Nitric oxide sensing in *Campylobacter jejuni* by NssR

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A Thesis submitted to the University of Kent at Canterbury for the degree of MSc in Microbiology

Department of Biosciences 2016

DECLARATION

No part of this thesis has been submitted in support of an application for any degree or qualification of the University of Kent or any other University or Institute of Learning.

James Froment 5th August 2016

Acknowledgements

I would like to thank my supervisor, Dr. Mark Shepherd, for all of the support and guidance over the past year.

I would also like to thank all the other members of the Shepherd, Blomfield and Robinson labs for all their help and copious amounts of cake.

Abstract

Pathogenic bacteria encounter the toxic free radical nitric oxide (NO) during infection via the activity of host nitric oxide synthases or via the reduction of nitrite and nitrate in the gut by commensal bacteria. Survival of bacterial pathogens during infection depends on various mechanisms for NO sensing and detoxification. The foodborne pathogen *Campylobacter jejuni* detoxifies NO to nitrate using a globin protein (Cgb), expression of which is induced strongly and specifically by the transcription factor NssR upon exposure to NO. Previous work indicates that nitric oxide does not directly modulate the interaction of NssR with target DNA, suggesting the existence of an indirect method for NO sensing. Bioinformatics analysis has revealed that NssR is likely to bind cyclic nucleotides, and cyclic-di-GMP (c-di-GMP) has previously been linked to NO sensing in other bacteria via a mechanism involving NO-responsive phosphodiesterase and cyclase enzymes. Structural modelling described herein is consistent with NssR binding to cyclic nucleotides, and tryptophan fluorescence quenching confirms the ability of NssR to bind to c-di-GMP, cAMP and cGMP. Subsequently, electrophoretic mobility shift assay (EMSA) suggests that that binding of c-di-GMP to NssR leads to increase in affinity for the *cgb* promoter. Finally, an *in vivo* reporter assay provides direct evidence that c-di-GMP can influence NssR activity. Taken together, these data are consistent with an *in vivo* mechanism whereby NO elevates c-di-GMP levels in the cell, which in turn activates NssR activity resulting in Cgb expression and NO detoxification. This work provides potential molecular explanation for how NO is sensed and detoxified in *C. jejuni*.

Contents

ABBREVIATIONS

Ap - Ampicillin

APS - Ammonium Persulfate

Cα - Alpha Carbon

cAMP - Cyclic adenosine monophosphate

C-di-GMP - Cyclic diguanylate; 3',5'-Cyclic diguanylic acid

C-GMP - Cyclic guanosine monophosphate

Cgb - Campylobacter haemoglobin

Crp - cAMP receptor protein

Cm - Chloramphenicol

Ctb - Campylobacter truncated haemoglobin

DGC - Diguanylate cyclase

DNA - Deoxyribonucleic acid

DNR - Dissimilative nitrate respiration regulator

EDTA - Ethylenediaminetetraacetic acid

EIC - Extracted ion chromatogram

FNR - Fumarate and nitrate reductase regulator

FnrP - Paracoccus denitrificans fumarate and nitrate reductase regulator homologue

GBS - Guilliane-Barre syndrome

GMP - Guanosine monophosphate

Gm - Gentamycin

GSNO - S-Nitrosoglutathione

GST - Glutathione S-transferase

GTP - Guanosine triphosphate

HD-GYP - HD superfamily hydrolase

HDOD – HD related output domain

HPLC - High Performance Liquid Chromatography

HPLC/MS - High performance Liquid chromatography–Mass spectrometry

IPTG - Isopropyl β-D-1-thiogalactopyranoside

IscR - HTH-type transcriptional regulator IscR

LB - Lysogeny broth

LPS - Lipopolysaccharide

MRM - Multiple reaction monitoring

mRNA - Messenger ribonucleic acid

NATA - N-acetyl-tryptophanamide

NO - Nitric oxide

NO+ - Nitrosonium ion

NO2- - Nitrite

NO3- - Nitrate

NorR - Anaerobic nitric oxide reductase transcription regulator NorR

NsrR - Nitric oxide-sensing Rrf2 repressor

NssR - Campylobacter nitrosative stress-responsive regulator

nrfA - Nitrite reductase

ONOO- - Peroxynitrite

ONPG - ortho-Nitrophenyl-β-galactoside

PAGE - Polyacrylamide gel electrophoresis

PCR - Polymerase chain reaction

PDB - RCSB protein data bank

PDE - Phosphodiesterase

RNA - ribonucleic acid

RNS - reactive nitrogen species

SDS - Sodium dodecyl sulphate

SOB - Super Optimal Broth

SOC - Super Optimal broth with Catabolite repression

SoxR - Redox-sensitive transcriptional activator SoxR

TAE - Tris/acetic acid/EDTA

TBE - Tris/Borate/EDTA

UV/VIS - Ultraviolet–visible spectroscopy

CHAPTER 1 - Introduction

Nitric Oxide is a free radical that has many important and diverse biological roles such as a cell signalling including elevation of cGMP levels (via inhibition of guanylate cyclase) which plays an important role in smooth muscle relaxation and vasodilation [1,2]. However, NO also plays a major role in the immune system in the defence against invading bacteria. There are a variety of cellular targets for NO in bacteria, and most pathogenic bacteria display a response to this nitrosative stress, which results in the circumvention of the toxic effects of NO and it's downstream products referred to as reactive nitrogen species (RNS). This study focusses on the enteric pathogen *C. jejuni* and the transcriptional regulator NssR (**N**itrosative **s**tress **s**ensing **R**egulator) that controls the major response to NO.

1.1 Nitric oxide and nitrosative stress in bacteria

Nitric oxide (NO) is a free radical that readily reacts with a wide variety of biological molecules. As a result there are a complex array of reactive nitrogen species (RNS). NO has many diverse and important biological functions in the human body such as playing a major role in the immune system in the defence against pathogen bacteria by damaging bacteria through nitrosative stress. This can occur directly from NO but also from other RNS that are formed by NO (e.g. nitrosonium ion NO^+, peroxynitrite $ONOO^-$). NO is generated by macrophages, monocytes and neutrophils via the action of inducible nitric oxide synthases, which is induced by bacterial LPS [3] and converts L-arginine into citrulline and NO. NO is highly lipophilic and can cross of the cell membrane of bacteria, and there are many cellular targets for RNS. For example, NO can cause DNA damage and can inhibit DNA repair and synthesis [4]. NO stress can also cause damage to proteins via *S*-nitrosylation [5], tyrosine nitration [6] or ADP ribosylation [7]. NO also has broad-acting toxicity by binding to haem groups, Fe-S clusters and zinc fingers [8,9].

As well as being a signal NO has a potent antimicrobial effect, in the presence of dioxygen or superoxide NO can be further oxidised to nitrite (NO_2^-) or peroxynitrite ($ONOO^-$) which are strong oxidising agents which can damage DNA, protein and cell walls. Interestingly, some bacteria are known to have their own nitric oxide synthase

enzymes and purposefully synthesise their own NO when in their host. This is due to NO being a known modulator of proteins for instance some bacteria have proteins that when S-nitrosylation of specific cysteine thiols occur specific genes related to NO tolerance and the detoxification of NO are upregulated [10]. Also, it has been reported that NO can diminish the efficacy of certain antibiotics [11], which could provide a growth advantage to NO-producing bacteria if their environmental niche is shared by antibiotic-producing bacteria or fungi.

1.2 - Campylobacter jejuni and disease

C. jejuni is a motile microaerophilic zoonotic Gram-negative bacterium with curved bacilli morphology. As an enteric pathogen, *C. jejuni* is often found in faeces. It has clinical importance in the pathology of gastroenteritis and is one of the most common causes worldwide. Around 50% of patients are considered asymptomatic which may increase the spread of the pathogen especially in unsanitary conditions. Gastroenteritis is characterised by crippling abdominal cramps, diarrhoea and fever however most cases are usually resolved in under a week without the treatment with antibiotics. *C. jejuni* is also implicated in the triggering of more severe diseases such a Gulliane-Barre syndrome (GBS) and is a common trigger in around 30% of all cases. GBS is an acute demyelinating disease of the peripheral nervous system and can lead to extensive axonal injury and even irreversible neurological damage. Significantly GBS resulting after *C. jejuni* infection is usually more severe [12].

1.3 - Bacterial NO-sensing transcription factors

There are several bacterial transcription regulators that sense NO, these sense NO using various molecular mechanisms. These can then up regulate expression genes involved in NO detoxification [13]. This prevents build-up of NO to toxic concentrations and can also up-regulate genes involved in DNA repair from damage that has already occurred [14]. Bacterial NO-sensing transcription factors are divided into 2 main groups, those with a primary role in NO sensing such as *Pseudomonas aeruginosa* DNR which senses NO via a prosthetic haem group, and those with secondary role such as FNR in *E. coli* [15–17]: FNR senses NO with an iron-sulphur

cluster group which has a primary role in sensing oxygen [16,17]. There are other regulator genes that have a secondary role sensing NO such as SoxR and IscR which have primary roles as redox stress sensors. Bacterial NO-sensing in pathogens is suggested to have gradually evolved from soil denitrifying bacteria through horizontal gene transfer [13]. An example of a potential FNR ancestral protein is FnrP found in the soil denitrifying bacteria *P. dentrificans* [18]. *E. coli* is a good model for NO-sensing transcriptional regulators as it has 2 well-characterised NO-sensing and detoxification systems. The first of which is the NorR regulator that senses NO and up-regulates the NorVW detoxification system [19]. The second of which is the NsrR transcription factor which has a widely accepted role as a global regulator of the bacterial NO stress response. NsrR is a member of the Rrf2 superfamily of transcriptional repressors and regulates a large regulon of around 60 genes via de-repression in the presence of NO [20,21]. This regulon includes the flavohaemoglobin Hmp enzyme which is a common NO detoxifying enzyme that detoxifies NO to nitrate or nitrous oxide via well-characterised mechanisms both under aerobic and anaerobic condition [22].

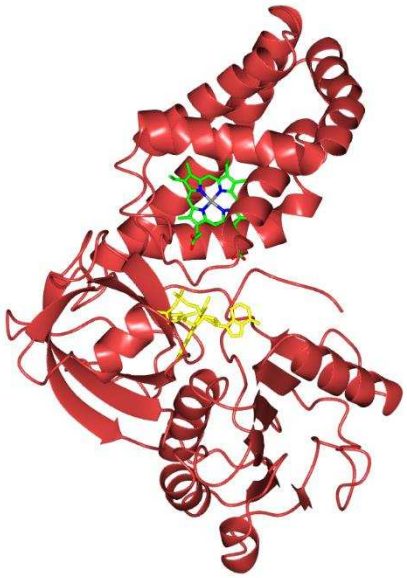

Figure 1.1 – Structures of Hmp of *E.coli.* Crystal structural model of NO detoxification enzyme Hmp with reduction domains highlighted, b-type haem cofactor (green) and bound oxidoreductase FAD (yellow) (PDBid = 1GVH)

1.4 – The NssR regulon of *C.jejuni*

Campylobacter jejuni lack the majority of NO sensing and detoxification systems in *E. coli*, but has a small regulon that is positively-regulated by NssR in response to NO [23]. This regulon incudes genes encoding two globin proteins, Cgb and Ctb (Cgb single domain globin, Ctb truncated globin). Cgb is thought to detoxify NO directly, whereas the role of Ctb is more poorly understood and has been implicated in oxygen metabolism [24,25]. The interplay between NssR-mediated NO sensing and Cgb-mediated NO detoxification is shown in Figure 1.2 NO is indirectly sensed by NssR which leads to up-regulation of Cgb expression leading to detoxification of NO.

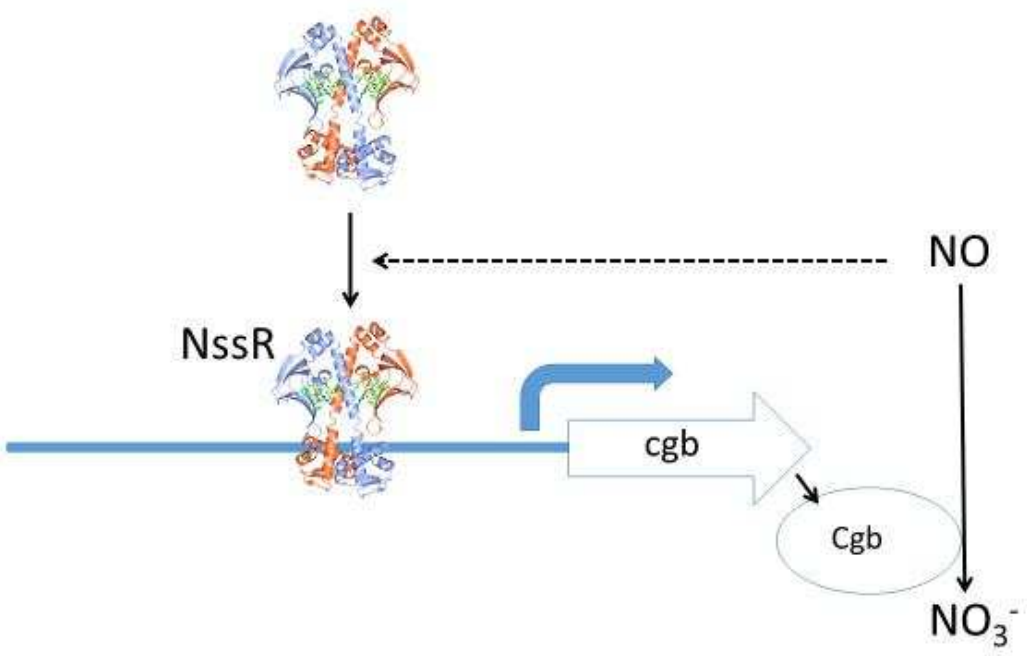

Figure 1.2 - Simplified model of mechanism of NO sensing and detoxification in *C. jejuni by the NssR detoxificstion system*. NssR activity is positively regulated indirectly by NO. The increased activity of NssR positively modulates Cgb expression leading to the NO detoxification to ni*trate. adapted from Monk et al(2008)*[26].

NssR is distinct from NsrR of *E. coli* and is a part of the Crp-Fnr superfamily [13,23]. However, the molecular mechanism for NssR remains elusive as NssR does not appear to exhibit enhanced DNA binding in the presence of its cognate signal (NO) [25]. NssR controls a small regulon involved in responding to nitrosative stress, other genes upregulated by this response have been implicated through proteome and

transcriptome profiling and include heat shock protein, thioredoxin reductase and alkylhydroperoxide reductase [26]. It is thought that Hsp plays a role in responding to unfolded and damaged proteins that have resulted from nitrosative stress. The seven remaining genes of the NssR regulon are involved in iron metabolism and transport [26], which is unsurprising given the importance of Fe-S clusters and haem as targets for NO binding.

1.5 - NO detoxification by *C. jejuni*

C. jejuni possesses two globin proteins involved in nitric oxide detoxification (Figure 1.3), both of which are up-regulated by the NO sensing transcription factor NssR [25]. Cgb is a single-domain globin implicated in the detoxification of NO radicals and Ctb is a truncated globin with function that is not fully understood, although it is suggested that plays a role in oxygen metabolism [24,25].

It has been suggested that Cgb detoxifies NO through the well-characterised dioxygenase and denitrosylase reactions (shown below), where O_2 and NO bind first to Cgb, respectively.

Dioxygenase reaction

$$Cgb - Fe(II) + O_2 \leftrightarrow Cgb - Fe(II)O_2 + NO^{\circ} \rightarrow Cgb - Fe(III) + NO_3^-$$

Denitrosylase reaction

$$Cgb - Fe(II) + NO^{\circ} \leftrightarrow Cgb - Fe(II)NO + O_2 \rightarrow Cgb - Fe(III) + NO_3^-$$

Which reaction predominates during infection is contentious. It is predicted that during the initial phase of infection that there is enough O_2 present that the dioxygenase reaction is more likely, whereas during the late stages of infection anoxic conditions and elevated NO would favour the denitrosylase reaction [24].

Ctb is a truncated globin that does not directly detoxify NO but has been implicated in roles supporting this via oxygen metabolism and also delivery of oxygen to Cgb under hypoxic conditions[26–28]. More recently it has been proposed that

Ctb dampens the response to NO under hypoxic conditions, this would suggest that NO detoxification has an oxygen dependent mechanism[25].

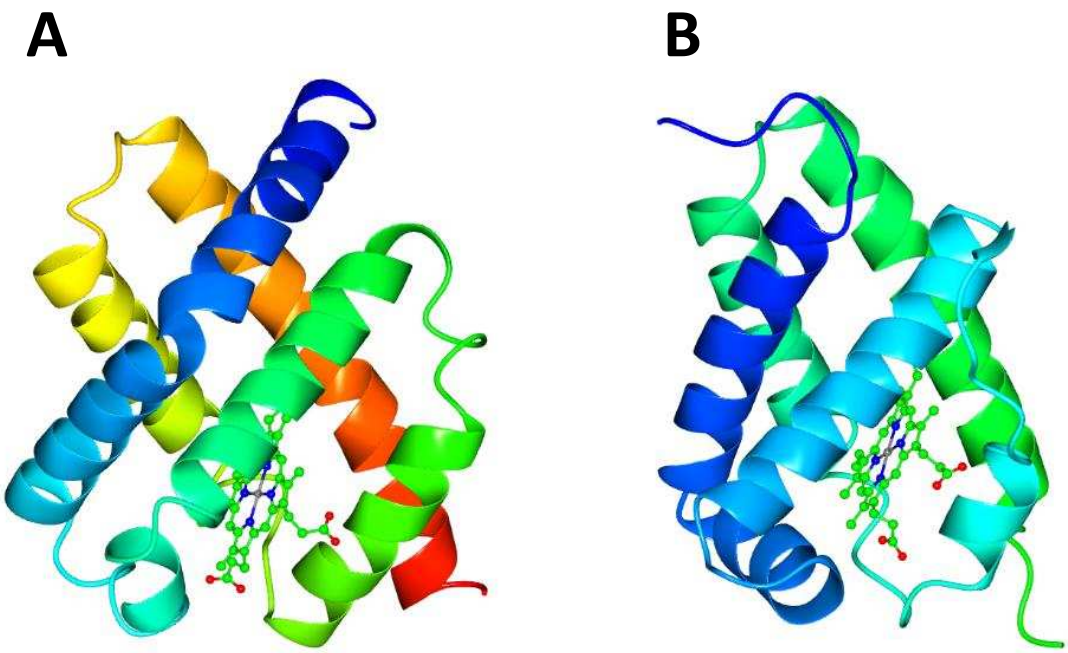

Figure 1.3 – Structures of the globin proteins of *C. jejuni.* A) The 'three over three' α-helical fold of Cgb (PDBid = 2WY4) with bound haem cofactor [29] B) The 'two over two' α-helical fold of Ctb (PDBid = 2IG3) with bound haem cofactor [30]

C. jejuni is also known to possess a NrfA nitrite reductase, with homologues of this enzyme being found in a large range of bacteria [31,32]. The primary role of NrfA is in anaerobic respiration, but NrfA of *C.jejuni* has been shown to provide tolerance to NO independent of the NssR regulated detoxification system [23] However, mutation of *cgb* and *nssR* genes have been shown to cause severe growth inhibition in the presence of NO whereas mutation of *nrfA* had only resulted in slightly slowed growth. This suggests that the NssR-regulated NO detoxification system plays a more important role in detoxification of NO [31].

1.6 - Cyclic nucleotides as bacterial signals

NssR has been shown to possess a cyclic nucleotide binding domain and contains sequence similarities to the helix turn helix DNA binding domain of the transcription factor Crp [33]. Proteins the have a cyclic nucleotide binding domain are likely to bind cAMP or cGMP and possess a common domain of around 120 amino acids composed of three alpha-helices and a distinctive eight-stranded, antiparallel beta-barrel structure. The archetypal Crp family member is the cAMP receptor protein Crp which is a known global regulator of genes largely involved in energy metabolism. c-GMP is a well-characterised signal in eukaryotic cells and is an excellent signalling molecule having a much higher stability than cAMP. However, in bacterial cells there is little evidence of its use as a signal. Conversely, the dimerised form of c-GMP, c-di-GMP, has a well-characterised role in NO sensing in *P. aeruginosa* where phosphodiesterase and diguanylase cyclase enzymes, which control c-di-GMP levels, have previously been shown to be modulated by NO [34,35]. Hence, c-di-GMP is a strong candidate for being a second messenger in NO-mediated activation of NssR activity. It was therefore hypothesised that increased levels of c-di-GMP will elevate NssR activity increasing Cgb expression (Figure 1.4).

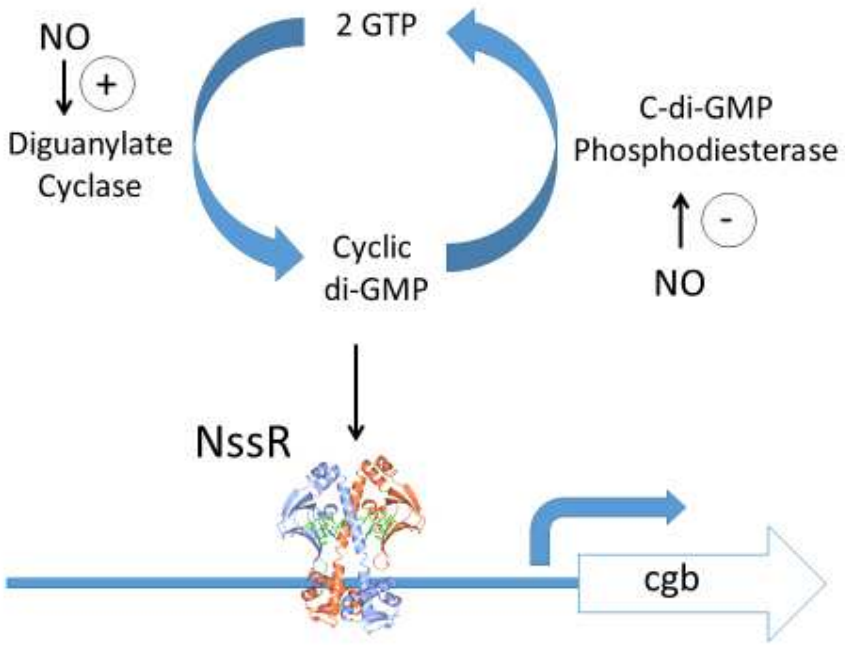

Figure 1.4 – Hypothesis for the regulation of NssR by nitric oxide and cyclic-di-GMP.

1.7 Project aims

This work aims to confirm NssR as a cyclic nucleotide binding protein through structural modelling and biochemical/spectroscopic binding experiments. Further aims include identification of the exact nucleotide that binds to NssR *in vivo*, and to test the hypothesis that c-di-GMP stimulates NssR-mediated transcriptional activation via *in vitro* and *in vivo* approaches.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 2.1.

Strain description	**Plasmids**	**Antibiotic resistance**	**Source**
E. coli TOP 10	-	-	Invitrogen
E. coli TOP 10	pGEM-T-*cgb* promoter	Ap	Smith et al (2011)
BL21 DE3	pGEX-KG-N-nssR	Ap	Smith et al (2011)
E. coli TOP 10	pSB1C3-Prom/RBS	Cm	Shepherd lab
E. coli TOP 10	pSB1C3-*cgb* promoter	Cm	This work
E. coli MC1000	-	-	Spiro & Guest (1987)
E. coli MC1000 Δ*yfgF*	-	Cm	Lacey et al (2010)
E. coli TOP10	pRS415-*cgb* promoter -*lacZ*	Ap	Shepherd lab
E. coli TOP10	pSU2718-G-nssR	Gm	Shepherd lab
E. coli MC1000	pRS415	Ap	Shepherd lab
E. coli MC1000	pRS415-*cgb* promoter-*lacZ*	Ap	This work
E. coli MC1000	pSU2718-G-nssR	Gm	This work
E. coli MC1000	pRS415	Ap	This work
E. coli MC1000	pRS415-*cgb* promoter-*lacZ* pSU2718-G-nssR	Ap, Gm	This work
E. coli MC1000 Δ yfgF	pRS415-*cgb* promoter-*lacZ*	Ap, Cm	This work
E. coli MC1000 Δ*yfgF*	pSU2718-G-*nssR*	Cm, Gm	This work
E. coli MC1000 Δ yfgF	pRS415	Ap,Cm	This work
E. coli MC1000 Δ yfgF	pRS415-cgb promoter-*lacZ* pSU2718-G-nssR	Ap, Cm, Gm	This work

***Table 2.1*. Bacterial strains and plasmids used in this work.** Working concentrations: Ap, Ampicillin (100µg/ml); Cm, Chloramphenicol (25µg/ml); Gm, Gentamycin (20µg/ml).

Bacterial strains were streaked out on LB Agar plates supplemented with appropriate antibiotic at working concentrations displayed below table 2.1 and grown overnight in a 37^{o}C incubator. For preparation of starter cultures generally 1 colony was inoculated in a sterile 50ml conical flask containing 10 ml of LB media supplemented with appropriate antibiotics as previously mentioned and then placed in a 37^{o}C shaking water bath for growth overnight.

2.1.2 Preparation of Growth Media

2.1.2.1 Luria Bertani (LB) medium

LB medium contained 10g tryptone, 5g yeast extract and 5g NaCl per litre. pH was adjusted to 7.0 using NaOH then medium was autoclaved.

2.1.2.2 Super optimal broth (SOB)

To prepare SOB medium, 20g tryptone, 5g yeast extract, 0.584g NaCl and 0.186g KCl was added to 990ml of milli-Q water and autoclaved. Once cooled to below 60^{o}C, 10ml of sterile 2M Mg^{2+} stock was added. To prepare the 2M Mg^{2+} stock, 20.33g MgCl2 and 24.65 g $MgSO_4$ were brought 100 ml with milli-Q water then filter sterilized with 0.2μm filter.

2.1.2.3 Super optimal broth with catabolite repression (SOC)

To prepare SOB medium, 20g tryptone, 5g yeast extract, 0.584g NaCl and 0.186g KCl was added to 970ml of milli-Q water and autoclaved. Once cooled to below 60^{o}C, 10ml of sterile 2M Mg^{2+} stock and 20ml of 1M glucose stock was added. To prepare the 2M Mg^{2+} stock, 20.33g MgCl2 and 24.65 g $MgSO_4$ were brought 100 ml with milli-Q water then filter sterilized with 0.2μm filter.

2.1.2.4 2X YT media

To 900ml distilled H_20 the following was added. 16g Bactotryptone, 10g yeast extract, 5g NaCl. pH was then adjusted to 7 10M NaOH and then distilled H_2O was added to 1L total volume.

2.2 Genetic methods

2.2.1 Isolation of plasmid DNA

A single colonies of E. coli strains harbouring plasmid required were picked to inoculate 10ml of LB supplemented with appropriate antibiotics of their respective antibiotic resistances to working concentration as illustrated in table 1 and incubated overnight at 37^{o} C and 180rpm. Plasmids were miniprepped following QIAprep Spin Miniprep Kit (from QIAGEN) protocol using a microcentrifuge with 4ml of each overnight culture.

2.2.2 Confirmation of plasmid integrity

Plasmid lengths were confirmed by linearizing the plasmids by restriction digestion. For a single digest (20 µl volume):
In a 1.5 ml eppendorf tube the follwing were added, 2 µL 10X appropriate enzyme buffer, 1.5 µL appropriate restriction enzyme and purified plasmid DNA. Generally 3µL of plasmid was used but the volume will depend on the concentration of your DNA. H_2O was then added to bring the final volume up to 20 µl.

For a double digest (20 µl volume): In a 1.5 ml eppendorf tube the follwing were added, 2 µL 10X appropriate enzyme buffer, 1.5 µL restriction enzyme 1, 1.5 µL restriction enzyme 2 and purified plasmid DNA. Generally 3µL of plasmid was used but the volume will depend on the concentration of your DNA. H_2O was then added to bring the final volume up to 20 µl.

Incubation times and temperatures varied for use with different restriction enzymes. Digestion was confirmed using agarose gel electrophoresis.

2.2.3 Oligonucleotides

The oligonucleotides used in this work are detailed in Table 2.2.

Name	5'-3' Sequence	Use	Direction
pRS415_seqFW	CCGCCATAAACTGCCAG GAATTGGG	Colony PCR pRS415-*cgb*	Forward
pRS415_seqREV	CGCGTCGCCGCTTTCAT CGG	Colony PCR pRS415-*cgb*	Reverse
pSU2718nssR_seqF	AAAAGCACCGCCGGAC ATCA	Colony PCR pSU2718-G-*nssR*	Forward
pSU2718nssR_seqR	CGAATTCGAGCTCGGTA CCC	Colony PCR pSU2718-G-*nssR*	Reverse
SP6 R	TATTTAGGTGACACTAT AG	Amplification of *cgb* promoter for gel shift assays	Forward
T7	TAATACGACTCACTATA GGG	Amplification of *cgb* promoter for gel shift assays	Reverse
Cgb_fwEcoRI	CCCGAATTCAAGCTTTA AAATTTGCCATAG	Amplification of *cgb* promoter fragment for cloning into pSB1C3 backbone for IV transcription	Forward
Cgb_rvPSTI	CCCCTGCAGTCAGGAAG ATCGCACTCCAGCCAGC TT	Amplification of *cgb* promoter fragment for cloning into pSB1C3 backbone for IV transcription	Reverse

***Table 2.2*. Oligonucleotides used in this work.** Oligonucleotides were designed and then checked using the primer design feature of sequence analysis and design software Vector NTI to ensure that G/C content of primer was appropriate. Oligonucleotides were produced by MWG-biotech.

2.2.4 Agarose gel electrophoresis

Electrophoresis was performed in a gel apparatus at a constant voltage of 150v in 1X TAE buffer (40mM Tris-acetate and 1mM NaEDTA). Gels were generally composed of 1% agarose dissolved in 1X TAE, however if fragments were expected to be below 0.5kb a 2% agarose gel was used. 1µL of 6X Blue/Orange load dye (Promega) was mixed with 5µL of sample before loading in gel wells. A 1kb or 100bp ladder (NEB) was resolved on each gel to determine the size of fragment samples. Gel were stained in ethidium bromide diluted in water 1:20000 dilution (5µL in 100ml) rocking for 30 minutes before visualising bands on a Syngene G:BOX gel dock.

2.2.5 Preparation of chemically competent cells

100ml of sterile LB with appropriate antibiotics for was inoculated with 1mL of overnight culture in a 250mL sterile flask and incubated at 37 ^{o}C and 200 rpm until an OD_{600} of 0.4-0.5. When the correct OD_{600} was reached, cultures were incubated on ice for 10 min prior to being harvested by centrifugation at 3000 rpm and 4 ^{o}C for 8 min in falcon tubes. The supernatant was then removed and remaining pellet was resuspended in 25mL of ice cold $CaCl_2$ prior to being incubated on ice for a further 10 min. The cells were then harvested again under the same conditions then the pellet resuspended in 2mL of 100mM ice-cold $CaCl_2$ and 2ml of 30% (v/v) glycerol and aliquoted 100µL per Eppendorf tube prior to storage at -80^{o}C.

2.2.6 Transformation of *E. coli* strains

50-100ng of plasmid DNA was added to 100 µl competent cells mixed gently by tapping on the tubes and then incubating on ice for 1h. Cells were then heat shocked at 42 °C for 2 min before being placed back on ice for a further 15 min. 1ml of LB was added to the cells and then incubated at 37 °C, shaking at 200 rpm for a further 1 h. Cells were then harvested by centrifugation at 5000 rpm for 5 min. 1mL of the

supernatant was gently removed and pellet was resuspended in 100 µl LB that remained in the tube. The resultant culture was then plated onto agar plates with appropriate antibiotics then incubated at 37 °C overnight.

2.2.7 Preparation of electro-competent cells

50 ml SOB was inoculated in a 250 ml flask with 1mL of overnight culture (LB) and then grown at 37°C and 180rpm to an OD_{600} of approximately 0.4-0.8. Cells were then kept on ice from this point on and transferred to two 50 ml sterile Falcon tubes. The culture was then centrifuged for 10 min at 4°C (3000 x g) and the pellet was resuspended in 5 ml ice-cold 10% glycerol by gently pipetting up and down. The centrifugation and washing with 10% glycerol was repeated three times. The cells were then centrifuged and the pellet was resuspended in 200 µl 10% glycerol and transferred to a cold 1.5 ml eppendorf tube and kept on ice.

2.2.8 Electroporation of plasmid DNA

500-1000ng of purified plasmid or water for the control were added to the side of a sterile electro-cuvette on ice. 40 µl of electro competent cells were then added to the electro-cuvette and electroporated using the following settings: Voltage (V) 2450V, Capacitance 25µF, Resistance 200 Ω and Cuvette 2mm. 1 ml of SOC was then added to the electro-cuvette and culture was transferred to a new sterile 1.5mL Eppendorf tube before being subjected to gentle agitation for approximately 1 hour at 37°C. The culture was then centrifuged for 5 min at 5000 rpm and 940 µL of the resulting supernatant was removed. The pellet was then resuspended gently in the remaining 100 µL of culture then plated onto a LB agar plate with antibiotic resistance of respective plasmid and incubated overnight at 37°C.

2.2.9 Screening transformants via colony PCR

Individual colonies from transformation plates were patched onto new plates with appropriate antibiotics. Antibiotics were at at working concentrations indicated in Table 2.1. From these patched plates Colony PCR was carried out to confirm the identity of plasmids by performing PCR with plasmid-specific primers and then resolving the fragments via agarose gel (1 % TAE) electrophoresis.

The cell suspension was prepared by suspending a single colony into 50µL of sterile water in an Eppendorf tube. In sterile PCR tubes, the following solutions were added: 9.5uL sterile water, 0.5uL forward primer from 10 µM stock, 0.5µL reverse primer from 10µM stock , 2µL of cell suspension and 12.5uL 2x PCRBIO Taq Mix Red (primers used for pRS415, pRS415-*cgb* and pRS415-*ctb* plasmids were pRS415-seqF and pRS415-seqR and for pSU2718-G-nssR plasmid primers pSU2718nssR_seqF pSU2718nssR_seqR). The following programme was then ran on the PCR machine:

95°C for 4 min

95°C for 15 s
57°C for 15 s - 35 cycles
72°C for 30 s

72°C cycle for 2 min

PCR products were analysed on a 1% (v/v) agarose gel (Section 2.2.4).

2.2.10 cloning of *cgb* promoter

A 364 bp fragment of 'pRS415-*cgb* promoter' (Appendix A2) containing the *cgb* promoter and a portion of the *lacZ* gene was cloned via standard PCR using Q5 high fidelity polymerase from NEB (2X master mix) and the primers given in Table 2.2. The generated insert was purified using QIAquick PCR purification kit (QIAGEN) following their provided protocol. As the psB1C3 backbone already had an insert (Appendix A4) the plasmid had to be digested by restriction enzymes EcoRI and PstI as described in (2.2.2) and resultant desired backbone fragment had to be purified by gel extraction from a 1% gel as described in (2.2.4). QIAquick Gel extraction kit (from QIAGEN) protocol was followed using a micro centrifuge to isolated the

anticipated fragment. DNA concentrations of insert and backbone were quantified using a NanoDrop to determine required quantities for ligation. The *cgb* promoter fragment was then ligated into the EcoRI/PstI sites of the pSB1C3 vector upstream of the rrnB terminator (Figure 3.10A). Ligations were performing using T4 DNA ligase from promega following their protocol and then where transformed into chemically competent TOP 10 cells prepared as indicated in section 2.2.5 to complete the ligation reaction. Plasmid was then checked by restriction digestion using EcoRI/ PstI as described earlier.

2.2.11 IV transcription experiment

In a 20µL reaction 5µM NssR was incubated with 8nM pSB1C3-*cgb* promoter plasmid, 0.5mM of each ribonucleotide (4µL of a ribonucleotide solution mix from NEB containing 25mM of each nucleotide), 50µM of each cAMP, cGMP and c-di-GMP and 1X reaction buffer provided with the RNA polymerase. After a 20 minute pre-incubation at 37^{o}C, 2 units of RNA polymerase holoenzyme from NEB saturated in sigma factor 70 was added and a further incubation of 20 minutes at 37^{o}C was carried out. To stop the reaction 60µL of Ambion NorthernMax formaldehyde Load Dye was added and then incubated for 15 minutes at 65^{o}C to denature any secondary RNA structures. 40µL of this sample was loaded onto a 5.5% TBE gel run at 150V and resultant gel was visualised with Sybr green II RNA stain.

2.3 Biochemical methods

2.3.1 Purification of NssR

2.3.1.1 Protein expression

A 10ml Starter culture of BL21 strain containing the pGEX-kG-nssR plasmid were transferred into 2L baffled flasks containing 1L of 2X YT growth media supplemented with Ampicillin (100µg/ml) under sterile conditions. The culture was then grown in shaker at 30^{o}C at 200 rpm until an OD of 0.4-0.5 had been reached. When the correct OD of the cultures had been reached IPTG was added to a final concentration of 0.4mM to induce overexpression of NssR. The cultures were then

incubated for a further 5 hours. After 5 hours the cells were then harvested by centrifugation at 6000Rpm for 20 min and 4°c. The resulting pellets were frozen at -20°C in preparation for future purification.

2.3.1.2 Purification with GSTrap column

A pellet from 1L culture was resuspended in 30 ml cold PBS pH 7.3 (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4) and then sonicated on ice for 6x30 seconds at an amplitude of 10 microns to release the overexpressed protein from the cell. Between each 30 second burst, sonication was stopped for 30 seconds to prevent heat damage of protein. A 20µL sample was taken for an SDS PAGE gel labelled as Sn. To remove cell debris the sonicated sample was then centrifuged at 15000rpm for 20 minutes at 4°c and then kept on ice. In preparation for purification by affinity chromatography a 5ml GSTrap column from GE lifecare was equilibrated with 5 column volumes of PBS at flow rate 1.5ml/min using a peristaltic pump. After the column was equilibrated the Sn was applied and a sample of the flow was taken for SDS page (labelled FT), the column was then washed with 100 ml of PBS to remove any protein or debris not bound to GST beads.

To elute the protein from the column 20 units of thrombin were added to 5ml PBS applied to the column flow stopped and left overnight to fully digest the GST tag. NssR was then eluted with 15ml of PBS into 1ml aliquots. Location of protein in these aliquots was determined using the Bradford reagent. If it was necessary to keep the GST tag intact when eluting NssR rather than thrombin 15ml of 10mM reduced glutathione, 50mM Tris-HCL pH 8.0 was used and no overnight digestion was necessary.

2.3.1.3 Heparin column removal of bound nucleotide

Sample was buffer exchanged using a 10Kda vivaspin concentrator then diluting out with Heparin equilibration buffer (50mM NaH_2PO_4 pH 7.0). 5ml Heparin column from GE lifecare was equilibrated with 10 column volumes of the phosphate buffer at 1-5ml/min using peristaltic pump. The sample was then applied and column washed with 5-10 column volumes of phosphate buffer. The initial 10ml of this flow through was caught in 1ml aliquots and put aside for later spectral analysis to identify location of eluted bound substance. The unbound NssR was then eluted with 10ml of high salt buffer (50mM NaH_2PO_4, 1M NaCl pH 7.0) a 20µL sample was taken for SDS PAGE labelled Heparin elution.

2.3.2 SDS PAGE

SDS PAGE was used to confirm purity of NssR at the various stages of purification as described in 2.3.1. A 4% stacking gel was cast on top of a 15% resolving gels (once the resolving gel had set for approximately 45- 60 minutes. Samples were prepared by adding loading dye in a 1:1 ratio. Loading dye used had the following composition, 50mM Tris, 2 % SDS, 0.1 % Bromophenol blue, 10 % Glycerol and 100mM DTT which was added just before use at pH 6.8. Gels were run at 150v in 1X SDS running buffer (0.1% w/v SDS, 25mM Tris, 250mM glycine, pH 8.3) until the dye front was approximately 1cm from the bottom of the glass plate. Gel were then stained in instant blue for 20 – 30 minutes with no distaining required. The recipe for SDS PAGE gel is shown in table 2.3 below.

	Volumes requires (ml)	
Chemicals	**15%**	**4%**
Acrylamide 40%	3.8ml	1ml
H_2O	3.5ml	6.3ml
1.5M Tris pH 8.8	2.5ml	-
0.5M Tris pH 6.8	-	2.5ml
10% SDS	0.1ml	0.1ml
10% APS	0.1ml	0.1ml
TEMED	0.004ml	0.01ml

Table 2.3 Recipes for 15% and 4% SDS PAGE gel. *TEMED is added immediately before casting

2.3.3 Mass spectrometry identification of NssR bound ligand

HPLC/MS analysis was performed using HPLC Agilent 1100 system coupled to micrOTOF-Q11 Mass spec from Bruker on negative electrospray mode. Separations occurred on Nucleosil 120A c18 column with 3µm (bead size) and dimension 150mm x 2mm. The protocol was adapted from (Van Damme 2012) in which cAMP and cGMP were identified from human plasma samples[36]. Flow rate was adjusted to 5ml/min from to compensate for using a 150mmx 2mm column rather than 150mm x 3mm. Solvent A was 0.1% formic acid and solvent B acetonitrile/methanol/water in volume ratio 1/2/4. A linear gradient was used with initial conditions 100% A and increasing solvent B from 0% to 50% in 10 min. Solvent B then went to 100% B in 1 min to clean the column and maintained for 1 min, followed by column regeneration for 10 min. Sample injection size was 20µL and 3 replicates were carried out. Rather than using negative-ion multiple reaction monitoring mode (MRM) which is time consuming and costly to set up a more generic fragmentation method was used. 25eV alternating between fragmented and intact and measured in the range of 50 – 1250m/z. Negative electrospray was used with capillary voltage 2300v. For identification of Cyclic nucleotides EIC

(extracted ion chromatograms) were used to look for qualified ions identified in the previously mentioned paper.

2.3.4 Co-purification of NssR with cAMP and cGMP

NssR was purified with GST tag still intact as described in 2.3.1.2 was then concentrated using 10kda viva spin concentrator and protein concentration identified using a NanoDrop. To 10µM NssR, 500µM cAMP or cGMP were added incubated for 30 minutes the sample was then added to an equilibrated GST column, which was then washed then left overnight to digest with thrombin and then eluted the next day as described in purification protocol 2.3.1. A control experiment was also carried out adding no substrate.

2.3.5 Tryptophan fluorescence assay

Fluorescence Titrations were performed in a Fluorimeter using 2ml of 0.2µM NssR protein unbound by nucleotide (diluted in 50mM NaH_2PO_4 pH 7.0) in a 2ml quartz fluorescence cuvette. NssR stocks were made and quantified as described in section 2.3.1 and 1mM Stocks of cyclic nucleotides cAMP, cGMP and c-di-GMP were prepared for use in the titrations. The protein was scanned for fluorescence over a wavelength range of 310nm – 400nm with an excitation wavelength of 295nm to avoid the shoulder of fluorescence readings of cyclic nucleotides as they absorb at around 260nm. The ligand of interest was then slowly titrated into the sample in fractions from 0.2µM to 10µM. Between each titration the sample was gently mixed by pipetting up and down then left briefly to equilibrate (few seconds) before another scan being taken over the same wavelength range. Increasing concentrations of ligand was successively added in the same increments until there was no apparent change in fluorescence. The titration was then repeated using the same ligand stocks and the fluorescent compound N-acetyl-tryptophanamide (NATA) as a control reaction toaccount for any change in fluorescence caused by the added ligand. The NATA was added until the fluorescence at 360nm was roughly equal to that of the original protein before adding the same quantities of ligand as before.

2.3.6 Electrophoretic mobility shift assay

Colony PCR with primers designed to anneal to the T7 and SP6 sites on the pGEM-T vector (table 2.2.) was used to amplify a 171 bp fragment containing the *cgb* promoter NssR-binding site. Target DNA (25 nM) and purified NssR were pre-incubated in binding buffer (20mM Tris–HCl, pH 7.8, 1mM EDTA, 3% glycerol) for 30 min at room temperature, in the presence and absence of 0.2mM c-di-GMP, cAMP and cGMP. The reaction mixtures were then re- solved on an 8% non-denaturing TBE polyacrylamide gel with a 4% stacking gel, and stained with ethidium bromide. Densitometry software (ImageJ) was used to quantify the relative abundance of NssR:DNA complex and un- bound target DNA.

2.4 β-galactosidase *in vivo* reporter system

To prepare Z buffer, the following were added to a 500mL Duran bottle: 300ml of miliQ water, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$. This was then stirred with a magnetic stirrer and adjusted pH to 7. The Duran bottle was then filled to 500 mL with milli-Q water and wrapped in foil to protect it from light and stored at 4^{o}c. Cells were grown anaerobically overnight by inoculating 45 ml of LB in a 50 ml falcon tube with a single colony with and incubated at 110 rpm and 28^{o}C. Immediately before experiments were conducted OD_{600} was measured.

The following was the added to glass test tubes and then vortex mixed thoroughly: 450µL Z buffer, 50µL of 1:100 dilution of culture, 5µL of 0.1% and 10µl CHCl. Tubes were then incubated at 28^{o}C for 5 min and then the reactions were started by adding 100 µL of 4mg/ml ONPG dissolved in Z buffer. When a pale yellow colour had developed reactions were stopped by adding 250 µL of 1M Na_2CO_3, making sure to take note of stop and start times for the assay. The OD_{420} and OD_{550} of these samples was then measured then miller equation used to work out miller units of expression

CHAPTER 3 - RESULTS

3.1 Structural modelling of NssR

To further investigate the hypothesis that NssR can bind cyclic nucleotides, structural modelling was carried out. The amino acid sequence of NssR was obtained from Uniprot and a structural model for NssR was generated using the online modelling program RaptorX [37]. RaptorX identifies protein crystal structures from the RCSB Protein Data Bank (PDB) that display structurally biased sequence homology to the input protein, and these templates are then used to model the desired structure. Table 3.1 shows the top 5 hits in rank order for proteins identified as suitable templates for structural modelling of NssR. These proteins are predominantly CRP-family transcriptional regulators that have been shown to bind cAMP, suggesting a potential role for cyclic nucleotide binding for the NssR mechanism.

Model Rank	Organism	Protein name	PDBid	Bound Ligand
1	*Thermus thermophilus*	Transcription regulator, Crp family	4EV0	cAMP
2	*Porphyromonas gingivalis*	Crp/Fnr transcription regulator	2GAU	cAMP
3	*Escherichia coli K-12*	cAMP-activated global transcriptional regulator CRP	4HZF	cAMP
4	*Ruminococcus obeum*	Cyclic nucleotide-binding domain protein	3DV8	cAMP
5	*Pseudomonas aeruginosa*	Cyclic AMP receptor-like protein	2OZ6	cAMP

Table 3.1 - PDB structures identified as suitable templates for NssR structural modelling. Model rank provides a measure of structural homology to NssR.

The most homologous of these template structures, CRP-like protein from *T. thermophilus* (3.1A) was therefore used as a comparison to ascertain the likeliness of cyclic nucleotides binding to NssR in the molecular graphics program CCP4MG. CCP4MG was used to initially identify the possibility that NssR could accommodate binding of cyclic nucleotides (figure 3.1). To achieve this, Cα atoms for template

and model were superimposed to model the cAMP ligand from the template into the binding cleft of the NssR model (Figure 3.1B). This initial investigation suggested binding pocket of NssR was sufficiently large enough to accommodate cyclic nucleotide binding. Subsequently CCP4MG was then used to identify whether important sidechains in the template (3.2A) were conserved in the modelled binding pocket of NssR (3.2B)

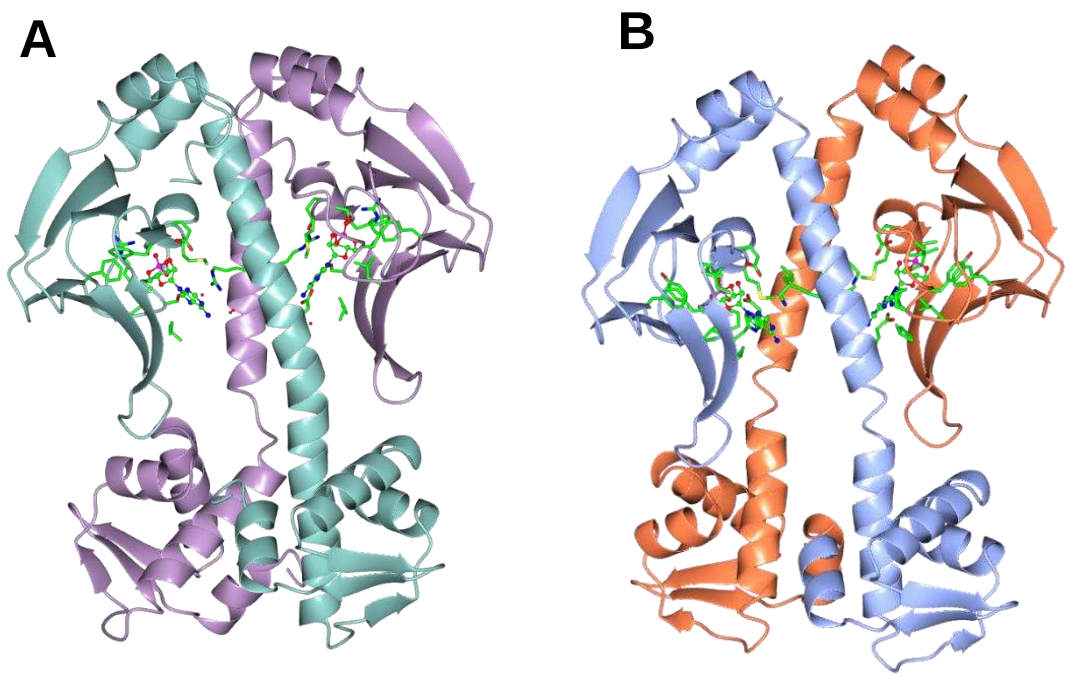

Figure 3.1 - Structural modelling of NssR. (A) Catabolite activator protein (PDB id = 4EV0). 4EV0 was used as a template to generate this structural model of NssR (B). The cAMP of 4EV0 is shown modelled into the binding cleft of each monomer of NssR shown in 3.1B.

Figure 3.2B displays a view of the NssR model looking into the binding cleft along the plane of the adenine ring of cAMP, with adjacent amino acid sidechains highlighted. This analysis suggests that the binding pocket of NssR may provide a suitable environment for cyclic nucleotide binding as many of the side chains are conserved between the 2 proteins in the same orientation and position. Also notably all interior

hydrophobic residues of binding pocket of Crp are conserved in NssR. The majority of substitutions that have occurred are between side chains which have similar characteristics or size, for example Leucine 53 in Crp being substituted for Isoleucine 43 in NssR Both are small hydrophobic residues with similar function. There are however notable exceptions to this, the most significant being both Arginine residues of Crp (residue 85 and 126) have been substituted with Tyrosine 75 and Lysine 116 respectively. This is notable as arginine residues are frequently found in binding sites and are especially are proficient in binding the negatively charged groups on phosphates. This is important for binding of cAMP as when it binds (shown in fig 3.1 and 3.2) its phosphate residues are buried deep within the protein. Lysine can also interact with phosphates in a similar way to arginine also being a positively charged residue however this interaction is more limited as it can form less H bonds with the negatively charged phosphate groups. There are 3 additional binding residues predicted in NssR not in positions found in Crp. These are glutamic acid, alanine and isoleucine. Both isoleucine residues and alanine are fairly non consequential for binding however glutamic acid residues are frequently involved in binding due to their short rigid nature and negative charge. These significant residue differences between the binding sites may result in cyclic nucleotides binding in a different conformation to NssR. [38].

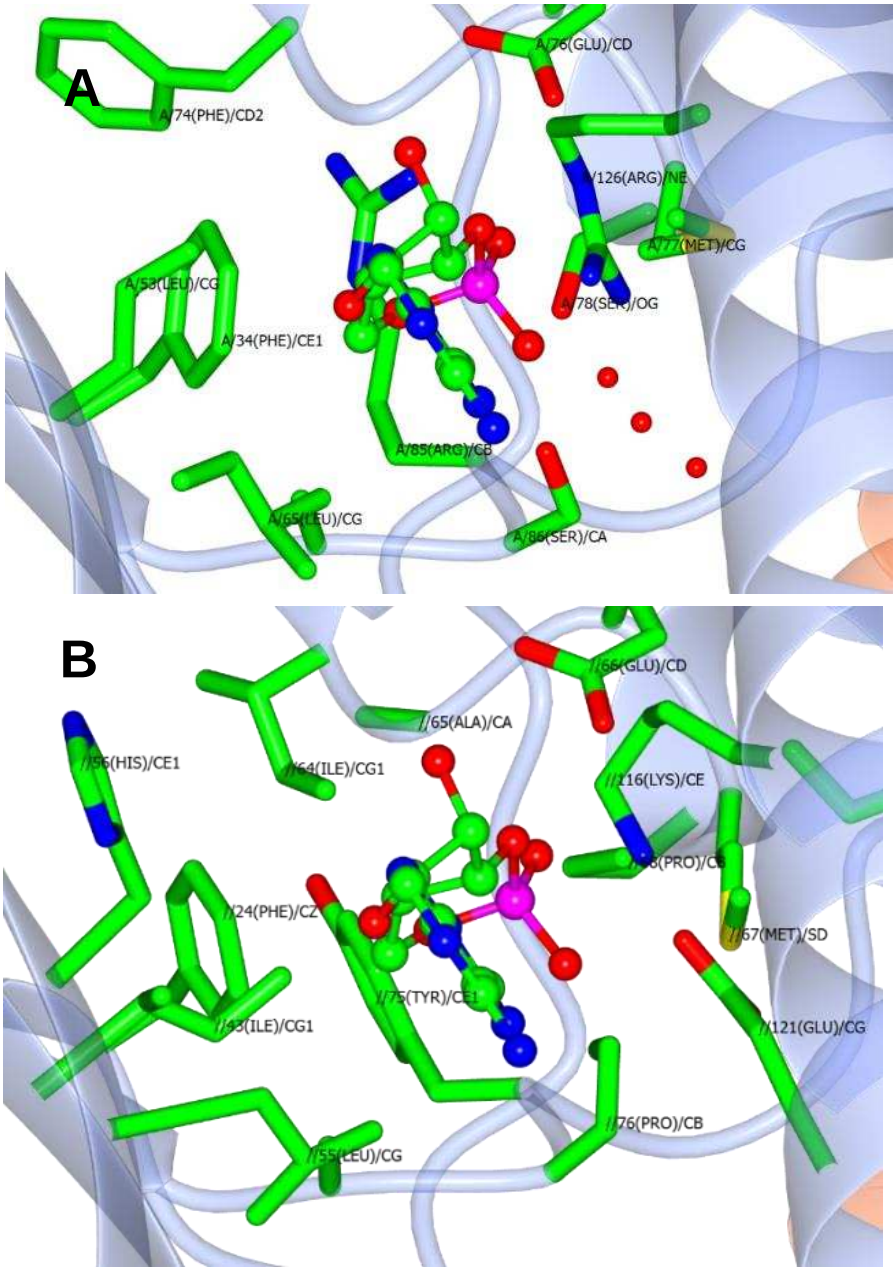

Figure 3.2 – Cyclic nucleotide-binding clefts of Crp and a NssR structural model. A) The cAMP-binding cleft of 4EV0 looking into nucleotide binding cleft along the plane of adenine ring of bound cAMP. B) The cAMP-binding cleft of the NssR model looking into nucleotide binding cleft along the plane of adenine ring of bound cAMP.

3.2 - Purification of NssR

NssR of *C. jejuni* was overexpressed and purified by affinity chromatography as described in Section 2.3.1. To determine purity, SDS-PAGE was carried out as described in Section 2.3.2 Results from the resolved gels (example in figure 3.3) show that a protein corresponding to the predicted molecular weight of untagged NssR (approximately 23kDa) was present in the elution fraction fractions from the GST and Heparin columns.

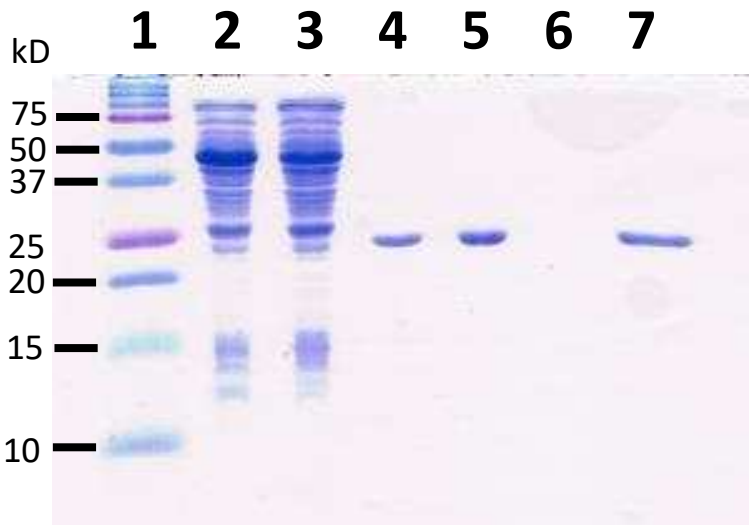

Figure 3.3 - Purification of NssR. NssR was purified via GST affinity chromatography followed by removal of bound nucleotides/DNA using a heparin column. Lane 1 Protein Marker, Lane 2 supernatant from pelleted sample, Lane 3 flow through from GSTrap column, Lane 4 1st protein elution from GSTrap column, Lane 5 2nd elution from GSTrap column, Lane 6 flow through from Heparin column, Lane 7 eluted Heparin treated protein.

Analysis of the purified NssR via UV/Vis spectroscopy in figure 3.4(A) shows that the purified protein has a sharp peak at 258nm typical of nucleotide. This would suggest that the protein was purified with the nucleotide bound. There is a clear observable shift to 280nm in fig 3.4 (B) after the protein has been treated in the heparin column as described in 2.3.1 confirming that the bound nucleotide has been removed and the protein is now unbound.

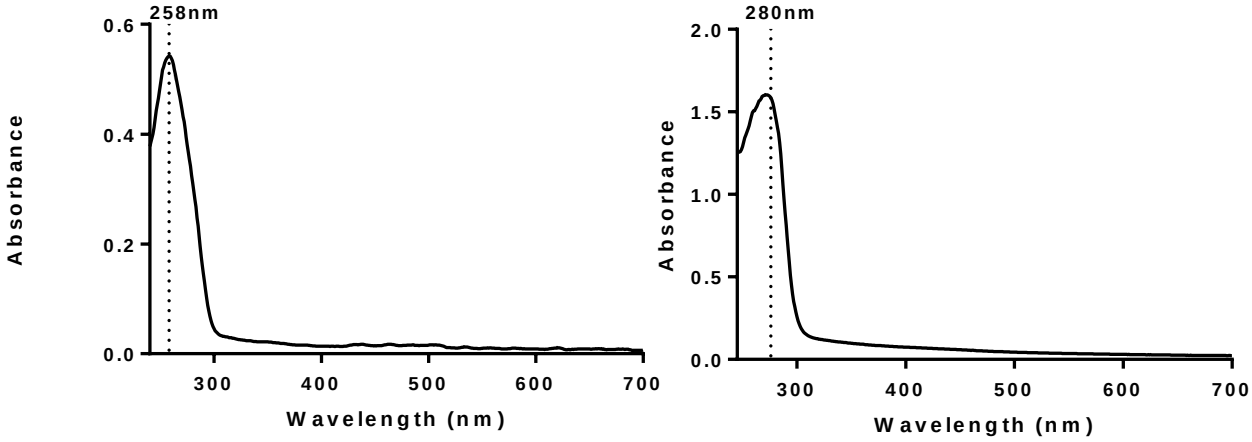

Figure 3.4 – UV-visible absorption spectroscopy of purified NssR. Absorption spectra for NssR eluted from the GST and heparin columns demonstrate that the heparin column removed bound nucleotides co-purified with NssR. (A) (absorbance maximum = 260 nm) associated with nucleotide (B) (absorbance maximum = 280 nm) associated with protein unbound by nucleotide.

3.3 Analysis of nucleotide co-purified with NssR via mass spectrometry

In an attempt to identify the nucleotides that were co-purified with NssR (Section 3.2), the heparin flow through fraction (Figure 3.3) was collected for further analysis via mass spectrometry. This sample was confirmed to have the expected absorbance characteristics of a peak around 255-260nm characteristic of nucleotides (Figure 3.5).

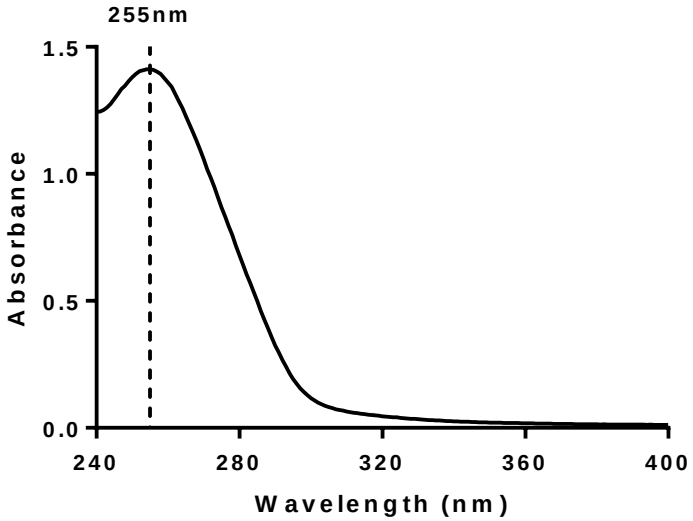

Figure 3.5 – UV-visible absorption spectroscopy of nucleotide that is co-purified with NssR. An absorption scan was recorded for the heparin flow through fraction (Figure 3.3).

A HPLC/MS approach (Section 2.3.3) was undertaken in an attempt to identify the nucleotide that is co-purified with NssR. This approach is based upon a previously characterised technique that has been used to identify cGMP and cAMP from human blood plasma[36]. This work identified a species with a mass peak at 154.9 Da (Figure 3.6), which could potentially be a protonated guanine base, possibly resulting from the fragmentation of c-di-GMP during the mass spectrometry process. Other notable peaks at 112.9 and 248.9 are due to sodium trifluoracetate that was in solvent A (0.1% formic acid).

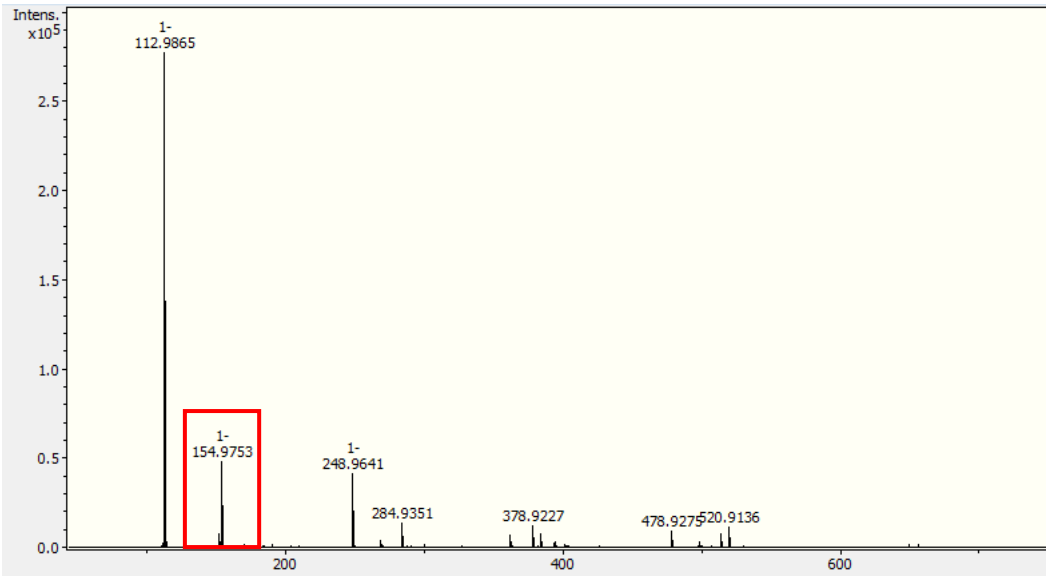

Figure 3.6 – HPLC/Mass spectrometry analysis of nucleotide that is co-purified with NssR. HPLC/MS analysis was performed on the heparin flow through fraction (Figure 3.3). Figure shows mass peaks from an extracted ion chromatogram at 150m/z looking for qualifier daughter ions found in Van damme 2012 paper.

3.4. Co-purification of cAMP and cGMP with NssR

To demonstrate that NssR can directly bind cyclic nucleotides, purified unbound GST-tagged NssR was isolated via GST affinity chromatography and eluted with reduced glutathione. Bound nucleotide was then removed via using a heparin column. Tagged NssR (10 µM) was then mixed with cAMP (500 µM) or cGMP (500 µM), followed by GST affinity chromatography and elution via cleavage of the GST tag with thrombin (Section 2.3.1). A negative control experiment was performed with NssR alone, but an experiment was not conducted with c-di-GMP due to the costly nature of the nucleotide. If nucleotide was bound to the eluted fraction, a shift in absorption maximum from 280 nm (protein) to 260 nm (nucleotides) would be expected. Absorption spectra of eluted fractions from this experiment (Figure 3.7) clearly show that NssR can bind both cAMP and cGMP.

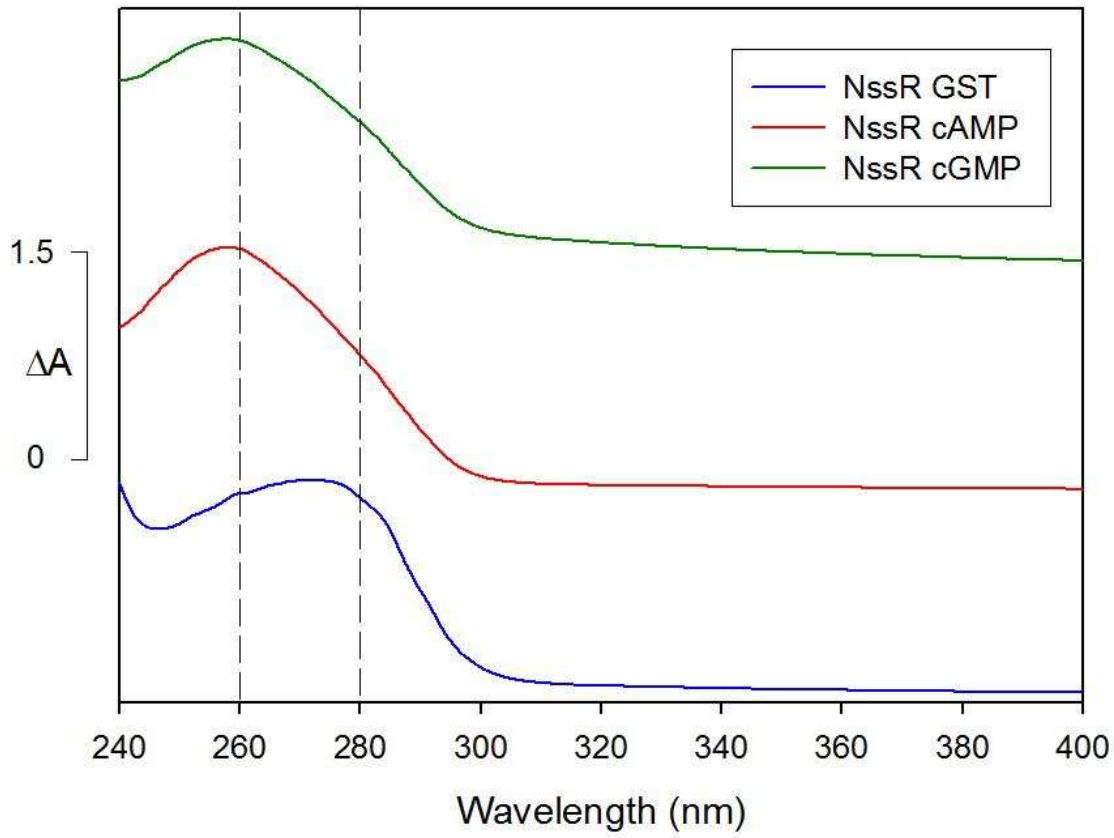

Figure 3.7 – Absorption spectra of NssR bound to cyclic nucleotides. NssR was preincubated for 20 min in the presence of cAMP or cGMP, bound to a GST affinity column and washed, and then eluted via cleavage of the GST tag with thrombin. Absorption spectra show that eluted NssR was bound to cAMP (red trace) or cGMP (green trace). A control experiment was also performed without nucleotide (blue trace).

3.5. Measurement of binding affinities of NssR for cyclic nucleotides

To gain an insight into the relative affinity of NssR for various cyclic nucleotides, tryptophan fluorescence quenching titrations were performed (Section 2.3.5). This technique exploits changes in fluorescence yield of protein tryptophan residues that result from conformational changes upon ligand binding. NssR has a single tryptophan residue at position 198, so it was decided that this was a sensible approach to analyse ligand binding. In brief, NssR (0.2 µM) was excited at 280 nm and fluorescence emission scans were recorded (Appendix A1) following titration of this solution with small volumes of cAMP, cGMP, and c-di-GMP. Control titrations were also performed using the fluorescent small molecule N-acetyl tryptophanamide (NATA) in place of NssR, which provide a measure of signal changes that can result

from the optical properties of the ligand. The fluorescence maxima at 360 nm were plotted against ligand concentration, and binding curves were fitted to single rectangular hyperbolae via nonlinear regression using Sigmaplot (Figure 3.8). These data reveal dissociation constants of 0.9 µM, 1.6 µM, and 0.8 µM for cAMP, cGMP, and cdi-GMP, respectively.

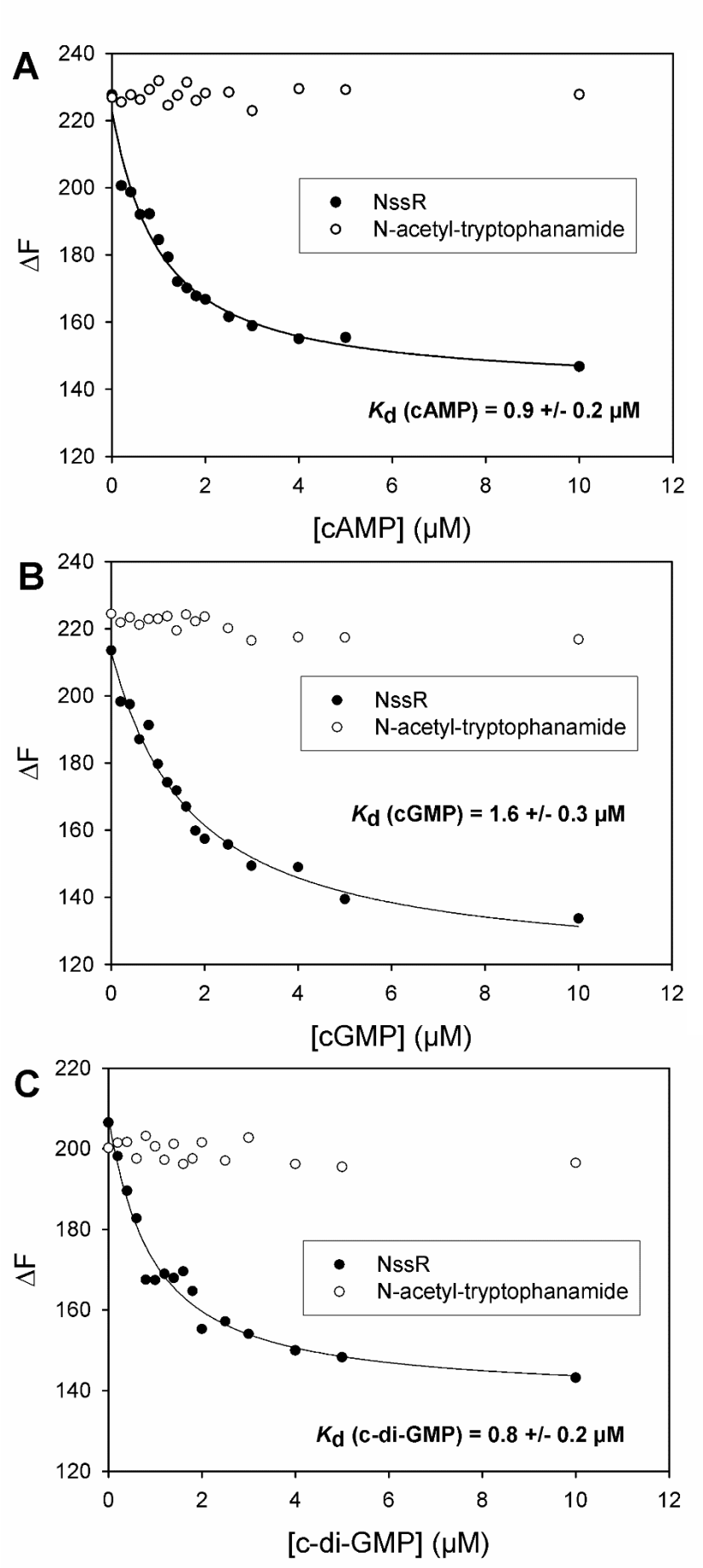

Figure 3.8 - Tryptophan fluorescence quenching titrations of NssR with cyclic nucleotides. To a fixed concentration of 0.2μM NssR, cAMP, cGMP, and c-di-GMP were titrated over a concentration range of 0.2μM to 10μM. Samples were excited at 295nm and fluorescence emission scans were recorded from 300-400nm at 200nm/min. The fluorimeter had excitation slits set to 5 nm and the emission slits were set to 10 nm. To determine whether the ligand absorbance directly affected fluorescence values control titrations were carried out for each nucleotide using the small fluorescent molecule N-acetyl-tryptophanamide.

3.6 - Electrophoretic mobility shift assay to investigate how cyclic nucleotides effect NssR affinity for the *cgb* promoter

After confirming that cAMP, cGMP, and c-di-GMP can bind to NssR, it was of interest to determine whether these ligands affect NssR binding to the target promoter. Gel shift assays were carried out essentially as described previously[25]. Briefly, a DNA fragment containing the *cgb* promoter was amplified via PCR and 25 nM of this was incubated with various concentrations of NssR in the presence of cAMP, cGMP, and c-di-GMP. To measure the fraction of DNA that was bound to NssR, electrophoretic mobility shift assays were perfomed (Section 2.3.6): binding of NssR to the *cgb* promoter was expected to slow migration of the DNA resulting in a band shift.

These data in figure 3.9 demonstrate that binding of c-di-GMP to NssR increases affinity for the *cgb* promoter (3.9B) whereas cAMP and cGMP (3.9C and 3.9D respectively) appear to cause a decrease in affinity with complete inhibition of binding in the case of c-GMP. There is substantial binding of NssR to the promoter in 3.9A when no ligand is present which is only bettered when c-di-GMP is bound.

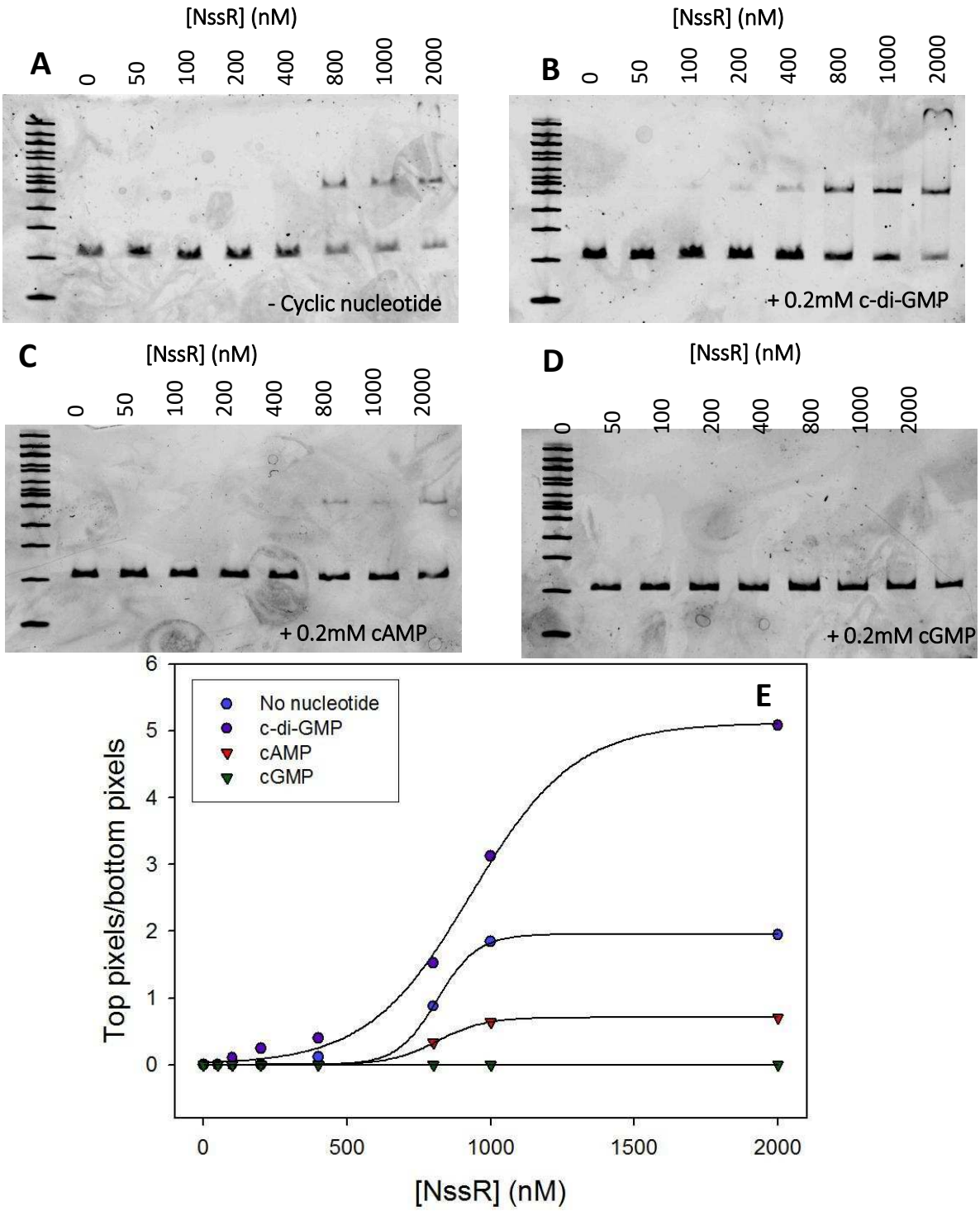

Figure 3.9 – Gel Shift analysis of NssR binding to the *cgb* promoter in the presence of cyclic nucleotides. Target DNA (25 nM) was incubated with varying concentrations of NssR in the absence (panel A) and presence of 0.2mM c-di-GMP (B), cAMP (C) and cGMP (D). Panel E shows intensities of the gel bands at increasing concentrations of NssR in the absence of presence of the cyclic nucleotides (0.2mM)

3.7 – Attempts to measure NssR activity via *in vitro* transcription assays

Since the gel shift assays suggested that the affinity of NssR for the *cgb* promoter was still at significant levels when not in the presence of c-di-GMP, it was hypothesised that NssR binds permanently to the target promoter and nucleotide binding causes a conformational shift to promote transcriptional activation. In an attempt to measure transcriptional activation by cyclic nucleotide, *in vitro* transcription studies were carried out (Section 2.2.11) following the generation of a suitable target DNA fragment.

3.7.1 Construction of template DNA for in vitro transcription

In vitro transcription assays involve the production of mRNA from a target DNA template of a convenient size that is upstream of a strong transcriptional terminator. This target DNA template was engineered via amplification of a 364 bp fragment of 'pRS415-*cgb* promoter' (Appendix A2) containing the *cgb* promoter and a portion of the *lacZ* gene (Section 2.2.10). This was then cloned into the EcoRI/PstI sites of the pSB1C3 vector upstream of the rrnB terminator (Figure 3.10A).

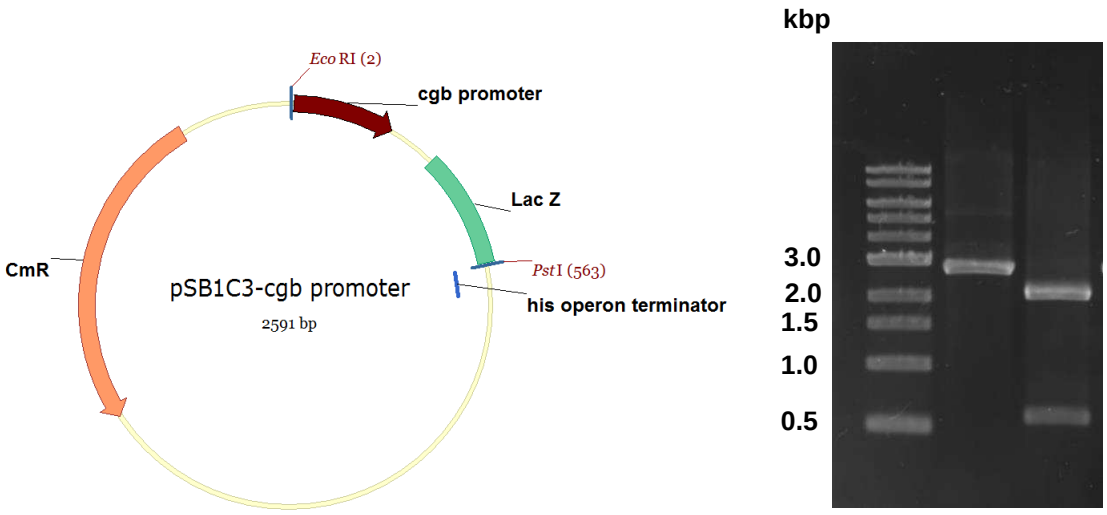

Figure 3.10 – Generation of a DNA template for *in vitro* transcription. A) Map of the desired 'pSB1C3-*cgb* promoter' vector containing the *cgb* promoter and a portion of the *lacZ* gene. B) Agarose gel electrophoresis of restriction-digested 'pSB1C3-*cgb* promoter' plasmid. Lane 1, marker; Lane 2, EcoRI single digest; EcoRI/PstI double digest. Expected fragment sizes for the double digest were 561 bp and 2030 bp.

3.7.2 *In vitro* transcription assays to measure NssR activity

In an attempt to measure the effect of cyclic nucleotides upon NssR activity, *in vitro* transcription assays were performed (Section 2.2.11) using 'pSB1C3-*cgb* promoter' as a template and c-di-GMP as the nucleotide. Transcriptional activity would result in the amplification of mRNA of approximately 500 bases in length. Attempts to amplify mRNA in the presence and absence of NssR were unsuccessful (Figure 3.11), and unfortunately the lanes in the nondenaturing gel electrophoresis did not migrate vertically.

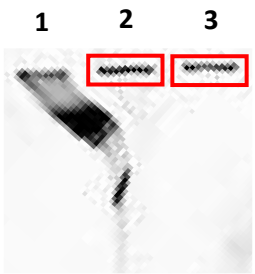

Figure 3.11 – Nondenaturing PAGE of *in vitro* transcription assays. Lane 1, RNA marker lane 2; negative control (+c-di-GMP, - NssR); Lane 3 test, (+c-di-GMP, - NssR). Template DNA is highlighted by the red boxes, and no mRNA product could be detected.

3.8. *In vivo* B-galactosidase reporter system to measure the impact of c-di-GMP upon NssR activity

Since the *in vitro* transcription approach was unsuccessful (Section 3.7), an *in vivo* approach (Section 2.4) was employed to investigate the effect of c-di-GMP upon NssR activity. Briefly, a *yfgF* strain of *E. coli*, lacking a c-di-GMP phosphodiesterase and therefore retaining higher levels of c-di-GMP, was used alongside the isogenic wild type strain. These strains were transformed with both an NssR expression plasmid (PSU2718-G-*nssR* Appendix A3) and a reporter plasmid with the *cgb* promoter upstream of the *lacZ* gene encoding β–galactosidase (Appendix A2). Briefly, cells were grown to stationary phase at 110 rpm as expression of *yfgF* has previously been shown to require low oxygen conditions, and NssR expression was induced with IPTG.

Transcriptional activity from the *cgb* promoter was measured via β–galactosidase assays (Section 2.4), and the data (Figure 3.13) show that the loss of *yfgF* and consequent increase in cellular c-di-GMP elevates transcription initiation at the *cgb* promoter only when NssR is expressed. This observation indicates that c-di-GMP is a positive regulator of NssR.

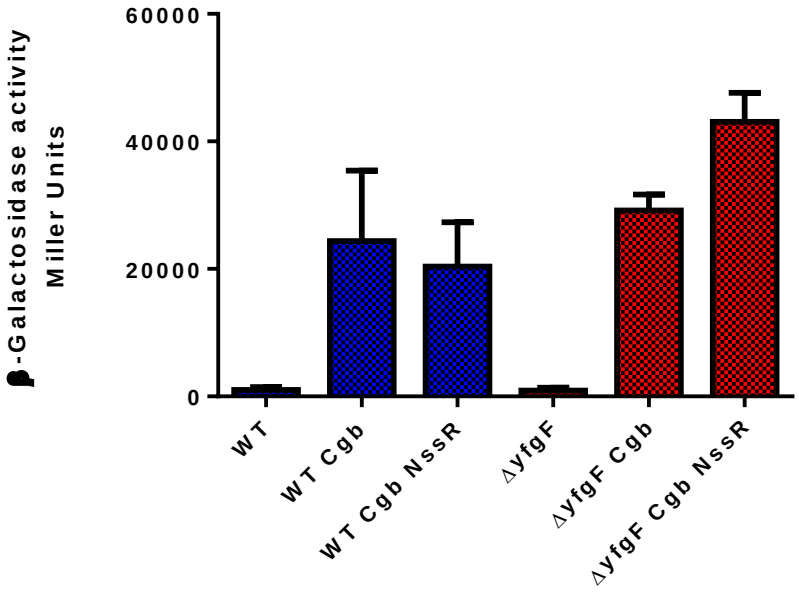

Figure 3.12. **NssR activity is enhanced by cyclic-di-GMP**. Wild type (blue bars) and *yfgF* mutant strains (red bars) were transformed with 'pSU2718-G-nssR' and 'pRS415-*cgb* promoter': strains containing these plasmids are annotated as Cgb and NssR, respectively. All strains grown at 28 °C and 110 rpm to stationary phase in LB with appropriate antibiotics. Expression of NssR was induced with IPTG. Transcriptional activity of the *cgb* promoter was measured via β-galactosidase assays.

CHAPTER 4 - DISCUSSION

NssR is a transcriptional regulator of *C. jejuni* shown to have function in NO detoxification through control of a small regulon containing globins Cgb and Ctb [26]. The molecular mechanism for how NssR senses NO has remained elusive and previous studies have shown NssR not to be directly modulated by Nitric oxide [25]. NssR does not possess any NO-binding cofactors (e.g. haem or iron-sulphur clusters) but has previously been identified as a member of the CRP family of transcription factors which commonly bind cyclic nucleotides. Phosphodiesterase and diguanylase cyclase enzymes, which control c-di-GMP levels, have previously been shown to be modulated by NO [34,35]. Hence, c-di-GMP is a strong candidate for being a second messenger in NO-mediated activation of NssR activity. This lead to the hypothesis that increased levels of cyclic di-GMP will elevate NssR activity increasing Cgb expression (Figure 1.4).

Structural modelling using the online RaptorX program in this work is consistent with NssR having a binding cleft that can accommodate cyclic nucleotides. Analysis of the surface of the NssR model shows a large binding pocket, large enough to accommodate cGMP or cAMP. However, the binding of cAMP requires the phosphate end of cAMP to be buried deep within the protein with the adenine ring exposed. This might pose a problem for c-di-GMP binding as the two GMP moieties are linked by the phosphates, so c-di-GMP would have to bind in an alternative orientation to the modelled cAMP in Figure 3.1. However analysis of side chain binding of NssR carried out in this work has revealed the substitution of both Arginine residues found in Crp, which are heavily associated with the binding of negative phosphate residues by formation of multiple hydrogen bonds[38]. This would suggest that an alternative binding orientation for c-di-GMP would be highly likely. Additionally recent research on CRP family transcription factors has reported that members of this family that can bind both c-di-GMP, which fits well with the observations in the current study[39].

The co-purification of NssR with cAMP and cGMP provides robust biochemical evidence that NssR can bind cyclic nucleotides. This experiment was not performed with c-di-GMP due to cost issues, but it is anticipated that a similar result would be obtained. The tryptophan fluorescence experiments are consistent with NssR being able to bind all three cyclic nucleotides *in vitro*, with tighter binding observed for c-di-GMP and cAMP.

As NssR has previously been shown to bind to the *cgb* promoter fairly tightly when not in the presence of a ligand it was initially predicted that binding of cyclic nucleotides would not increase affinity for the promoter but induce a conformational change to the protein leading to increased transcriptional activation [25], Gel shift assays carried out in this work show that c-di-GMP does in fact increases NssR affinity for the *cgb* promoter and lead to the possibility of both scenarios being true.

To test the activity of NssR, *in vitro* transcription experiments were undertaken to directly measure mRNA transcript levels from a *cgb* promoter (Figure 3.12). Unfortunately, these experiments were unsuccessful, possible due to detection levels using the gel staining method. More sensitive methods for detecting mRNA transcript levels are available, such as ^{35}P-labelling of mRNA and autoradiograph detection. This may provide a sensible future strategy for measuring NssR activity *in vitro*. The *in vivo* approaches were more successful (Figure 3.13). The wild type and mutant strains lacking plasmids exhibited low levels of β-galactosidase activity, which was expected as these strains do not possess the *lacZ* fusion and the MC1000 background strain lacks the chromosomal *lac* operon. However, in all strains that contain the *cgb-lacZ* reporter plasmid, there is a fairly high residual level of LacZ expression, presumably due to leaky expression by the *E. coli* transcriptional machinery or via activation by an *E. coli* transcription factor. Expression in the absence of NO could be due to differences in how *E. coli* and *C. jejuni* respond to NO: this seems likely as *C. jejuni* lacks the NsrR transcription factor of *E. coli* that controls the major transcriptional responses to NO, and *E. coli* lacks NssR. Nonetheless, despite this high background signal, this remains a useful tool to investigate NssR activity *in vivo*. In the mutant strains harbouring the *cgb-lacZ* reporter plasmid, LacZ expression is not significantly increased when compared to wild type strains with *cgb*.

There is a slight increase in levels of β-galactosidase activity between WT Cgb and Δ*yfgF* Cgb strains, although this difference is not significant at the 95% level (*t*-test). Expression in WT Cgb and Δ*yfgF* Cgb is still at a basal level in the absence of the transcriptional regulator NssR. However, upon introduction of the NssR expression plasmid, the ΔyfgF Cgb nssR strain displayed significantly increased expression compared to the ΔyfgF Cgb control strain, suggesting that cyclic-di GMP is enhancing NssR activity.

Herein, it is shown that c-di-GMP enhances NssR-mediated transcriptional activation of the *cgb* promoter. However, it is still unclear how NO modulates c-di-GMP levels in *C. jejuni.* We can hypothesise that NO is either positively regulating DGC activity or negatively regulating PDE activity (Figure 4.1). It is also possible that both routes are active and occur simultaneously as both routes have the same outcome of increased levels of c-di-GMP which enhances NssR activity which leads to upregulation of expression of Cgb for NO detoxification. *C. jejuni* has been found to encode proteins with the diguanylate cyclase domain GGDEF but do not have proteins with either of the EAL or HD-GYP phosphodiesterase domains which are common in bacteria [40]. Hence, it has been suggested that c-di-GMP in *C. jejuni* may be degraded by a general PDE with relaxed substrate specificity [40]. More recently, a protein of *C. jejuni* has been identified as a potential c-di-GMP PDE [41]. Bioinformatic analyses identified a domain with distant homology to the conserved HD-GYP domain, which has been named 'HD related output domain' (HDOD). Confident assignment of this protein as a phosphodiesterase has not been made due to lack of a conserved metal binding residue found in typical HD domains [41].

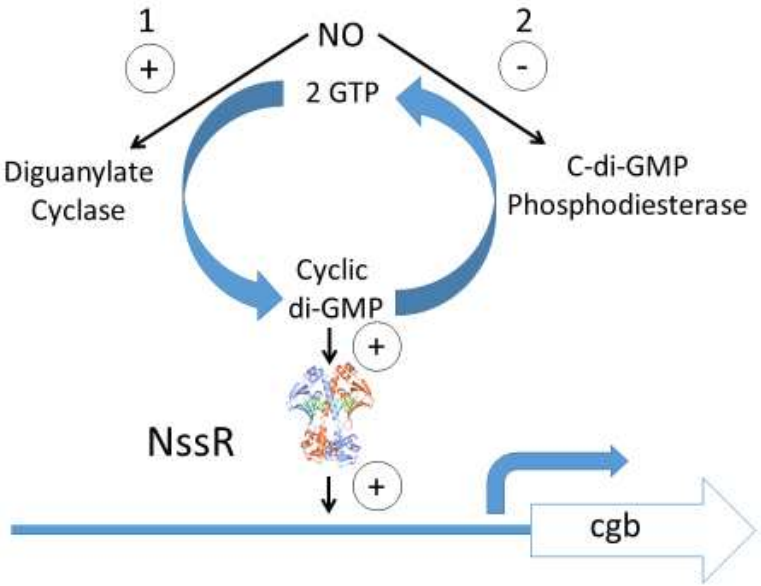

Fig 4.1 Model for NssR mechanism in *Campylobacter jejuni*. There are 2 possible routes via which NO may increase cyclic-di-GMP levels. Route 1: NO is positively regulating diguanylate cyclase. Route 2: NO is negatively regulating C-di-GMP phosphodiesterase activity.

In conclusion, this work shows that c-di-GMP elevates NssR activity, which is likely to be the molecular signal that controls NssR activity in *C. jejuni*. In essence, this work provides important insights into the molecular mechanism of NssR and provides a robust model for how *C. jejuni* senses and responds to NO *in vivo*. This has important implications for how *C. jejuni* survives nitrosative stress during infection, and provides a molecular explanation for how this zoonotic pathogen can persist in the avian gut.

Future work in this area might include development of the in vitro transcription assays via radiolabelling of the mRNA for increased sensitivity, deletion of cyclase and phosphodiesterase genes in the native host followed by physiological experiments on NO tolerance, and determination of the crystal structure of NssR with bound cyclic nucleotides.

Appendix

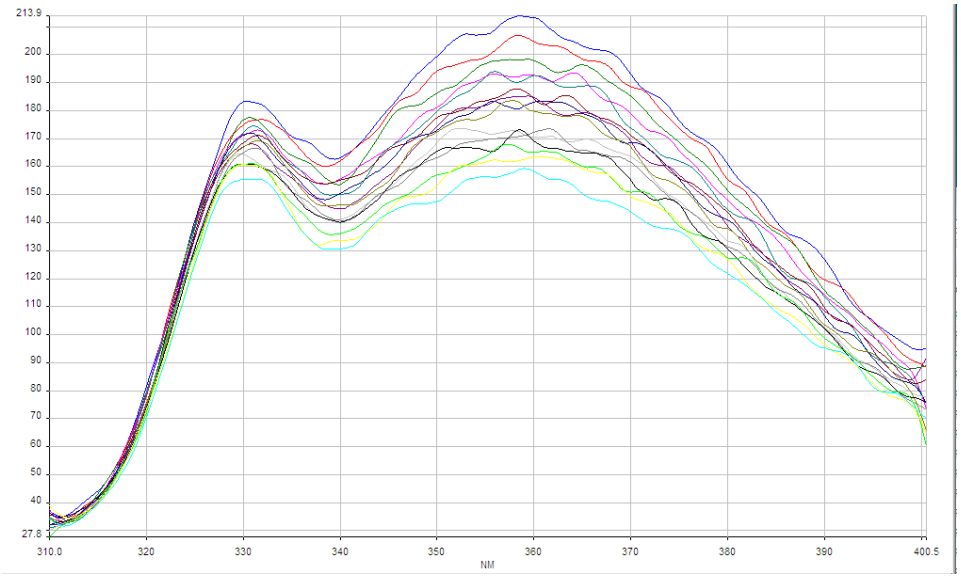

Figure A1 – Fluorescence emission scans of NssR titrated with c-di-GMP

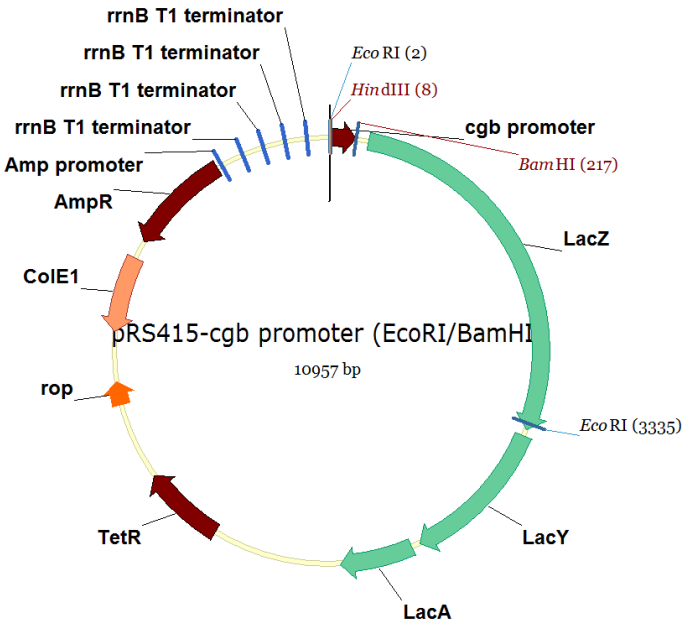

Figure A2 – The pRS415-*cgb* promoter plasmid. This was used to amplify target DNA for NssR *in vitro* transcription experiments and as a reporter construct in *in vivo* measurements.

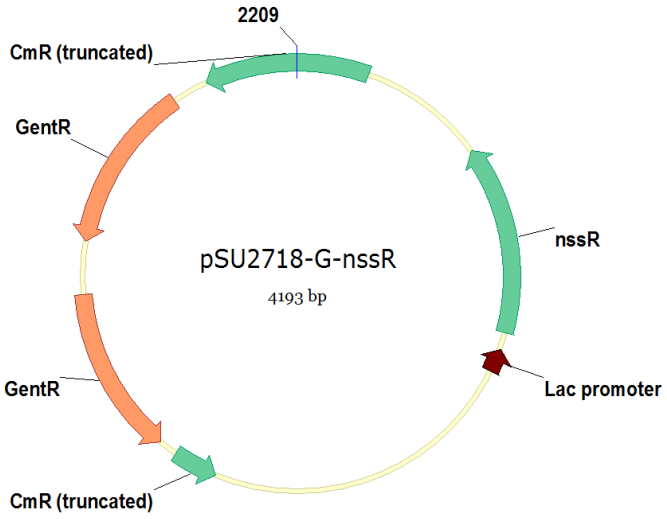

Figure A3 – Map of the pSU2718-G-NssR plasmid used to express NssR in *E. coli*. This plasmid was used in the *in vivo* NssR reporter assay experiments.

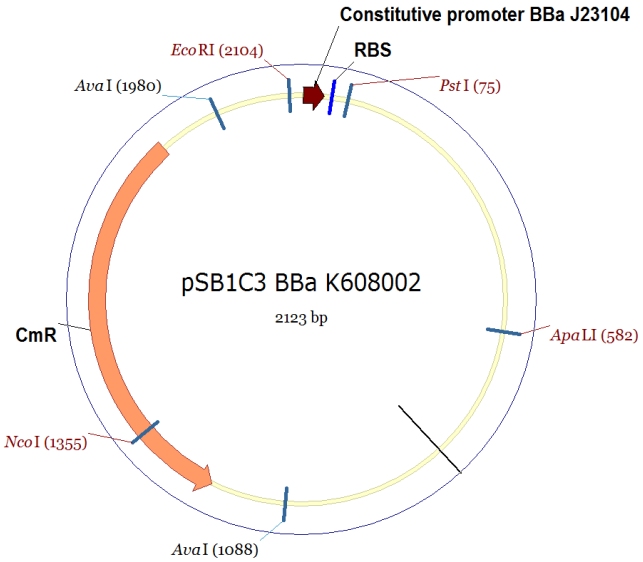

Figure A4- Map of the pSB1C3 BBa plasmid. The BBa promoter was removed and this plasmid was used as the backbone to insert the cgb promoter fragment for IV transcription experiments.

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