIHF (Figures 3.14 and 3.15). LpRpoS appears to be required for optimal expression of *ihfA* as the expression level in post-exponential phase in the absence of cellular RpoS was decreased approximately by half which may be attributed to the negative regulatory effects of LpIHF, as maximal levels of this protein have been observed in this growth phase (Figure 3.16) (Morash *et al.*, 2009). Decreased expression levels are observed in lag phase as plate-grown *L. pneumophila* cells are in stationary phase and therefore they will have a high amount of cellular LpIHF when the cells resuspended in broth at the start of the microplate fluorometer assay. However, the reduced but significant expression levels in the absence of LpIHF and LpRpoS suggest the involvement of another unidentified regulatory factor or basal level expression (Figure 3.17).

Of the four putative LpIHF binding sites identified by bioinformatics analyses (Figures 3.4 and 3.5), microplate fluorometer assays indicate that site #4 appears to functionally bind LpIHF imparting negative regulatory effects on *ihfA* expression as indicated by depressed expression levels during lag and post-exponential growth phases,

which correlate with maximal cellular LpIHF protein levels observed during these growth phases (Morash *et al.*, 2009) (Figures 3.14 and 3.15). The identification of site #4 functionally binding IHF was further supported by the EMSAs of the truncated *ihfA* promoter region segments. Full band shifts were achieved with promoter region segments containing site #2, whereas shifting of only a subpopulation was achieved with the exclusion of site #2 from the promoter region segment (Figure 3.29). The shifting of only a subpopulation exhibited between LpIHF and the *ihfA* promoter region was observed for all subsequent constructs including $\alpha 8$ which includes site #4. Thus, site #4 appears to be essential for the repression of *ihfA* transcription (Figure 3.29 and 3.15). Together these results indicate that site #4 when bound by LpIHF is crucial for the inhibition of *ihfA* transcription, and site #2 may assist in mediating the repression although this function appears to be dispensable as observed in the microplate fluorometer assays (Figure 3.15).

Positive regulation of *ihfA* expression seems to be largely controlled by LpRpoS in *L. pneumophila* and the site for binding LpRpoS appears to be restricted to the $\alpha 8$ promoter region fragment of the *L. pneumophila ihfA* promoter region (Figure 3.16). A site (CTATAAT) matching the *E. coli* RpoS consensus binding site sequence (CTAcacT) was identified located just within the 5' end of the $\alpha 8$ promoter region fragment (Lee and Gralla, 2001) (Figures 3.4 and 3.5). Although highly similar in sequence to the -10 box (TATAAT) recognized by σ^{70} , it has been reported elsewhere that the σ^{70} -35 box is not utilized by RpoS in recognition of the -10 region sequence in *E. coli* (Tanaka *et al.*, 1995; Colland *et. al.*, 1999; Lee and Gralla, 2001). This fact supports the functionality of the putative LpRpoS site in the $\alpha 8$ promoter region fragment as the σ^{70} -35 box is located

upstream and beyond the 5' border of the $\alpha 8$ promoter region fragment. Interestingly, the putative LpIHF binding site #4 overlaps the putative LpRpoS site upstream of *ihfa* (Figures 3.4 and 3.5) suggesting competition between LpRpoS and LpIHF in binding for the activation and repression of *ihfa* expression, respectively. Similarly, *himA* expression is positively and negatively regulated by RpoS and IHF, respectively, in *E. coli* (Aviv *et al.*, 1994). Analysis of the sequence upstream of *himA* revealed a site (GTAAACT) matching the RpoS consensus sequence overlapping the sole identified IHF binding site. Taken together, LpIHF and LpRpoS regulate *ihfa* expression in *L. pneumophila* in the manner similar to the expression profile determined with *E. coli himA*.

4.2 Regulation of *ihf β* expression by LpIHF and LpRpoS

Similar to the alpha subunit (encoded by *himA*), the beta subunit (encoded by *himD*) of *E. coli* is also found to be negatively and positively regulated by IHF and RpoS, respectively (Aviv *et al.*, 1994). In this study with *L. pneumophila*, microplate fluorometer assays of *ihf β* were comparable to that of *ihfa* as they indicated that expression of *ihf β* was upregulated upon transition from exponential to post-exponential phase and this upregulation was maintained in the absence of cellular LpIHF (Figure 3.18 and 3.19). Moreover, LpRpoS also appeared to be required for optimal expression of *ihf β* as the expression level in post-exponential phase in the absence of cellular RpoS were also decreased approximately by half, again contributing to the negative regulatory effects of LpIHF as maximal levels of this protein have been observed in this growth phase (Figure 3.20). Lastly, the significantly reduced expression levels in the absence of

LpIHF and LpRpoS indicate the involvement of another unidentified regulatory factor, analogous to what was observed with *ihf α* (Figure 3.21).

Microplate fluorometer assays indicated that of the two sites identified by the bioinformatic analysis (Figures 3.4 and 3.6), site #2 appeared to functionally bind LpIHF and convey negative regulatory effects on *ihf β* indicated by increased expression levels observed for β 1 through β 3 during post-exponential phases which again, correlate with maximal cellular LpIHF protein levels (Morash *et al.*, 2009) (Figures 3.18 and 3.19). The identification of site #2 was further supported by the EMSAs of the truncated *ihf β* promoter region segments with both the pre- and post-HiTrapTM EMSAs where full bands were achieved with promoter region segments containing both sites #1 and #2 and shifting of only a subpopulation was observed with β 4 which could be simply explained by a lack of docking space available for LpIHF to securely bind (Figures 3.4 and 3.6), like observed with the α 5 fragment in the *ihf α* promoter constructs (Figures 3.27, 3.28 and 3.30). However, the post-HiTrapTM EMSAs suggest the possibility of an additional binding site located within the β 5 – β 7 region as shifting of only a subpopulation were achieved. Pre-HiTrapTM EMSAs with the fluorometer assays indicate that site #2 is crucial for the inhibition of *ihf β* transcription, and that site #1 may also inhibit *ihf β* transcription but that site may be a weaker affinity for binding LpIHF. Post-HiTrapTM supports site #2 that when bound by LpIHF inhibits transcription of *ihf β* ; however, another possibility is the presence of an additional LpIHF binding site within the β 5 – β 7 region that may be crucial for the inhibition of *ihf β* . Taken together the *ihf β* promoter region contains at least two functional binding locations. Similarly the *himD* promoter

region of *E. coli* is found to contain two IHF binding locations used for regulation of *himD* expression (Aviv *et al.*, 1994).

Similarly to *ihf α* , positive regulation of *ihf β* expression seems to be largely controlled by LpRpoS in *L. pneumophila* and the site for binding LpRpoS appears to be contained within the β 3 – β 4 region (3.20). Located at the 5' end of the β 4 promoter region fragment is a site (CTATACT) matching the *E. coli* RpoS consensus binding site sequence (CTAcacT) (Lee and Gralla, 2001) (Figures 3.4 and 3.6). The lack of fluorescence seen with the β 4 expression profile can be explained by the lack of docking space left available for RpoS binding based on the location of the putative RpoS site.

4.3 Proposed mechanistic model of LpIHF regulation by LpIHF and LpRpoS

IHF protein forms a complex with 35 bp of DNA such that DNA is wrapped around the protein in a U-turn shape, bent by $>160^\circ$ (Rice *et al.*, 1996). It has been shown that when the *E. coli* IHF protein binds to the *nir* promoter, IHF binding induces a bend at the -88 location upstream of the start codon (McLeod and Johnson, 2001). This bend is thought to possibly prevent RNA polymerase from making contact with the promoter and thus repress transcriptional activation (McLeod and Johnson, 2001). Based on this rationale this may be a possible behavior being displayed by LpIHF on the *ihf α* and *ihf β* promoter regions, to repress expression by preventing LpRpoS binding. More specifically, as post-exponential phase is reached, the concentration of LpRpoS increases and binds to the promoter regions of both *ihf α* and *ihf β* thus resulting in expression of LpIHF. As post-exponential phase continues on LpIHF continues to be produced and in turn regulates virulence genes. Once LpIHF reaches a high enough concentration, a

negative feedback loop occurs where LpIHF binds to both *ihf α* and *ihf β* promoter regions, bending the DNA and thus preventing LpRpoS binding. Post-exponential phase comes to an end and both LpRpoS and LpIHF are produced at basal levels entering lag and exponential phases.

4.4 Regulation of *rsmY* and *rsmZ* by LpIHF

The two-component signal transduction regulator LetA directly regulates the expression of non-coding RNAs RsmY and RsmZ (Sahr *et al.*, 2009). However, basal expression of *rsmY* and *rsmZ* still remained in the absence of cellular LetA suggesting the involvement of another regulatory factor (Sahr *et al.*, 2009). Surprisingly, the LetA consensus binding site sequence was highly similar to the *E. coli* IHF binding site sequence. Interestingly, the expression profile of *rsmY* reflected maximal cellular LpIHF protein levels in lag and post-exponential growth phases indicating the LpIHF may positively regulate *rsmY* expression (Morash *et al.*, 2009) (Figure 3.32). Moreover, the expression of *rsmY* was solely dependent on the presence of cellular LpIHF as expression was completely abolished in absence and presence of LpIHF and LetA, respectively. The site binding LpIHF appears to be restricted to the region defined by the 5' ends of Y1 and Y2 promoter region segments and therefore not overlapping the LetA binding site located in the Y2 promoter region fragment as originally hypothesized (Figure 3.31). Sequence analysis of the Y1 promoter region fragment sequence revealed a putative site (TATCCACTGTATT) with three mismatches to the *E. coli* IHF binding site sequence. EMSA of LpIHF binding to the promoter region of *rsmY* further supports that LpIHF does in fact bind and regulate *rsmY* expression (Figure 3.34 A). Thus, in contrast to the

finding reported by Sahr *et al.* (2009), it appears that LetA is not directly responsible for *rsmY* expression and that LpIHF is solely responsible for the expression of *rsmY*.

Likewise, RsmZ expression was also positively regulated by LpIHF without any contribution by LetA. The expression level was not affected in the absence of LetA but was significantly affected by the absence of LpIHF such that the expression level decreased to approximately a third of the expression level observed in the wild-type strain background (Figure 3.33). However, different from *rsmY* promoter region, the site binding LpIHF appears to be restricted to the region defined by the 5' ends of Z2 and Z3 promoter region segments and therefore overlapping the LetA binding site located in the Z2 promoter region fragment as originally hypothesized (Figure 3.31). EMSA of LpIHF binding to the promoter region of *rsmZ* further supports that LpIHF does in fact bind and regulate *rsmZ* expression (Figure 3.34 B). Interestingly, the similar trends observed between the three constructs where all Z2 constructs (wt, $-\Delta ihf$, and $-\Delta letA$) have approximately 50% lower expression levels than all Z1 constructs (wt, $-\Delta ihf$, and $-\Delta letA$) respectively, indicating the possibility of an additional transcriptional activator. Thus, in contrast to the finding reported by Sahr *et al.* (2009), it appears that LetA is not directly responsible for *rsmZ* expression and that LpIHF and another transcriptional activator or basal level transcription are responsible for the expression of *rsmZ*.

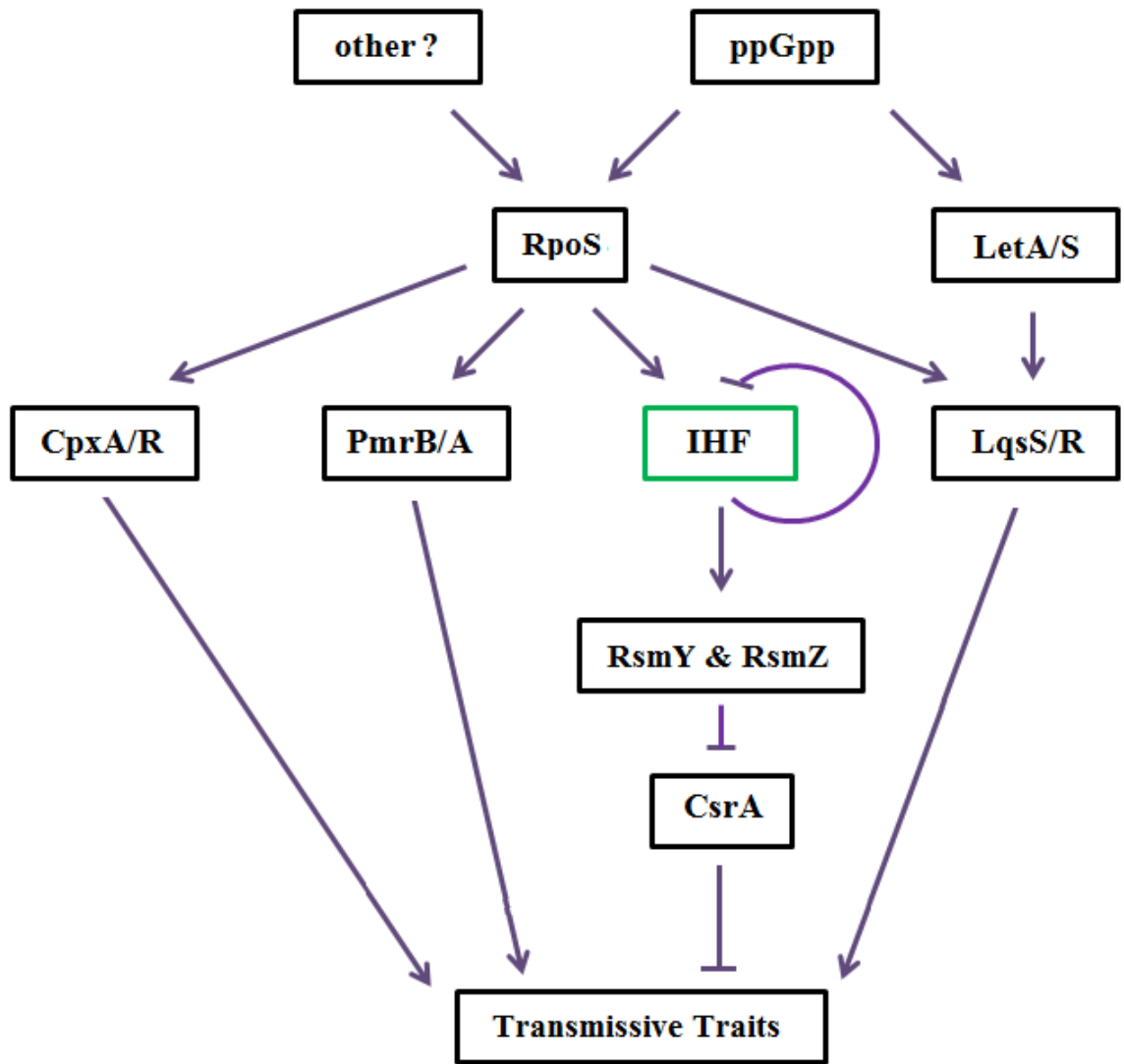
The results of the GFP reporter assays and EMSA indicate that LpIHF is the major transcriptional activator of both *rsmY* and *rsmZ* expression in *Legionella pneumophila*. Similarly for *P. fluorescens*, EciHF has been observed to bind to the promoter region of *rsmZ* (Humair *et al.*, 2010). Hence, IHF may also act as a regulator of *rsmZ* expression in *P. fluorescens*. In addition the identified palindromic sequence

TGTAAGcNNNNtCtTACA within the *rsmY* promoter region displayed similarity to the sequence element found in the promoter upstream of *rsmZ* and thus, IHF may also act as a regulator of *rsmY* expression in *P. fluorescens* (Valverde *et al.*, 2003). With respect to LetA, homologous to GacA found in *P. fluorescens*, similarity is only seen with LetA regulating *rsmY* expression as this study has identified LetA to not be a regulator of *rsmZ* (Valverde *et al.*, 2003).

4.5 Summary

This study revealed that the promoter regions of *ihf α* and *ihf β* are negatively autoregulated by LpIHF, where LpIHF interacts with a minimum of two separate sites within both *ihf α* and *ihf β* promoter regions. Of the four putative sites identified within the *ihf α* promoter region, sites #4 and #2 were confirmed to be functional in binding LpIHF. Whereas only one of the two putative sites identified in the *ihf β* promoter region, site #2, was confirmed to be functional in binding LpIHF, with an additional LpIHF functional binding site not identified from the bioinformatic search. This study also revealed that LpRpoS plays a major role in activating the transcription of both *ihf α* and *ihf β* . Furthermore, this study also determined that LpIHF positively regulates expression of non-coding RNAs RsmY and RsmZ. Thus, LpIHF appears to be an integral part of the global regulatory cascade governing genes associated with morphological differentiation and/or virulence traits (Figure 4.1).

Figure 4.1: Inclusion of LpIHF within the global regulatory cascade. Proposed location of LpIHF in the current schematic of the regulatory cascade as supported by thesis research conclusions. See Chapter 2 for details.



4.5 Future directions

Future prospects of this work could include DNaseI protection or footprint assays to precisely define the binding site sequences of LpIHF and LpRpoS within the upstream promoter regions of *ihf α* and *ihf β* . Similarly, footprint assays of LpIHF could also be employed with the promoter regions of *rsmY* and *rsmZ* to locate the specific LpIHF binding site sequences. Additionally, a consensus binding site sequence for LpIHF can be generated from the collection of characterized LpIHF binding site sequences. The mechanistic dynamics of LpIHF and LpRpoS binding the upstream promoter regions of *ihf α* and *ihf β* can be investigated via DNaseI footprint competition assays. Likewise, competition DNaseI footprint assays can be employed to further investigate the binding activities of LpIHF and LetA within the upstream promoter regions of *rsmY* and *rsmZ* in the attempt to resolve the contradictory result of LpIHF, rather than LetA as reported elsewhere, as the major transcriptional activator of RsmY and RsmZ expression.

Chapter 5: Appendix

5.1 Double knock out strategy (*ΔihfaletA*)

In order to ascertain whether LetA and LpIHF are the sole regulatory factors responsible for the regulation of *rsmY* and *rsmZ* expression, a double gene deletion Lp02 *ΔihfaletA* was initially proposed to be constructed. pKBXR::*ihfα*-gent plasmid was isolated and electroporated into *Legionella pneumophila* Lp02 *ΔletA* (see section 2.13). After incubation colonies were struck onto BCYE (supplemented with gentamycin, kanamycin, thymidine) and BCYE (supplemented with metronidazole and thymidine) for selecting. Colonies that were resistant to gentamycin, kanamycin and metronidazole were subjected to genomic isolation (see section 2.3) and PCR verification (see section 2.4). PCR verification included the use of primers *ihfα*(Int) and *ihfα*(Ext) (Table 2.3) to check for the absence of an internal band and the presence of an external band that is approximately 3 kb as it should include the kanamycin cassette. Unfortunately after many trials the knockout was never successfully created.

5.2 Triple knockout (*ΔihfaihfβrpoS* & *ΔihfaihfβletA*)

Previous assays and knockout strategies involved knocking out *ihfα* to investigate whether LpRpos and LpIHF were the sole regulatory factors responsible for the regulation of *ihfα* and *ihfβ* expression and whether LetA and LpIHF were the sole regulatory factors responsible for the regulation of *rsmY* and *rsmZ* expression. These assays/strategies were performed with the single knockout of *ihfα* due to the lack of antibiotic cassettes available. However, a new strategy was attempted involving

complete gene deletion with the use of Flp recombinase targets (FRTs). For this strategy, both *letA* and *rpoS* were PCR amplified using primers RpoS(FR) for the *rpoS* KO and LetA(FR) for the *letA* KO (Table 2.3) with PhusionTM polymerase (see section 2.4). Both amplicons and vector pBluescript SK+ (pBS) (Table 2.2) were digested with restriction digest enzymes specified in Table 2.4. Once digested the *letA* amplicon and the *rpoS* amplicon were ligated into pBS separately (see section 2.5). A chloramphenicol cassette plus FRT sites was PCR amplified from pKD3 with primers Cm(FRT+RpoS) for the *rpoS* KO and Cm(FRT+LetA) for the *letA* KO (Table 2.3) with PhusionTM polymerase (see section 2.4). To promote insertion of the antibiotic cassette between the two flanking regions, 3 μ L of pBS containing gene amplicon and 7 μ L of amplified CmFRTgene were mixed and electroporated into 50 μ L of both induced and uninduced *E. coli* recombineering strain DY330 (Table 2.1) (see section 2.6.4), after which 100 μ L aliquots were plated onto LB plus chloramphenicol and incubated for 24 – 48 h at 30°C. Three separate trials of these electroporations generated no colonies. Therefore the entire protocol was repeated where the electroporation was done with the *E. coli* recombineering strain DY331 (Table 2.1) (see section 2.6.4), after which 100 μ L aliquots were plated onto LB plus chloramphenicol and incubated for 24 – 48 h at 30°C. The first trial yielded 2 colonies for the *letA* knockout strain. Unfortunately when the colonies were restreaked and left to incubate for 24 – 48 h at 30°C no growth occurred. At this point the protocol was discontinued, however, if colonies grew from the induced cells, they would then have to be checked with internal primers specific to gene of interest [RpoS(Int) for RpoS KO and LetA(Int) for LetA KO] to check the absence of the gene and therefore confirm the presence of the antibiotic cassette alone. Once desired

construct was attained the plasmid would be isolated and transformed into *E. coli* DH5 α to recheck the construct with the internal primers [RpoS(Int) and LetA(Int)]. If construct is reconfirmed then the plasmid would then electroporated into *L. pneumophila* Lp02 and recombination would be checked via PCR with the internal primers [RpoS(Int) and LetA(Int)] and sequenced (as per protocol in section 2.8).

5.3 Construction of pDuet vector with *ihf* α and *ihf* β insertions

As LpIHF is a heterodimeric protein, the His₁₀-tagged α and β subunits were individually expressed and purified separately on nickel-charged gravity columns, and then combined. Therefore the construction from the pDuet vector was undertaken in order to express both Ihf α and Ihf β subunits together. This would reduce the amount of time required to express and purify the protein, in addition it would ensure a 1:1 ratio of the two subunits. For this strategy both *ihf* α and *ihf* β were PCR amplified using primers pDuet::*Ihf* α and pDuet::*ihf* β (Table 2.3) with PhusionTM polymerase (see section 2.4) . Both the *ihf* α and *ihf* β amplicons and the pDuet vector (Table 2.2) were digested with restriction digest enzymes specified in Table 2.4. Once digested the *ihf* α amplicon was ligated into pDuet (see section 2.5) and transformed into *E. coli* DH5 α (see section 2.6.2). Transformants were selected on LB amp plates. Colonies which grew were tested for the presence of the *ihf* α amplicon using pooled colony and individual PCR with the use of primers pDuet::*Ihf* α (Table 2.3) (see section 2.7). Once a colony was identified to contain the pDuet plasmid with the *ihf* α amplicon, the colony was subjected to plasmid DNA extraction via QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA). The isolated plasmid was then digested with restriction digest enzymes specified in Table 2.4. Once

digested the *ihfβ* amplicon was ligated into the pDuet plasmid containing the *ihfα* amplicon (see section 2.5) and transformed into *E. coli* DH5α (see section 2.6.2). Transformant colonies were selected on LB amp plates. Colonies which grew were tested for the presence of the *ihfβ* amplicon underwent colony and individual PCR with the use of primers pDuet::*Ihfβ* (Table 2.3) (see section 2.7).

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