



**Gene Regulation in Baculovirus-infected Insect
Cells: Application in the Development of a
Budded Virus Free Expression
System**

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A thesis submitted in partial fulfilment of the requirements of
Oxford Brookes University for the award of Doctor of Philosophy

December 2022

Abstract

The baculovirus expression vector system (BEVS) is a valuable tool for the production of recombinant proteins including complexes such as virus-like particles (VLPs) for vaccine production. A significant limitation of the BEVS, however, is the co-production and subsequent contamination of VLPs and other biopharmaceuticals with the baculovirus budded virus (BV). This is of concern to regulatory bodies as BVs are enriched with the glycoprotein GP64, which is immunogenic, so must be removed from biopharmaceutical preparations. However, the cost of downstream processing to remove these particles is considerable. Consequently, this thesis aimed to develop an upstream solution that inhibits BV production, thus eliminating the need for downstream processing. A novel, selectively repressible system was developed employing components of the *Escherichia coli lac* operon to block expression of essential genes required for BV production during VLP/protein production (the LacI/O Repressor System).

Initially, RNA interference was used in a screening process to inhibit expression of a large number of structural genes associated with BV. Five “key” structural genes (*gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*) were identified as integral to successful assembly and release of BVs, and which could have potential to reduce BV production if repressed by the LacI/O Repressor System. The effects of paired gene silencing by RNAi was also assessed. Data demonstrated that silencing of two “key” genes could further significantly reduce ($p<0.05$) BV production.

For the development of the LacI/O Repressor System, multiple single and dual gene expression promoter constructs (based on the *chitinase/cathepsin* (*chiA/cath*) promoter) were produced. Each contained the *E. coli lac* operator (*lacO*) at different positions relative to the transcription initiation site (TAAG), and through fluorescence assays and western blotting, their function was confirmed. Basal gene expression from both the single and dual expression *chiA/lacO* promoters was affected by *lacO* positioning. Insertion of *lacO* after the TAAG significantly ($p<0.01$) increased promoter activity, whilst insertion before the transcription initiation site significantly ($p<0.01$) decreased activity. Insertion of one *lacO* after the TAAG motif delayed promoter activity, with the increase in promoter activity due to an increase in promoter strength. Addition of a second *lacO* after TAAG did not increase promoter activity by further increasing promoter strength. Instead, by extending the length of time the promoter was active.

A stable cell line (SfLacI) constitutively producing the *E. coli* repressor protein, LacI, was established. Infection of these cells with a recombinant virus expressing the “key” genes under the *chiA/lacO* promoter would allow LacI to bind *lacO* and block gene expression, resulting in a reduction in BV synthesis.

Flow cytometry experiments showed that all single expression *chiA/lacO* promoter constructs were capable of downregulating gene expression in the presence of LacI. However, only insertion of *lacO* after the TAAG motif yielded significant levels ($p<0.05$) of gene inhibition. Increased promoter activity seemed to correlate with greater levels of gene downregulation, which was likely due to the increased rates of RNAP clearance, which increased the opportunity for LacI binding to *lacO* in the promoter. Addition of a second *lacO* sequence after the TAAG motif made gene repression more consistent between experimental replicates, possibly by increasing the rate of repressor/operator binding, or by increasing the stability of the repressor-operator complex. The presence of two *lacOs* spaced further apart enhanced gene repression, possibly by enabling the formation of DNA loops. Significant downregulation of two genes was possible from the dual *chiA/lacO* promoter construct when in the presence of LacI. When employing the LacI/O Repressor System to downregulate the “key” gene, *gp64*, BV titres were significantly reduced ($p<0.05$) by 50%. This is a significant starting point for the development of a BV free expression system and further work is required to enhance the current levels of gene/BV repression.

Presentations

The work described in this thesis has been presented at international conferences and seminars in the form of posters and oral presentations.

Posters

Backhouse, V., Possee, R. and King, L. (2022). Designing a novel, repressible system for the downregulation of native virus genes through modification of the late chitinase gene promoter. 54th Annual Meeting of the Society of Invertebrate Pathology, Nelson Mandela Bay, South Africa (Online).

Backhouse, V., Possee, R. and King, L. (2022). Designing a novel, repressible system for the downregulation of native virus genes through modification of the late chitinase gene promoter. 8th European Congress for Virology 2023, Gdansk, Poland.

Oral Presentations

Backhouse, V., Possee, R. and King, L. (2019). Gene Regulation in Baculovirus Infected Insect Cells: Application in the Development of a Budded Virus Free Expression System for the Production of VLP Based Vaccines. Oxford University Doctoral Training Programme Career Development Series, Oxford University, Oxford, UK.

Backhouse, V., Possee, R. and King, L. (2022). Gene Regulation in Baculovirus Infected Insect Cells: Application in the Development of a Budded Virus Free Expression System for the Production of VLP Based Vaccines. Oxford University Doctoral Training Programme Annual Symposium, Oxford Brookes University, Oxford, UK.

Acknowledgements

I can't believe the moment is finally here, when everything is done and the last thing to do is to write my acknowledgements. There have been many moments when I was not sure this would happen. However, I always knew that I could and now my determination has paid off. I have learnt so much over the last 4 years, not only about the BEVS, virology and genetics, but about myself. I am a more confident, resilient, and resourceful person and have learnt life skills that will serve me well in the future.

To start with I would like to thank all of the people who made this opportunity possible: Dave Carter, Oxford Brookes University, Oxford University DTP, and my supervisors Robert Possee and Linda King. Thank you for seeing my potential. And thank you to my financial backers Oxford Brookes University, Oxford University, the BBSRC and Chris Backhouse, without whom this would not have been possible.

I would like to say thank you to all those from the IVRG and OET who supported me, made me feel welcome and helped answer my many questions. Particular thank you to my favourite brunch group, Cassie Rigby, Laura Swan, and Carina Bannach, whose encouragement kept me going throughout the years.

Next I would like to thank all my friends who have been there to lend an ear and encourage and support me when things were hard. Whether with hugs, wine, a good chat or a great night out your support has meant a lot. I would like to give a special mention to Natalie Curtis. You have been the most supportive, encouraging, reassuring, and all round amazing friend. Your advice as someone who has just gone through the same experience, and offers of dinner and movie nights to make me take a break has meant so much to me.

Thank you to my dad for always being there if I needed a chat. Thank you for surviving the COVID lockdown with me and providing many cuddles which made the writing of Chapter 1 possible. Thank you for all the food, wine, music, and love.

Finally, I would like to say a huge thank you to my mum. Without you this never would have been completed. Your determination has been my perfect example of how to never give up. The many hours of phone calls when all seemed too much and your encouraging texts have meant more to me than you can imagine. Thank you for the regular visits and cuddles and for pretending like you know what I'm on about when I tell you about my work. And finally thank you for literally living in a holiday destination and providing a perfect place, complete with an emotional support cat, for writing up!

XOXO

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Abbreviations

α	Alpha
A	Adenosine
Ac	<i>Autographa californica</i>
AcMNPV	<i>Autographa californica nucleopolyhedrovirus</i>
APS	Alkaline Phosphatase
BAC	Bacterial artificial chromosome
BEVS	Baculovirus expression vector system
<i>Bm</i>	<i>Bombyx mori</i>
bp	Base pair
BV	Budded virus
C	Cytosine
<i>cath</i>	<i>Cathepsin</i>
<i>chiA</i>	<i>Chitinase</i>
CRE	cAMP response element
DAPI	4',6-diamidino-2-phenylindole, fluorescent stain that binds the DNA
DPBS	Dulbecco's PBS
d.p.i.	Days post-infection
d.p.t	Days post-transfection
dsRNA	Double stranded RNA
<i>E. coli</i>	<i>Escherichia coli</i>
<i>egfp</i>	Enhanced green fluorescent protein gene
EGFP	Enhanced green fluorescent protein
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence-activated cell sorting
FCS	Foetal calf serum
PIF	<i>Per os</i> infectivity factor
G	Guanine
<i>gfp</i>	Green fluorescent protein gene
GFP	Green fluorescent protein
GP64/<i>gp64</i>	Glycoprotein 64 (protein/gene)
GVs	Granuloviruses
HEK	Human embryonic kidney
h.p.i.	Hours post-infection
h.p.t.	Hours post-transfection
<i>hrs</i>	Homologous regions
HRP	Horseradish peroxidase
ie	Immediate early
<i>KanR</i>	Kanamycin resistance gene, confers resistance to G418
kbp	Kilobase pair
kDa	KiloDalton
<i>LacI/lacI</i>	<i>lac</i> inhibitor/repressor (protein/gene)
<i>lacO</i>	<i>lac</i> operator
<i>LacZ</i>	β -galactosidase gene (from <i>E. coli</i>)
miRNA	MicroRNA
mRNA	Messenger ribonucleic acid
MOI	Multiplicity of infection
NAE	Nucleocapsid assembly-essential element

NEB®	New England Biolabs® Inc.
neo	Neomycin resistance gene, confers resistance to G418
NLS	Nuclear localisation signal
NPVs	Nucleopolyhedroviruses
NP40	Nonidet™ P 40 (lysis buffer)
nt	Nucleotide
OBs	Occlusion bodies
ODV	Occlusion-derived virus
OET	Oxford Expression Technologies Ltd
Op	<i>Orgyia pseudotsugata</i>
orf	Open-reading frame
P	Passage
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PIPES	10mM piperazine-N,N'-bis(2-ethanesulfonic acid)
Polh/polh	Polyhedrin (protein/gene)
Pfu	Plaque-forming unit
qPCR	Quantitative polymerase chain reaction
RISC	RNA-induced silencing complex
RNAi	RNA interference
RNAP	RNA polymerase
<i>S. frugiperda</i>	<i>Spodoptera frugiperda</i>
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacryamide gel electrophoresis
SEM	Standard error of the mean
SfC1B5	A clonal cell line derived from Sf21 cells
SfLacI	Cell line derived from Sf9 cells that constitutively expresses <i>lacI</i>
Sf9	<i>Spodoptera frugiperda</i> 9, a clonal cell line derived from Sf21 cells
Sf21	Cell line derived from <i>Spodoptera frugiperda</i> ovarian cells
siRNA	short interfering RNA
SOB	Super optimal broth
T	Thymine
TAE	Tris-acetate
TEM	Transmission electron microscopy
TF	Transcription factor
<i>T. ni</i>	<i>Trichoplusia ni</i>
TPA	12-O-tetradecanoylphorbol 13-acetate
TRA	12-O-tetradecanoylphorbol 13-acetate response element
UTR	Untranslated region
VLF	Very late factor
VLP	Virus-like particles
WB	Western blot
wt	Wild-type

Chapter 1:

Introduction

1.1 Introduction

The large *Baculoviridae* family is composed of over 600 arthropod infecting members (King *et al.*, 2011), which are sub-divided into four genera comprising of both nucleopolyhedroviruses (NPVs) and granuloviruses (GVs): *Alphabaculovirus* (Lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPVs) and *Deltabaculovirus* (dipteran-specific NPVs) (reviewed by Kelly *et al.*, 2016). The NPVs produce large (0.5-1.5µm in diameter) occlusion bodies (OBs) composed of polyhedrin protein that contain multiple virions. The largest of the genera, *Alphabaculovirus*, plays host to the widely studied *Autographa californica* Multiple NPV (AcMNPV), which was exclusively used throughout this research study.

Baculoviruses play an important role in biocontrol as their infection of natural insect populations results in the death of the insect hosts. Consequently, baculoviruses have been employed for the suppression of pests, mainly to protect crops but also forests and pastures (reviewed by Szewczyk *et al.*, 2006). Furthermore, due to its ability to produce high yields of multiple foreign proteins, the AcMNPV has been readily used as a vector for the expression of recombinant proteins and protein complexes such as virus-like particles (VLPs) for vaccine production including GlaxoSmithKline's Cervarix™ against human papilloma virus, Belyaev and Roy (1993) Bluetongue VLPs and the Vaxine/ Oxford Expression Technologies Ltd SARS-CoV-2 VLP-based vaccine (R. Possee, personal communication). Modifications of the AcMNPV genome has better enabled the virus to fulfil these roles. For example, the addition of multiple copies of strong promoters facilitated the production of multiple recombinant proteins at once (Weyer and Possee, 1991; Belyaev *et al.*, 1995).

However, the baculovirus expression vector system (BEVS) still has its limitations and more modifications could be done to improve the production of VLP vaccines. Baculovirus replication results in the production of budded virus (BV), which is often co-purified along with VLPs. This represents a particular concern to regulatory bodies due to the fact that BVs are enriched with the glycoprotein GP64, which is immunogenic in mammals (Luo *et al.*, 2013) and therefore undesirable in VLP vaccine preparations. The cost of downstream processing to remove contaminating BV particles in terms of both time and money is considerable. Consequently, further modifications to the BEVS that inhibit or, more

realistically, significantly reduce BV synthesis during VLP production would be desirable and is therefore the main focus of this research study.

1.2 Baculovirus structure

Baculoviruses possess a supercoiled, double-stranded DNA genome (80-180 kbp) enclosed within 40-60nm X 230-385nm nucleocapsids (reviewed by Rohrmann, 2019). The condensed packaging of the genome is facilitated by the core DNA-binding protein p6.9, composed of 54 amino acids and rich in arginine (Wilson *et al.*, 1987). The baculovirus nucleocapsid is mainly composed of a 39-kDa protein, VP39. This major capsid protein is distributed throughout the surface of the nucleocapsid (Thiem and Miller, 1989) and its interaction with a conserved domain of kinesin 1 is thought to be involved in the transport of budded virus nucleocapsids to the cell membrane after their assembly in the nucleus (Danquah *et al.*, 2012).

A characterising feature of baculoviruses is their production of two forms of progeny; the BV (Figure 1.1) and the occlusion-derived virus (ODV), packaged into occlusion bodies (OBs) and released upon nuclear lysis. Although BVs and ODVs are genetically identical, their morphology is quite different.

BV virions (Figure 1.1) were initially described as rod-shaped (Beaton and Filshie, 1976; Fraser, 1986) but recent results by cryo-electron microscopy (cryo-EM) have shown an elongated, ovoid shape and whilst most envelope proteins were found distributed on the apical ends of the virion, a few were also found on lateral regions (Wang *et al.*, 2016a). The BV envelope contains two layers ~6-7 nm thick acquired as the virions bud from the host cell plasma membrane (Braunagel and Summers, 1994; Wang *et al.*, 2016a). Along with VP39 and P6.9, the glycoprotein GP64 is one of the three most abundant AcMNPV BV proteins (Wang *et al.*, 2010). It is acquired during budding along with the host cell plasma membrane where it is enriched at localised areas (Blissard and Rohrmann, 1989; Washburn *et al.*, 2003).

For AcMNPV, OBs contain multiple ODVs which are encased within a polyhedrin matrix (Rohrmann, 2019). This crystalline matrix protects the virions from most environmental stress factors. OBs are surrounded by an envelope, which is mainly composed of the viral

PP34 protein (Rohrmann, 2019). These OBs are approximately 0.6–2 μM in diameter (Akermann and Smirnov, 1983).

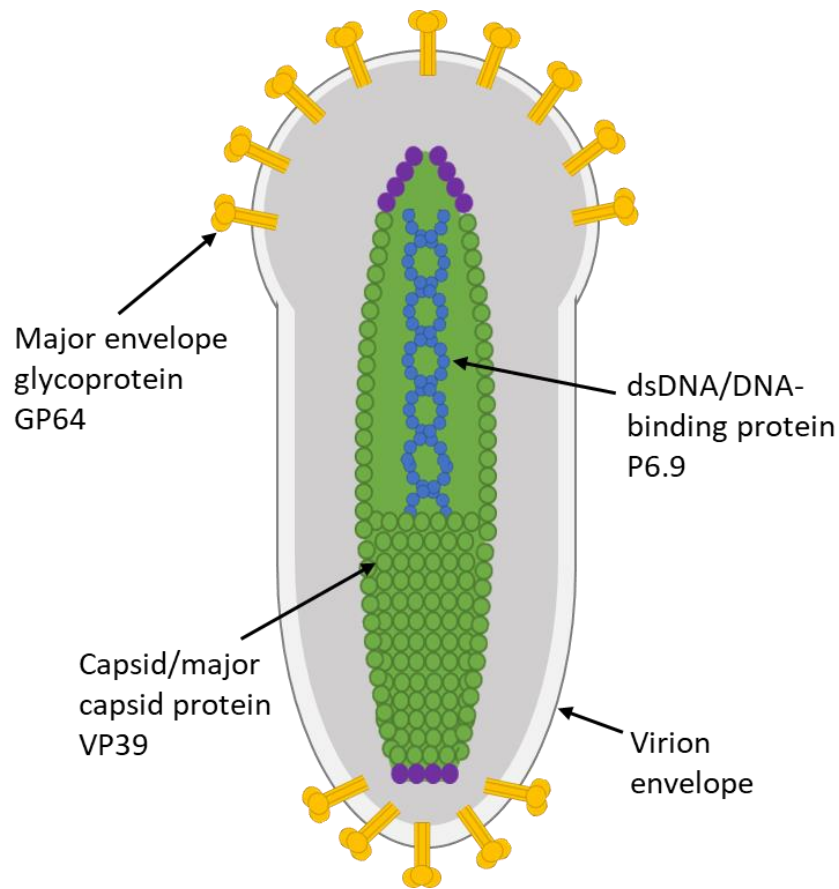


Figure 1.1- A simplified representation of the baculovirus budded virus (BV) structure.

1.3 Baculovirus replication cycle

Baculoviruses are characterised by their unique bi-phasic replication cycle that results in the production of two different forms of progeny, the occlusion derived virions (ODVs) packaged into occlusion bodies (OBs) and the budded viruses (BVs) (Figure 1.2; Kelly *et al.*, 2016). Whilst the OBs facilitate the horizontal transmission of baculovirus infection between insects via its dissemination throughout the local environment, the BVs are responsible for the spread of the virus between the insects own cells, *in vivo* and in cultured cells (Keddie and Volkman, 1985; Keddie *et al.*, 1989; Rohrmann, 2019). It is the production of the baculovirus BV during the late stage of infection that will form the focus of this research study.

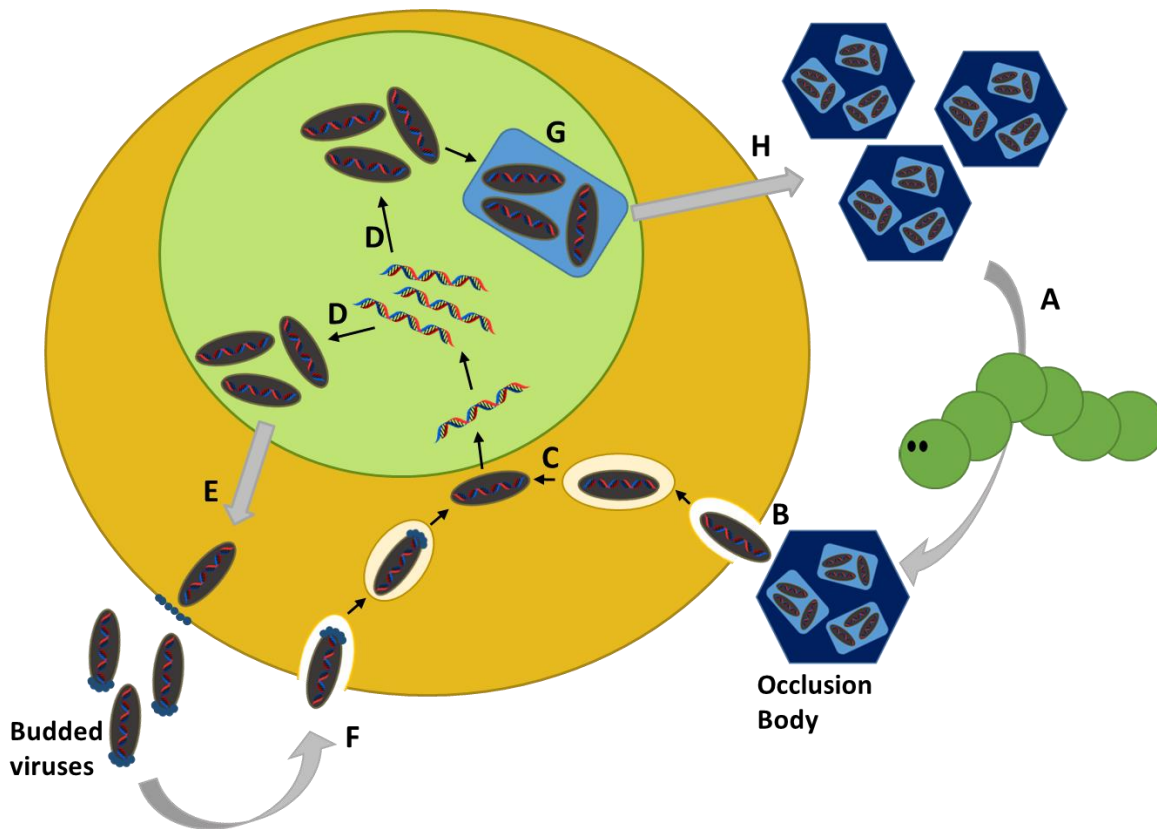


Figure 1.2- Baculovirus replication cycle.

Occlusion bodies (OBs) containing the occlusion derived virions (ODVs) are ingested by an insect **(A)**. The OBs dissolve in the larval midgut and the ODVs infect the midgut cells **(B)** and are transported to the nucleus **(C)**. The viral DNA is replicated and new nucleocapsids are assembled **(D)**. In the late phase of infection, virions (BVs) are trafficked to the cell basal membrane and are budded from GP64-enriched zones **(E)**. The BVs initiate a secondary systemic infection **(F)**. In the very late phase of infection, virions are encased within a polyhedrin matrix forming occlusion bodies **(G)** that are released into the environment upon cell death **(H)**.

The AcMNPV replication cycle is initiated when OBs are ingested by an insect (Figure 1.2, A). The OBs dissolve in the alkaline conditions of the larval midgut resulting in the release of the ODVs from their polyhedrin matrix (Rohrmann, 2019). These ODVs then infect the columnar epithelial cells of the midgut, initiating the primary infection (Figure 1.2, B). Actin filaments mediate the transport of the nucleocapsids to the cell nucleus where they enter via the nuclear pores (Granados and Lawler, 1981) (Figure 1.2, C). Viral DNA is replicated and new nucleocapsids are assembled (Figure 1.2, D). AcMNPV infection can be divided into four distinct phases: immediate early and early (0-6 hours post-infection; h.p.i.), late (6-24 h.p.i.), and very late phase (18-72 h.p.i.). It is during the late phase that nucleocapsids are trafficked to the cell basal membrane and are budded from GP64-enriched zones to

produce budded virus (Figure 1.2, E; Blissard and Rohrmann, 1989; Washburn *et al.*, 2003), initiating a secondary systemic infection. In the very late phase of infection nucleocapsids remain in the nucleus to form the occlusion derived virions that are encased within a polyhedrin matrix forming the occlusion bodies (Figure 1.2, G). This viral infection culminates in the liquefaction of the insect host and the dissemination of the OBs into the environment (Figure 1.2, H; Rohrmann, 2019).

1.4 Baculovirus budded virus: nuclear egress, trafficking and uptake

For AcMNPV, the late stage of infection initiates at approximately 6 h.p.i. with DNA replication within the host cell nucleus. This is then packaged into nucleocapsids before being trafficked to the plasma membrane. The envelope glycoprotein, GP64, localises to discrete areas of the plasma membrane, where budding of virions takes place (Blissard and Rohrmann, 1989; Washburn *et al.*, 2003). Indeed, localisation of GP64 is an essential step in the production of BVs as research has shown that reduced surface levels of GP64 results in a dramatic decrease in BV production (Oomens and Blissard, 1999). As the virions bud, they acquire the host cell plasma membrane as an envelope, which is enriched with the GP64 glycoprotein (Figure 1.1). GP64 also plays an essential role in the successful entry of BVs into new cells, and therefore enables the spread of infection both *in vivo* and in cell culture (Monsma *et al.*, 1996).

Two models have been proposed for the movement of nucleocapsids as they exit cells to form BVs. Firstly, VP39 and Ac141 have been shown to interact with a conserved domain of kinesin-1, which suggests that microtubule transport systems may be involved in baculovirus nucleocapsid egress and BV formation (Danquah *et al.*, 2012; Biswas *et al.*, 2016). Danquah *et al.* (2012) also found that BV production was significantly reduced from AcMNPV infected insect cells treated with colchicine (a microtubule-depolymerising drug). Further to this, Biswas *et al.* (2016) showed that downregulation of the *Drosophila melanogaster* kinesin-1 light chain by siRNA resulted in a significant reduction of BV production in *Spodoptera frugiperda* (Sf9) cells.

However, a second mechanistic model proposes that nucleocapsids undergo actin-based motility following escape from the nucleus. In contrast to Danquah *et al.* (2012), Ohkawa

and Welch (2018) found that colchicine did not affect viral titre. They did, however, find the rates of nucleocapsid movement to be consistent with actin-based motility as opposed to that of motor-driven transport. Further to this, research assessing the effect of actin-disrupting drugs has shown actin to be crucial for BV production (Ohkawa and Volkman, 1999). Overall, it is likely that both pathways may be involved in nucleocapsid exit with each involved at different stages; microtubule transport for nuclear egress and actin-based motility for transport to the cell surface and exit.

The endosomal sorting complex required for transport (ESCRT) machinery is involved in many different processes associated with BV production such as endosomal sorting, vesicular trafficking and virus budding (reviewed by Ahmed *et al.*, 2019). Importantly, Yue *et al.* (2018) have shown the ESCRT machinery to be necessary for successful AcMNPV BV egress from insect cells. RNA interference (RNAi) assays targeting individual ESCRT-III genes found ESCRT-III components to be required for efficient nuclear egress of progeny nucleocapsids. Several baculovirus conserved or core proteins such as Ac142, have also been shown to interact with Vps4 and other components of ESCRT-III, and may form an “egress complex” that recruits ESCRT-III components to a virus egress domain on the nuclear membrane (Li and Blissard, 2012; Yue *et al.*, 2018).

1.5 Baculovirus gene expression

Baculovirus gene expression is initiated following nucleocapsid uncoating and release of DNA in the cell nucleus. It is characterised by four distinct phases: immediate early, early, late and very late (Figure 1.3). The early phases of gene expression are mediated by the host cell RNA polymerase II, whilst the late genes are transcribed by the virus encoded RNA polymerase (reviewed by Berretta *et al.*, 2013; Rohrmann, 2019). Five genes have been identified as immediate early genes, which code for transcriptional regulators of the early viral genes and initiate the transcriptional cascade. These are *ie0*, *ie1*, *ie2*, *me53*, and *pe38* (Rohrmann, 2019).

The progression from the early to late phase of infection is marked by the start of viral DNA replication. The viral RNA polymerase recognises unique promoter motifs (TAAG) from which transcription of late and very late genes initiates (Rohrmann, 2019). In this phase,

some early gene expression is suppressed, whilst some genes, such as *ie1*, are not and instead contribute to the regulation of late gene expression (Kovacs *et al.*, 1991).

The late phase is primarily concerned with the assembly of nucleocapsids and production of BVs. Consequently, this is the phase in which the nucleocapsid structural protein genes are expressed. This includes the three most abundant AcMNPV BV proteins GP64, VP39 and P6.9 (Wang *et al.*, 2010) along with numerous other structural protein genes associated with the BVs. Finally, the hyper expression of *p10* and *polyhedrin (polh)* viral genes marks the transition to the very late phase.

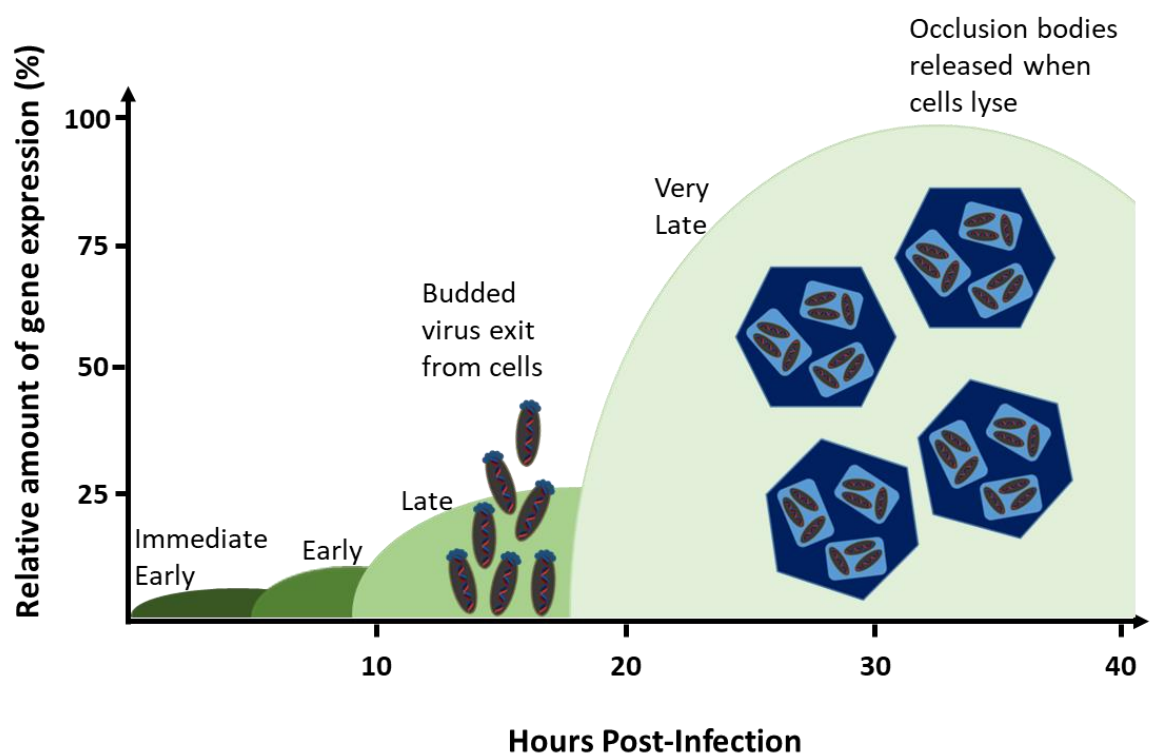


Figure 1.3- AcMNPV gene expression *in vitro*.

AcMNPV infection is divided into four distinct phases: immediate early and early, late, and very late phase. It is during the late phase that budded viruses are assembled and released from the cells. The very late phase culminates in the release of the occlusion bodies upon cell lysis.

1.6 Baculovirus Expression Vector System (BEVS)

Baculoviruses are employed as vectors for the expression of genes in both insect and mammalian cells. Indeed, multiple factors make baculoviruses well suited to their role as expression vector systems. For example, AcMNPV possesses the very late *p10* and *polh*, both of which are not only dispensable for virus replication in cell culture (Smith *et al.*,

1983; Rohrmann, 2019), but are also under the control of very strong promoters. The pairing of one of these promoters, therefore, with a foreign gene-coding region allows for the production of high yields of recombinant protein. In addition to this, the insect cells in which baculoviruses replicate are capable of carrying out complex eukaryotic post-translational protein modifications (Kelly *et al.*, 2016), which may be important for the correct self-assembly and release of some proteins. Furthermore, the BEVS is recognised for its inherent safety, large transgene capacity and its ease and speed of recombinant virus production (reviewed by Hu, 2008).

Additional research has also allowed for further improvements to the BEVS. For example, the addition of anti-apoptotic genes into the baculovirus vector extended the viability of infected cells. This extend the period for recombinant protein production thus increasing its yield (Steele *et al.*, 2017). Furthermore, deletion of *chiA* and *cath* from the baculovirus vector protected recombinant protein from degradation and enabled greater production (Kaba *et al.*, 2004). Deletion of other non-essential genes (*p26*, *p74* and *p10*) further increased the expression of foreign genes (reviewed by Hitchman *et al.*, 2009).

Further research has facilitated the production of multi-protein complexes, such as virus-like particles (VLPs). Initially, and still to this day, co-infection of cells with multiple recombinant viruses, each expressing different foreign genes, allows for the co-production of several proteins *in vitro* (St Angelo *et al.*, 1987; Dodson *et al.*, 1989). This method allows for flexibility of protein production through variations of the multiplicity of infection (MOI). However, the burden of copying genetic material from multiple different viruses can result in lower protein yields (Vieira *et al.*, 2005). Co-infection is further hindered by the uneven distribution of individual virus infections across the cell population. Consequently, co-expression (in which multiple genes are expressed from a single baculovirus vector) is becoming a popular alternative method for multi-protein production, as it ensures each infected cell expresses each foreign gene. Co-expression was facilitated by the construction of multi/dual-expression BEVS that contained multiple copies of the *p10* and *polh* promoters (Weyer and Possee, 1991; Belyaev *et al.*, 1995; Berger *et al.*, 2004). Currently, many dual expression vectors have been produced with some commercially available for expression in both insect (pOET5.1 (OET Ltd.), pFastBac™ Dual vector (Invitrogen™)) and mammalian cells (Zhang *et al.*, 2014). These developments have meant that recently, the

BEVS is increasingly being used to produce protein complexes such as VLPs for vaccine production (Belyaev and Roy, 1993; Zhang *et al.*, 2018; Mi *et al.*, 2021)

However, one limitation of many dual expression vectors, including those such as the MultiBac system, is their use of multiple copies of just the *p10* or *polh* promoters. This can sometimes cause instability in the recombinant virus, in the form of loss or rearrangement of the foreign gene sequences due to homologous recombination between the duplicated promoter sequences (R. Possee, personal communication). Consequently, production/discovery of other strong promoters for the expression of foreign genes is desirable as this would reduce the number of identical promoter sequences which facilitate foreign gene sequence loss or rearrangement as a result of homologous recombination.

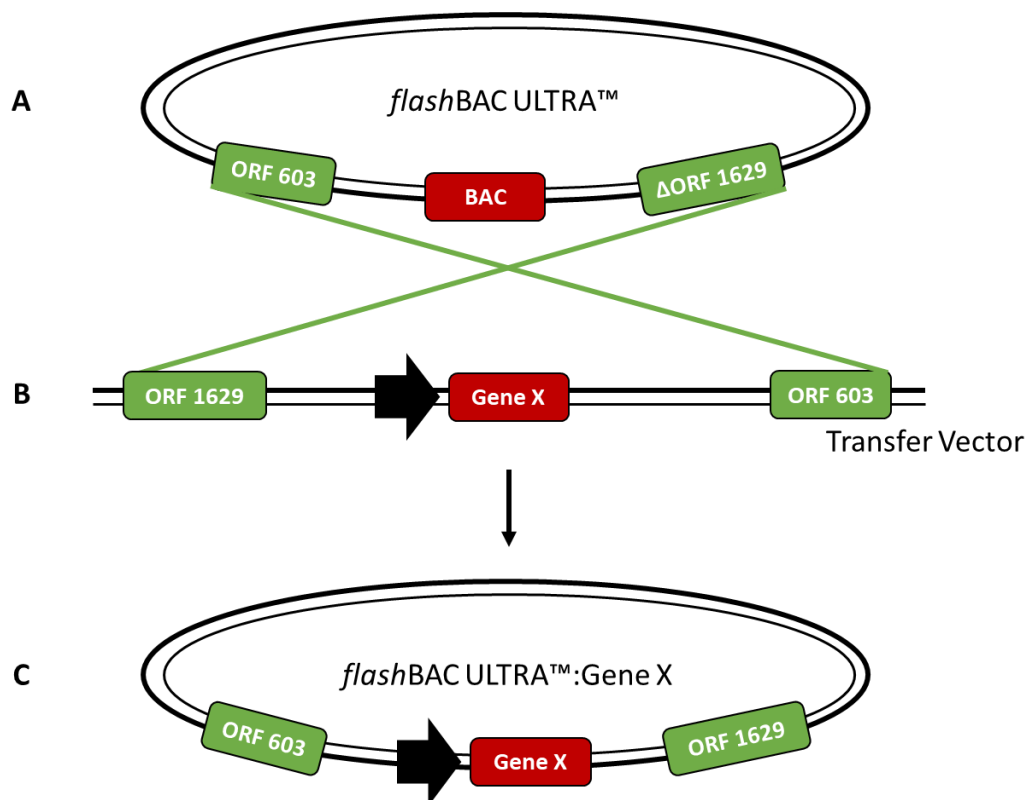


Figure 1.4- Overview of how the *flashBAC ULTRA™* system works to produce recombinant baculovirus. (A) *flashBAC ULTRA™* DNA containing a partially deleted copy of the essential gene *orf1629*, bacterial artificial chromosome (BAC) and complete *orf603* gene. (B) Sequence encoding the desired gene to be expressed is cloned into an appropriate transfer vector (containing the complete *orf1629* and *orf603*) under the *polh* or other appropriate promoter (black arrow). Homologous recombination removes the BAC and the incomplete *orf1629* gene, replacing them with the gene and promoter of interest and the complete *orf1629*. (C) A recombinant AcMNPV genome is produced. Restoration of the *orf1629* allows for replication within insect cells. Harvested recombinant budded virus can now be used to synthesise the target protein.

Much of the work in this thesis involves the production of recombinant viruses, made using the *flashBAC* ULTRA™ expression system (OET Ltd; Figure 1.4). Briefly, the *flashBAC* ULTRA™ system consists of an AcMNPV genome modified to contain a bacterial artificial chromosome (BAC) at the *polh* locus, replacing the *polh* coding sequence. It also lacks part of the essential gene *orf1629*. The presence of the BAC enables the genome to be maintained in bacterial cells as a bacmid, whilst the *orf1629* deletion prevents virus replication in insect cells. Insect cells are co-transfected with this genome and a transfer plasmid containing the gene of interest flanked by the complete *orf603* and *orf1629* (sequences that flank the *polh* in the virus genome). This results in homologous recombination within the insect cells that restores the *orf1629* allowing the recombinant virus to replicate and removes the BAC, which is replaced by the gene of interest under the *polh* (or other) promoter. The replication of this virus genome results in the production of recombinant virus, which can be harvested as BV from the cell culture medium. Use of this recombinant BV to infect insect cells then allows for the expression of the inserted foreign gene and production of the desired protein as the virus replicates.

1.7 BEVS for VLP production

The ability of the BEVS to produce high yields of multiple proteins has meant it is increasingly being used for the production of protein complexes such as VLPs for vaccine production (Liu *et al* 2012; reviewed by Liu *et al.*, 2013; Arevalo *et al.*, 2016; Zhang *et al.*, 2018; Mi *et al.*, 2021). Structurally, VLPs are multimeric protein complexes that mimic the conformation of virus particles but lack the viral genome. They are assembled from one or a few viral proteins and can be enveloped or non-enveloped depending on the virus and method of production (Liu *et al.*, 2013). They lack the ability to replicate and infect, and are capable of inducing an immune response due to their repetitive, high density display of epitopes (Bachmann *et al.*, 2004). This, therefore, makes them ideal vaccine candidates. Furthermore, VLPs are safer and often cheaper than live or attenuated vaccines. The mechanism of VLP production varies depending on the virus itself, as well as on the type of VLP being assembled (enveloped or non-enveloped). Usually, the assembly of an enveloped VLP culminates in the interaction of the matrix and/or capsid proteins with cellular protein complexes at the plasma membrane, which results in the budding of the VLP. Many VLP-

based vaccines have been developed using BEVS including GlaxoSmithKline's Cervarix™ against human papilloma virus and the Belyaev and Roy (1993) Bluetongue VLPs. In addition to their uses as vaccines, VLPs have also shown potential as surface display platforms and vehicles for gene delivery (reviewed by Roy and Noad, 2009; Koho *et al.*, 2015).

VLPs are considered to be strong candidates for a SARS-CoV-2 vaccine. To date, a number of VLP-based coronavirus vaccines have been developed using the BEVS (Lu *et al.*, 2007; Bai *et al.*, 2008; Lu *et al.*, 2010; Liu *et al.*, 2011). This is therefore promising for the production of a SARS-CoV-2 VLP-based vaccine produced in the same manner. Indeed, there are currently multiple SARS-CoV-2 VLP-based vaccines under development, including that of Premas Biotech (Arora *et al.*, 2020), Medigoaco Inc./ GSK (reviewed by Kaur and Gupta, 2020), Lanzhou University (China) (Mi *et al.*, 2021), Tampere University (Finland) (Kaur and Gupta, 2020), and Vaxine/Oxford Expression Technologies Ltd (R. Possee, personal communication). Those made by Lanzhou University, Tampere University, and Vaxine/Oxford Expression Technologies Ltd are all being produced in the BEVS.

A significant limitation of the BEVS, however, is the co-production and therefore contamination of VLP samples with the baculovirus BV, which can often reach titres of up to 10^8 /mL. Although AcMNPV cannot replicate in mammalian cells, BV contamination of biopharmaceuticals is still a particular concern to regulatory bodies as BVs are enriched with the glycoprotein GP64, which has been shown to be immunogenic in mammals (Luo *et al.*, 2013). Consequently, before VLP preparations can be used for scientific or medical purposes, they must be biophysically or biochemically separated from BVs. However, similarities in size, architecture, and physical properties (Wagner *et al.*, 2014) makes the consistent, scalable and economically feasible isolation of VLPs from BVs particularly difficult (reviewed by Pijlman, 2015). Furthermore, harsh purification can reduce VLP potency (particularly that of enveloped VLPs) and yield by harming their physical structure (Huhti *et al.*, 2010; Pijlman, 2015).

Currently, sucrose or caesium chloride gradient ultracentrifugation is the primary method for the purification of VLPs. However, this approach comes with numerous limitations including being labour-intensive, time-consuming and scale-restricted with batch-to-batch variation in production quality (Liu *et al.*, 2013). Ultracentrifugation has also often been

found to result in low VLP yield and failure to remove significant quantities of BV contaminants (Huhti *et al.*, 2010; Liu *et al.*, 2013). Consequently, additional steps are usually needed to achieve the high levels of VLP purity required. These can include ultrafiltration/diafiltration, size exclusion chromatography, or ion-exchange chromatography (Liu *et al.*, 2013; Smith *et al.*, 2013; Qu *et al.*, 2020; Boonyakida *et al.*, 2022). However, research has shown charge and size similarity between BVs and some VLPs to still pose a problem for separation by ion- and size exclusion chromatography (Wagner *et al.* 2014). Alternatively, purified VLPs can undergo chemical inactivation by detergent or low pH treatment to eliminate BV infectivity (Koho *et al.*, 2012; Liu *et al.*, 2013). However, enveloped VLPs can be sensitive to chemical treatment and therefore these methods can reduce their antigenicity (Liu *et al.*, 2013).

Both the biophysical and biochemical methods for VLP/BV separation come with significant limitations, which inherently reduces the attractiveness of the BEVS as a tool for VLP production. I therefore propose an alternative strategy which would reduce, and potentially eliminate, the requirement for these time consuming, multi-step VLP purification processes. A novel, repressible BEVS will be developed that employs components of the *Escherichia coli lac* operon to permanently switch off or down-regulate the expression of essential genes (identified using RNAi) required for BV production during VLP production (LacI/O Repressor System). An upstream solution like this, which inhibits BV production would eliminate the need for stringent downstream VLP processing, and would save significant time and money.

1.8 RNA interference

RNA interference (RNAi) is a natural phenomenon present in organisms such as fungi, plants, metazoans, insects and mammals (reviewed by Hammond *et al.*, 2001). It utilises microRNA (miRNA) to induce the degradation of the endogenous cellular mRNA homologue via an RNA-induced silencing complex (RISC), thus preventing translation of the mRNA to protein. This miRNA is derived from a longer precursor transcript of pre-miRNA, produced by the cell itself. Upon migration to the cytoplasm, the pre-miRNA is cleaved to mature miRNA by an endonuclease protein (Dicer). It is now widely accepted that for plants, RNAi may act as a defence mechanism against viral infections (reviewed by Waterhouse *et al.*,

2001 and Rosa *et al.*, 2018). The purpose of RNAi in animals, however, is yet to be determined, although roles in germ line fate, organism development, and defence against viruses and transposable elements have been suggested (reviewed by Finnegan *et al.*, 2001). The natural process of RNAi can be induced *in vitro* (Figure 1.5) by delivering double-stranded RNA (dsRNA) into cells experimentally (Fire *et al.*, 1998), and is a popular tool for the study of gene expression. The dsRNA is cleaved by Dicer, just as the endogenous pre-miRNA would be, to produce short interfering RNA (siRNA) ~20-25nt in length. Similarly to miRNA, the siRNA binds to RISC and induces the degradation of the endogenous cellular mRNA homologue.

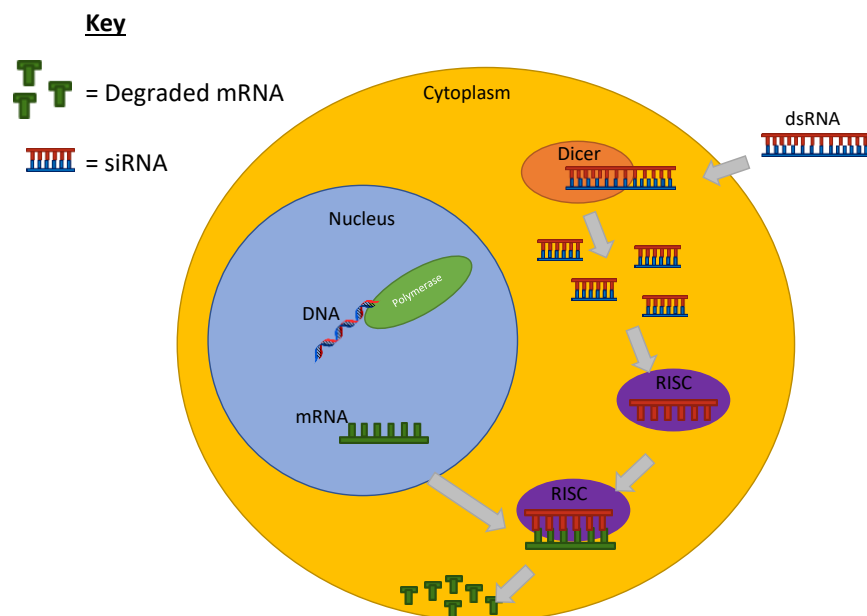


Figure 1.5- Schematic representation of the process of RNA interference (RNAi) induced *in vitro* by dsRNA transfection. dsRNA is introduced into the cell and is bound by an endonuclease protein known as Dicer, which cleaves it into shorter fragments about 20-25nt long. These fragments bind an Argonaut protein, which selects one of the strands to be degraded. This forms the RNA induced silencing complex more commonly known as RISC. RISC is guided by the siRNA to bind the corresponding mRNA. RISC induces the cleavage of the mRNA, which is then degraded, thereby preventing the translation of the mRNA into a polypeptide chain.

RNAi is a popular tool for the study of gene regulation. Indeed, rather than making time consuming gene deletions to silence gene expression, RNAi can be used to target the destruction of relevant mRNA. For example, Valdes *et al.* (2003) used RNAi to prevent AcMNPV infection *in vivo* and *in vitro* by targeting *gp64* and *ie1* mRNA. More recently, RNAi

has been used to improve the production of recombinant proteins from various expression platforms including insect cells (reviewed by Chavez-Pena and Kamen, 2018). For example, Kim *et al.* (2007) used RNAi to target caspase-1 in Sf9 cells, reducing its activity and introducing a cell line more resistant to apoptosis than its untransformed equivalent. By reducing apoptosis, Kim *et al.* (2007) were able to reduce the loss of viable cells capable of recombinant protein production, thus increasing protein yield. Further research has also shown potential for the reduction of BV production when individual genes were silenced by RNAi. Marek *et al.* (2011) prevented BV synthesis without affecting recombinant protein production when targeting the baculovirus *vp80* gene, whilst Lee *et al.* (2015) reduced BV production when targeting *gp64*.

The initial stages of research in this thesis will use this convenient RNAi tool in a screening process to knockdown numerous structural genes associated with the BV. This will allow for the identification of key genes integral to the successful assembly and release of BVs, and which therefore prevent or at least significantly reduce BV production upon silencing. Control transfections with a non-specific dsRNA targeting the firefly luciferase gene would ensure results reflect the true knockdown potential of each structural gene by accounting for any non-specific/off-target effects that may occur as a result of the transfection process. The identified key genes will then be targeted for controlled knockdown by a gene repressor system that employs elements of the *E. coli lac* operon.

1.9 The *Escherichia coli lac* operon and its uses in the development of a repressor system

The well characterised *E. coli lac* operon (Figure 1.6) is concerned with the metabolism of lactose. It consists of three structural genes: *lacZ*, coding for β -galactosidase, which acts to cleave lactose into glucose and galactose; *lacY*, coding for *lac* permease, a transmembrane protein necessary for lactose uptake; and *lacA*, which codes for a transacetylase that transfers an acetyl group from coenzyme A (CoA) to the hydroxyl group of galactosides (reviewed by Ramos *et al.*, 2013). These genes are under the negative control of a tetrameric repressor protein (LacI) encoded constitutively by the *lacI* gene. When lactose is present in high levels, the inducer allolactose, derived from lactose, binds to the LacI repressor subunits, and prevents their assembly into an active tetramer (reviewed by

Pelley, 2012). This allows for *lacZYA* gene expression and the subsequent metabolism of lactose. However, in the absence of lactose the LacI repressor protein binds specifically to the *lac* operator (*lacO*) sequence located 11bp downstream of the transcriptional start site (reviewed by Lewis, 2005) and inhibits transcription of the *lacZYA* genes. Repression from the *lac* operon has been found to be particularly strong in comparison to other negatively regulated bacterial systems. Indeed, in the absence of LacI repression, the level of the *lac* enzymes may increase approximately 1000-fold, whereas the *gal* operon is only induced <20-fold (Oehler *et al.*, 1990).

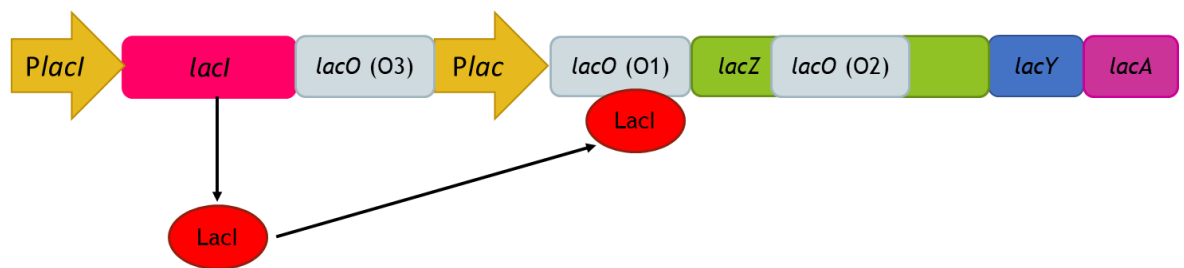


Figure 1.6- Schematic representation of the *Escherichia coli* *lac* operon.

The three structural genes (*lacZ*, *lacY*, and *lacA*) are controlled from the *P_{lac}* promoter. They are repressed by the *lac* repressor from three operators (light blue bars): O1 is centered at +11 with respect to the transcription start, O2 is located within the beta-galactosidase gene 401bp downstream of O1, O3 is located 92bp upstream of O1. The *lacI* gene codes for the LacI repressor protein, which can bind to the *lac* operators in the absence of lactose. This physically blocks RNA polymerase from transcribing the *lac* structural genes. Details are not drawn to scale.

Gilbert and Maxam (1973) found the *lacO* to be a pseudosymmetric sequence, 27bp in length, with a central G:C base pair. Later, the minimal sequence required for specific LacI binding was found to be only the 17bp at the centre of the Gilbert and Maxam 27bp sequence (Bahl *et al.*, 1977). In 1974 and 1976, the presence of two additional auxiliary operators was discovered. The O2 is located 401 bp downstream of the O1 (Reznikoff *et al.*, 1974) whilst the O3 is 92 bp upstream (Gilbert *et al.*, 1976). The *in vitro* affinity of O2 for LacI has been determined to be 10-fold less than that of O1 (Pfahl *et al.*, 1979; Winter and von Hippel, 1981) whilst the *in vitro* affinity of O3 is between 16- and 1000-fold lower than that of O1 (Pfahl *et al.*, 1979; Fried and Crothers, 1981; Winter and von Hippel, 1981). Due to their relatively low affinities for LacI and their distances from the promoter, it was

initially believed that the two “pseudo” operators played no significant role in repression from the *lac* operon. However, since then, research has found LacI capable of simultaneously binding to two operator sequences located on one DNA fragment and that binding of O1 and O2 or O1 and O3 forces the intervening DNA into a loop structure (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Oehler *et al.*, 1990). Loop formation from the two auxiliary operators has been shown to significantly increase repression up to 48-fold (Oehler *et al.*, 1990; Becker *et al.*, 2013).

In 1986, gene repression was shown to be possible from two synthetic *lac* operators flanking the *lac* promoter in an artificial system (Besse *et al.*, 1986). Since then, other research has used these *lac* operon elements to regulate gene expression from the vaccinia virus. Fuerst *et al.* (1989) saw a 97% reduction in β -galactosidase activity when the *lacO* was inserted into the F17R (P11) vaccinia virus late promoter. Zhang and Moss (1991) also reported a 95% reduction in gene transcription when *lacO* was inserted into the same promoter. Along with this, much other research has provided insights into the mechanics (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018) and design (Shore *et al.*, 1981; Shore and Baldwin, 1983; Krämer *et al.*, 1987; Oehler *et al.*, 1994; Becker *et al.*, 2013) of a synthetic *lac* repressor system.

To date, a selectively repressible LacI/O system has never been established in the baculovirus expression system. However, the promising results from the research in vaccinia virus, along with the high repression levels achieved in comparison to other negatively regulated bacterial systems, makes the elements of the *lac* operon ideal candidates for the production of a repressor system incorporated into the BEVS. I therefore intend to use the elements from the *E. coli lac* operon to develop a unique, selectively repressible BEVS in which BV production can be switched off or down-regulated during VLP/protein production (the LacI/O Repressor System). An AcMNPV promoter associated with identified genes of interest will be modified to contain *lacO*. A recombinant virus will be produced containing this modified promoter construct so that upon infection of a cell line expressing *lacI*, LacI will bind the *lacO* and block the expression of the associated genes. Whilst initially one gene associated with BV production will be targeted for knockdown, the system developed will have the ability to knock down expression of two genes for increased

BV inhibition. Further development of this system could allow for knockdown of three or more BV associated genes, which may allow for even an even greater reduction in BV production. Ultimately, this system will be used to block the expression of identified key BV structural genes to inhibit BV production, enabling the production of VLPs and other proteins in the absence of BVs. This would eliminate the need for downstream processing of VLPs/proteins to remove the contaminating BV particles, which would save considerable time and money during the production of biopharmaceuticals. Indeed, the recent event of the COVID-19 pandemic has highlighted the need for fast, safe methods for the production of vaccines. Successful development of a BV free BEVS would considerably reduce the production time of VLP vaccine in an event such as this.

Whilst the main objective of this thesis was to use the developed LacI/O Repressor System to switch off or down-regulate the expression of essential genes required for BV production during VLP/protein production, the system could also be utilised for other purposes. In theory, the system could be used to inhibit the expression of any of the AcMNPV genes for the investigation of many different viral processes. Furthermore, the repression system could be used for the regulation of foreign gene expression. This would be particularly useful for cytotoxic gene products, which can render making the original recombinant virus difficult. Infection of the cell line producing LacI would allow for downregulation of the cytotoxic gene during virus amplification. Subsequent infection of Sf9 cells would then allow for expression of the cytotoxic gene when required.

1.10 Aims

The BEVS has been shown to be a valuable system for the expression of VLPs and other recombinant proteins. A significant limitation of the BEVS, however, is the co-production and therefore contamination of VLPs with the baculovirus BV. This represents a particular concern to regulatory bodies due to the fact that BVs are enriched with the glycoprotein GP64, which is immunogenic in mammals (Luo *et al.*, 2013). Therefore, the presence of BVs would be undesirable in VLP vaccine preparations. Furthermore, the cost of downstream processing to remove contaminating BV particles in terms of both time and money is considerable, particularly because BVs are similar in size and density to VLPs. Consequently, this project aimed to develop an upstream solution that inhibited BV production, thus eliminating the need for downstream processing. A novel, selectively repressible system was developed that employed components of the *E. coli lac* operon to switch off or down-regulate the expression of essential genes required for BV production during VLP/protein production (the LacI/O Repressor System).

Initially, RNA interference was used in a screening process to knockdown a large number of different structural genes associated with the BV. This allowed identification of key genes integral to the successful assembly and release of BVs, and therefore prevent or at least significantly reduce BV production upon silencing. In contrast to previous research, this thesis assessed which combinations of these key genes, if silenced, would afford the greatest reduction in BV production, thus enabling the determination of an optimal knockdown combination. The identified key genes were then targeted for knockdown by the developed LacI/O Repressor System.

The development of the LacI/O Repressor System involved designing a promoter construct modified to contain the *lac* operator. The *chitinase/cathepsin* (*chiA/cath*) promoter acted as the promoter of choice. This first required assessment of the viability of the promoter upon insertion of *lacO*. Further work also assessed how the positioning of the *lac* operator within the promoter affected gene expression, with the intent of identifying insertion sites that would knockdown expression without affecting recombinant protein/ VLP production. This section of work yielded significant data on the functional structure of the *chiA/cath* promoter.

The next key step in the development of the LacI/O Repressor System was to produce an Sf9 cell line expressing *lacI*. Infection of these cells with a recombinant virus expressing the modified *lacO* promoter would result in the repression of BV production during VLP production, whereas infection of non-modified Sf9 cells would allow for BV production. Together, these elements formed a novel, repressible system that allows for the assembly of VLPs in the absence or significantly reduced production of BV when desired.

Chapter 2:

Materials and Methods

2.1 Materials

2.1.1 Insect cell lines and cell culture reagents

The experiments described in this thesis used the following insect cell lines: Sf21 cells, originally derived from the pupal ovarian cells of the fall army worm, *Spodoptera frugiperda* (Vaughn *et al.*, 1977), Sf9 (ATCC® CRL-1711™) and SfC1B5 cells (personal communication, R. Possee OET Ltd), clonal cell lines derived from Sf21 cells. The SfLacI cell line was established from Sf9 cells (Chapter 5) and both were maintained in ESF921 medium (Expression Systems, USA). The SfLacI cells were supplemented with 0.6mg/mL G418 (Gibco) until passage 12 and then reapplied after every 10 passages for 2 consecutive passages. Sf21 cells were grown in TC100 (Gibco) medium supplemented with 10% (v/v) foetal calf serum (FCS; Labtech, UK; (King and Possee, 1992)), whilst SfC1B5 cells were maintained in ESF-AF animal-free medium (Expression Systems, USA). Cells were maintained as shaking (Sf9, SfC1B5 and SfLacI; 135rpm) or spinner cultures (Sf21; 80-90rpm). Occasionally cells were adapted to different growth conditions as experiments required. All cell lines were grown at 28°C.

2.1.2 Viruses

All viruses and bacmid DNA generated or used in this thesis are presented in Table 2.1. Viruses were supplemented with 2% (v/v) FCS and working stocks were stored at 4°C. For long-term storage viruses were stored at -80°C.

Table 2.1- Viruses and bacmid DNA

Virus	Description	Source	Chapter
AcMNPV C6 (AcMNPV)	Plaque isolate of the wild type prototype type I alphabaculoviruses	R. D. Possee (OET Ltd.)	3,4, 5, 6
p35Δbacmid	AcMNPV bacmid, in which the <i>p35</i> gene was replaced with a <i>neo</i> expressing cassette	R. D. Possee (OET Ltd.)	3
AcPolh-EGFP	Recombinant AcMNPV expressing <i>egfp</i> from the <i>polh</i> promoter	This thesis	4, 6
AcChiA-EGFP	Recombinant AcMNPV expressing <i>egfp</i> from the <i>chiA/cath</i> ^(syn) promoter	This thesis	4, 6
AcLacO77-EGFP	Recombinant AcMNPV expressing <i>egfp</i> from the LacO77 promoter	This thesis	4, 6
AcLacO79-EGFP	Recombinant AcMNPV expressing <i>egfp</i> from the LacO79 promoter	This thesis	4, 6

Table 2.1 Continued- Viruses and bacmid DNA

AcLacO81-EGFP	Recombinant AcMNPV expressing <i>egfp</i> from the LacO81 promoter	This thesis	4, 6
AcLacO83-EGFP	Recombinant AcMNPV expressing <i>egfp</i> from the LacO83 promoter	This thesis	4, 6
gp64Δbacmid	AcMNPV bacmid, in which the <i>gp64</i> gene was replaced with a <i>neo</i> expressing cassette	R. D. Possee (OET Ltd.)	4
gp64ΔFBG	<i>flashBAC Gold™</i> with native <i>gp64</i> deleted and replaced with a <i>KanR</i> expressing cassette	This thesis	4
AcPolh-gp64	Recombinant AcMNPV expressing <i>gp64</i> from the <i>polh</i> promoter. <i>gp64</i> was deleted from its natural position and replaced with a <i>KanR</i> expressing cassette	This thesis	4, 6
AcChiA- gp64	Recombinant AcMNPV expressing <i>gp64</i> from the <i>polh</i> promoter. <i>gp64</i> was deleted from its natural position and replaced with a <i>KanR</i> expressing cassette	This thesis	4, 6
AcLacO77-gp64	Recombinant AcMNPV expressing <i>gp64</i> from the LacO77 promoter. <i>gp64</i> was deleted from its natural position and replaced with a <i>KanR</i> expressing cassette	This thesis	4, 6
AcLacO79-gp64	Recombinant AcMNPV expressing <i>gp64</i> from the LacO79 promoter. <i>gp64</i> was deleted from its natural position and replaced with a <i>KanR</i> expressing cassette	This thesis	4, 6
AcLacO81-gp64	Recombinant AcMNPV expressing <i>gp64</i> from the LacO81 promoter. <i>gp64</i> was deleted from its natural position and replaced with a <i>KanR</i> expressing cassette	This thesis	4, 6
AcLacO83-gp64	Recombinant AcMNPV expressing <i>gp64</i> from the LacO83 promoter. <i>gp64</i> was deleted from its natural position and replaced with a <i>KanR</i> expressing cassette	This thesis	4, 6
AcChiA_TAAG-EGFP/mCherry	Recombinant AcMNPV expressing <i>egfp</i> and <i>mCherry</i> from the ChiA_TAAG promoter	This thesis	4, 6
AcPOET5-EGFP/mCherry	Recombinant AcMNPV expressing <i>egfp</i> and <i>mCherry</i> from the <i>polh</i> and <i>p10</i> promoters respectively	This thesis	4, 6
AcLacO85-EGFP/mCherry	Recombinant AcMNPV expressing <i>egfp</i> and <i>mCherry</i> from the LacO85 promoter	This thesis	4, 6
AcLacI	Recombinant AcMNPV expressing <i>lacI</i> and a SV40 nuclear localisation signal (NLS) from the <i>polh</i> promoter	This thesis	5
AcLacI-ΔNLS	Recombinant AcMNPV expressing <i>lacI</i> from the <i>polh</i> promoter with SV40 NLS removed	This thesis	5

2.1.3 Plasmids

All plasmids used in this thesis are described in Table 2.2 and were stored at -20°C.

Table 2.2- Plasmids

Plasmid	Description	Source	Chapter
pPolh-EGFP	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>egfp</i> replacing <i>polh orf</i> driven by <i>polh</i> promoter	R. Arinto Garcia (OET Ltd.)	4
pChiA-EGFP	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>egfp</i> driven by <i>chiA/cath</i> ^(syn) promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO77-EGFP	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>egfp</i> driven by LacO77 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO79-EGFP	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>egfp</i> driven by LacO79 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO81-EGFP	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>egfp</i> driven by LacO81 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO83-EGFP	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>egfp</i> driven by LacO83 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pgp64_Twist	Transfer vector encoding <i>gp64</i> without a promoter	Twist Bioscience	4
pAcBgIII-E	Transfer vector encoding <i>gp64</i> flanked by regions from its native locus	R. D. Possee (OET Ltd.)	4
pPolh-gp64	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>gp64</i> replacing <i>polh orf</i> driven by <i>polh</i> promoter	This thesis	4
pChiA- gp64	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>gp64</i> driven by <i>chiA/cath</i> ^(syn) promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO77- gp64	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>gp64</i> driven by LacO77 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO79- gp64	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>gp64</i> driven by LacO79 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO81- gp64	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>gp64</i> driven by LacO81 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pLacO83- gp64	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>gp64</i> driven by LacO83 promoter replacing <i>polh</i> promoter and <i>orf</i>	This thesis	4
pOET5	Transfer vector encoding AcMNPV LEF-2, <i>polh</i> and <i>p10</i> promoters in opposite orientation	R. D. Possee (OET Ltd.)	4
pmCherry-Actin	Transfer vector encoding <i>mCherry</i>	C. Bannach (OET Ltd.)	4
pChiA_TAAG-EGFP/mCherry	Transfer vector encoding AcMNPV LEF-2 and <i>egfp</i> and <i>mCherry</i> replacing <i>polh orf</i> driven by the dual ChiA_TAAG promoter	This thesis	4

Table 2.2 Continued- Plasmids

pOET5-EGFP/mCherry	Transfer vector encoding AcMNPV LEF-2 and <i>egfp</i> and <i>mCherry</i> replacing <i>polh</i> orf driven by the <i>polh</i> and <i>p10</i> promoters, respectively	This thesis	4
pLacO85-EGFP/mCherry	Transfer vector encoding AcMNPV LEF-2 and <i>egfp</i> and <i>mCherry</i> replacing <i>polh</i> orf driven by the dual LacO85 promoter	This thesis	4
pOET2-LacI	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>lacI</i> and SV40 nuclear localisation signal (NLS), replacing <i>polh</i> orf driven by <i>polh</i> promoter	R. D. Possee (OET Ltd.)	5
pOET2-LacI-ΔNLS	Transfer vector encoding AcMNPV LEF-2, ORF603 and <i>lacI</i> without an NLS, replacing <i>polh</i> orf driven by <i>polh</i> promoter	This thesis	5
pIE1k-LacI+	Transfer vector encoding <i>lacI</i> and NLS driven by the <i>Ac-ie1</i> promoter	R. D. Possee (OET Ltd.)	5
pIEk1-1neo	Transfer vector encoding <i>KanR</i> (conferring resistance to the antibiotic G418) driven by the <i>Ac-ie1</i> promoter	R. D. Possee (OET Ltd.)	5
pOET6-GFP	Transfer vector encoding <i>gfp</i> driven by the <i>polh</i> promoter	R. D. Possee (OET Ltd.)	5
pOpIE1-neo	Transfer vector encoding the <i>Op-ie2</i> promoter and <i>neo</i> (conferring resistance to the antibiotic G418) driven by the <i>Ac-ie1</i> promoter	R. D. Possee (OET Ltd.)	5
pOpIE1-neo-LacI	Transfer vector encoding <i>lacI</i> driven by the <i>Op-ie2</i> promoter and <i>neo</i> (conferring resistance to the antibiotic G418) driven by the <i>Ac-ie1</i> promoter	This thesis	5

2.1.4 Competent bacterial strains

For generation, maintenance or amplification of plasmids, DH5α or DH10B bacterial strains were used (Invitrogen™). Bacterial stocks were stored in aliquots at -80°C.

2.1.5 Antibiotics

Antibiotics for maintenance of bacteria are presented in Table 2.3 and were stored at -20°C.

Table 2.3- Antibiotics

Antibiotic	Working concentration
Ampicillin	100 µg/ml
Chloramphenicol	15 µg/ml
Kanamycin	15 µg/ml
Tetracycline	3 µg/ml

2.1.6 Primers and Probes

The DNA oligos used in this thesis are presented in Table 2.4. Oligo primers were obtained from Eurofins MWG Operon (Germany) and dissolved in dH₂O to stock concentrations of 100 pmol/μL and stored at -20°C.

Table 2.4- Primers and Probes

DNA oligo name	Sequence (5' → 3')	Chapter
qPCR probe <i>gp64</i>	[FAM]AAAAGTCTACGTTACACACGCGCCAAA[TAM]	3
qPCR <i>gp64</i> R ^{OET}	CGGCGTGAGTATGATTCTCAA	3
qPCR <i>gp64</i> F ^{OET}	ATGAGCAGACACGCAGCTTTT	3
RDP213	TGAGACGCACAACTAATATCAC	4
RDP214b	ATACGTACAACAATTGTCTGTAAATCAAC	4
RDP1059	TTAATACGACTCACTATAGGGTTAGACGGCTATTCTCCACCTG	3
RDP1060	TTAATACGACTCACTATAGGGATGGCGCTAGTGCCCGTGGG	3
RDP1061	TTAATACGACTCACTATAGGGTTAATAGTAGCGTGTCTGTAAAC	3
RDP1062	TTAATACGACTCACTATAGGGATGGTTTATCGTCGCCGTCGCC	3
RDP1102	TCAGGATCCATAAATATGAGCCATATTCAACGGGAA	5
RDP1103	GACAGATCTTGCTCTGCCAGTGTTACAAC	5
RDP1104	TTAATACGACTCACTATAGGGGAAAACAGTCGTCGCTGTCAC	4
RDP1105	TTAATACGACTCACTATAGGGGAGTACATGAAGTTGCGCGTG	4
RDP1112	TTAATACGACTCACTATAGGATGTGTGTAATTTTCCGG	4
RDP1113	TTAATACGACTCACTATAGGGTTGAGTGCAGTAGTTACATTG	4
RDP1117	TTAATACGACTCACTATAGGGATGAACACGGACGCTCAGTC	3
RDP1118	TTAATACGACTCACTATAGGGTCCCGCTACACCAATAACC	3
RDP1119	TTAATACGACTCACTATAGGGATGTGTTTCGACCAAGAAACCG	3
RDP1120	TTAATACGACTCACTATAGGGCATCGATGCGGTCATCAAGTAC	3
RDP1121	TTAATACGACTCACTATAGGGCTACAGTTGTGTGCGTG	3
RDP1122	TTAATACGACTCACTATAGGGCCGAGATATCAACACGTTG	3
RDP1123	TTAATACGACTCACTATAGGGCGTCAAGTTTGTACCTCTG	3
RDP1124	TTAATACGACTCACTATAGGGCAGACGCAGATAGCGG	3
RDP1125	TTAATACGACTCACTATAGGGATTACACGAGCCGCAGC	3
RDP1126	TTAATACGACTCACTATAGGGTTCAACACCGTCGACC	3
RDP1127	TTAATACGACTCACTATAGGGTTGACGAGCAAATTGGAGG	3
RDP1128	TTAATACGACTCACTATAGGGCCGATATCGGCGACAC	3
RDP1129	TTAATACGACTCACTATAGGGACACGCCATGTATTGGC	3
RDP1130	TTAATACGACTCACTATAGGGCTGGTACGAATCGACAAACG	3
RDP1131	TTAATACGACTCACTATAGGGTGACGAGTTGATCGAATG	3
RDP1132	TTAATACGACTCACTATAGGGCATCGTTTGACTTTGACG	3
RDP1133	TTAATACGACTCACTATAGGGCACAATATTAAACGCGC	3

Table 2.4 Continued- Primers and Probes

RDP1134	TTAATACGACTCACTATAGGGAACCTTGCAATTGGACACGGC	3
RDP1135	TTAATACGACTCACTATAGGGCCCCAGCTATTTCTTTGC	3
RDP1136	TTAATACGACTCACTATAGGGCAGCACGTGACAAAC	3
RDP1137	TTAATACGACTCACTATAGGGTTGGGATGAAAGTCGATCG	3
RDP1138	TTAATACGACTCACTATAGGGCGCTCAGCAAATACATGG	3
RDP1139	TTAATACGACTCACTATAGGGTAAAAAGCGAATGGTCCG	3
RDP1140	TTAATACGACTCACTATAGGGTAACATCCAGCGACCTG	3
RDP1141	TTAATACGACTCACTATAGGGCAATGTTGTACAGCGGCG	3
RDP1142	TTAATACGACTCACTATAGGATGCCGTACGGCGAATAGTC	3
RDP1143	TTAATACGACTCACTATAGGCATGACCAACACGCTGTG	3
RDP1144	TTAATACGACTCACTATAGGGCTTGTATCGGCGTGCG	3
RDP1145	TTAATACGACTCACTATAGGGCGTTTTTGGGCGGGT	3
RDP1146	GTCGGCGGATGTTCTATATC	5
RDP1152	TTCGGATCCAGTCACTTGGTTGTTACGATC	5
RDP1230	TACAAGCTTGTGAGTTCAAGTCTCGCTGAG	4
RDP1332	Phos-CCTTGAGGATCCATTCTGCA	5
RDP1333	Phos-GAATGGATCCTCAAGG	5
RDP1376	ATCTTTTTACATAAAATAATTACAACATAAAATTAAGATAAAATTAAG	4
RDP1377	GATCCTTTAATTTATCTTAATTTTATGTTGTAATTATTTTATGTAAAAAAGAT	4
RDP1378	ATCTTTTTACATAAAATAATTACAACATAAAATTAAGATAAAATTAAGAAT TGTTATCCGCTCACAATTCCG	4
RDP1379	GATCCGGAATTGTGAGCGGATAACAATTCCTTAATTTATCTTAATTTTATGTT GTAATTATTTTATGTAAAAAAGAT	4
RDP1380	ATCTTTTTACATAAAATAATTACAACATAGAATTGTTATCCGCTCACAATTC CAAATTAAGATAAAATTAAGAATTGTTATCCGCTCACAATTCCG	4
RDP1381	GATCCGGAATTGTGAGCGGATAACAATTCCTTAATTTATCTTAATTTGGAATT GTGAGCGGATAACAATTCTATGTTGTAATTATTTTATGTAAAAAAGAT	4
RDP1387	GGATCCTAAGATACATTGATGAGTTTGGACAAACCACAAC	5
RDP1413	GATCTTTTTTACATAAAATAATTACAACCTAAAAATTAAGATAAAATTAAG	4
RDP1414	GATCCTTTAATTTATCTTAATTTTAAAGTTGTAATTATTTTATGTAAAAA	4
RDP1415	GATCTGGAATTGTGAGCGGATAACAATTCCTTTTTACATAAAATAATTACAAC TTAAAAATTAAGATAAAATTAAGAATTGTTATCCGCTCACAATTCCG	4
RDP1416	GATCCGGAATTGTGAGCGGATAACAATTCCTTAATTTATCTTAATTTTAAAGTT GTAATTATTTTATGTAAAAAAGAATTGTTATCCGCTCACAATTCCA	4
RDP1417	ATCTTTTTTACATAAAATAATTACAACATAAAATTAAGATAAAATTAAGAATT GTTATCCGCTCACAATTCGAATTGTTATCCGCTCACAATTCCG	4
RDP1418	GATCCGGAATTGTGAGCGGATAACAATTCGGAATTGTGAGCGGATAACAATT CTTAATTTATCTTAATTTTATGTTGTAATTATTTTATGTAAAAAAGAT	4
RDP1419	ATCTTTTTTACATAAAATAATTACAACATAGAATTGTTATCCGCTCACAATTCCA AATTAAGATAAAATTAAG	4
RDP1420	GATCCTTTAATTTATCTTAATTTGGAATTGTGAGCGGATAACAATTCTATGTTG TAATTATTTTATGTAAAAAAGAT	4
VEB2	TACGATGTCGAGAGTATGC	5
VEB3	GGTATCGATAAGCTTGGGC	5

2.1.7 Antibodies

Primary and secondary antibodies used in this thesis were diluted in PBS-Tween with 5% milk to the appropriate concentration (Table 2.5).

Table 2.5- Antibodies

Antibody	Species	Working dilution	Supplier
Anti-GP64 AcV5-tag	Mouse	1:2800 (WB)	Sigma-Aldrich
eGFP	Mouse	1:1000 (WB)	Thermo Fisher Scientific
Anti-LacI	Mouse	1:1000 (WB)	EMD Millipore Corp.
Anti-mCherry	Mouse	1:1000 (WB)	Novus Biologicals
Anti-mouse IgG conjugated to horseradish peroxidase (HRP)	Goat	1:50000 (WB)	Jackson ImmunoResearch

2.1.8 Buffers and solutions

General chemicals were purchased from the following companies unless otherwise stated: BDH Chemicals Ltd, Fermentas, Invitrogen™, Life Technologies, New England Biolabs® Inc. (NEB®), Promega, Sigma-Aldrich, Thermo Fisher Scientific or VWR. Reagent percentages are w/v unless otherwise stated. Cell culture media was supplied by Gibco or Oxford Expression Technologies Ltd (OET). Sterilisation was performed by autoclaving for 15 minutes, 15 pounds per square inch or by filtration (0.2µm). Enzymes and appropriate buffers were supplied by NEB® or Promega and manufacturers' recommendations followed for reaction optimisation.

Table 2.6- Buffers and solutions

Name	Composition
Alkaline Phosphatase (APS)	100mM Tris-HCl pH 9.2, 100mM NaCl, 5mM MgCl ₂
Cell lysis buffer (NP40)	150mM NaCl, 50mM Tris, 1% Nonidet™ P 40 (NP40)
Inoue transformation buffer	10mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) pH 6.7, 55mM MnCl ₂ •4H ₂ O, 15mM CaCl ₂ •2H ₂ O, 250mM KCl
PBS-Tween	PBS, 0.1% (v/v) Tween 20
PBS-T milk powder	PBS, 0.1% (v/v) Tween 20, 5% milk powder
Phosphate-buffered saline (PBS, 1x)	From tablets – 10mM sodium phosphate, 2.68mM KCl, 140mM NaCl
SDS loading buffer (6x)	250mM Tris-HCl pH 6.8, 10% SDS, 50% (v/v) glycerol, 0.05-0.5% bromophenol blue, 25% (v/v) β-mercaptoethanol (added fresh)
SDS running buffer (5X)	124.6mM Tris base, 0.8% SDS, 1.25M glycine (final pH ~8.3)
Super optimal broth (SOB) medium	2% tryptone, 0.5% yeast extract, 8.56mM NaCl, 2.5mM KCl, 10mM Mg ₂ Cl pH 7.0
TE buffer (1x)	100mM Tris-HCl, 1mM EDTA pH 8.0
Tris-acetate (TAE) buffer (1x)	40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA pH 8.0
Towbin buffer	25mM Tris base pH 8.3, 192mM glycine, 20% (v/v) methanol

2.2 Virus Methods

All virus work was performed in a sterile environment using aseptic techniques. All virus methods including virus amplification, small-scale virus infection of insect cells, production of recombinant viruses by co-transfection of insect cells, transfection of cells with viral or plasmid DNA, isolation of budded viruses from cell culture medium, virus titration by plaque assay and purification of genomic DNA from baculovirus BVs was performed as previously described (King and Possee, 1992; OET, 2020). Virus titration by BaculoQUANT™ qPCR was performed according to manufacturer's instructions (Hitchman *et al.*, 2007; OET Ltd.)

2.3 Bacterial Methods

All bacterial work was performed in a sterile environment using aseptic techniques.

2.3.1 Production and transformation of competent bacterial cells

Chemically (DH5α) or physically (DH10B) competent cells were generated for the heat-shock method or electroporation, respectively (as described in Sambrook and Russell, 2006). For transformation, competent cells were thawed on ice. 50μl of *E. coli* competent cells were transformed with 2-5μl of the ligation mix per reaction (see 2.4.5) and plated on Lysogeny broth agar (Thermo Fisher Scientific) Petri dishes, as described (Green and Sambrook, 2012). A non-digested vector (1-10ng) was treated in the same way to test cell efficiency.

2.3.2 Plasmid purification from bacteria and evaluation

Single colonies of transformed cells were diluted in 10μl of 1x TE and screened by PCR with appropriate primers for the presence of the insert (see 2.4.1). Appropriate colonies were grown overnight (12-15h) in shaking cultures (200rpm) of LB Broth (Thermo Fisher Scientific) supplemented with the appropriate antibiotic. Plasmid DNA from bacterial cultures was purified using DNprep Spin Miniprep kit (Qiagen®) following the manufacturer's protocol. Alternatively to the PCR screening, plasmid DNA was digested with appropriate restriction enzymes to confirm the presence and orientation of the insert.

All plasmid DNA presenting the right insert was sequenced by the Sanger method (see section 2.4.6).

2.3.3 Red®/ET® Recombination

Homologous recombination by Red®/ET® for the production of gene deletion bacmids was carried out according to the manufacturer's instructions (Gene Bridges GmbH). Bacmid DNA was purified according to the manufacturer's instructions (Gene Bridges GmbH).

2.4 Molecular biology methods

2.4.1 Polymerase chain reaction (PCR)

DNA amplification or screening by PCR was performed using *Taq* polymerase (NEB® Inc.) and reactions were performed according to the supplier's protocol. Amplified DNA was purified using QIAquick PCR purification kit (Qiagen®) according to the supplier's instructions.

2.4.2 Agarose gel electrophoresis

For the separation of DNA or RNA according to its size, agarose gels (1%, Thermo Fisher Scientific) were prepared as described (Green and Sambrook, 2012) and submerged in 1x TAE buffer. DNA electrophoresis were run horizontally at 80-120 volts for 45min-1.5h and visualised using a UV transilluminator (Bio Rad, ChemiDoc™ Imaging Systems). For DNA purification by agarose gel electrophoresis, the appropriate band was excised using QIAquick gel extraction kit (Qiagen®) as recommended by the supplier.

2.4.3 Restriction enzyme digestion

Restriction enzyme digestion of DNA was performed as recommended by the manufacturer (NEB® Inc.).

2.4.4 Dephosphorylation of DNA

For cloning purposes, previously digested vector DNA was dephosphorylated using Antarctic Phosphatase (NEB® Inc.) according to the supplier's instruction. Samples were purified using the QIAquick PCR purification kit (Qiagen) and stored at -20°C for the ligation step (section 2.4.5).

2.4.5 DNA ligation

The ligation of DNA with complementary ends was performed using T4 DNA Ligase (Promega or NEB® Inc.) according to the supplier's recommendations.

2.4.6 DNA sequencing

DNA sequencing was performed by Source Bioscience (U.K). The resulting data was analysed using SnapGene® software (version 5.0.8).

2.4.7 RNA synthesis

For dsRNA synthesis, the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB® Inc.) was used according to the supplier's protocol.

2.4.8 RNA interference (RNAi)

For the transfection of cells with RNA for RNAi, the appropriate cells were incubated with 5µg (or alternative during titration) of the appropriate dsRNA and 7µL of Lipofectamine® RNAiMAX (Thermo Fisher Scientific) for 5h at 28°C in a 35mL dish. The transfection mix was removed and cells infected with AcMNPV MOI (multiplicity of infection) 1 (e.g., 1 plaque forming unit (pfu) per cell) for 1h. The inoculum was removed and cells washed three times with PBS before replacing the appropriate growth medium and incubating at 28°C for 48h. Media containing BV was harvested and clarified as described (section 2.2).

2.5 Protein Analysis

SDS-PAGE was utilised for protein separation (Green and Sambrook, 2012) and following supplier's information (Bio-Rad Laboratories, Inc.). Separated proteins were visualised by immuno- (western) blotting against the protein of interest as described (Green and Sambrook, 2012). Images were acquired using the ChemiDoc™ imaging system (Bio-Rad Laboratories, Inc.).

For the analysis of protein location within a cell, cell fractionation with a lysis buffer (Table 2.6) to lyse the cell membrane and release cytoplasmic proteins was performed. Pelleted cells were resuspended in 200µl lysis buffer and incubated at 4°C rotating for 1h. Cytoplasmic and nuclear fractions were separated by centrifugation at 15,000rpm for 10mins at 4°C.

2.6 Flow cytometry

For analysis of cells producing a fluorescent protein (EGFP), cells in suspension (Sf9 or SfLacI) were infected at the appropriate MOI. At the appropriate time point, 2mL of each cell sample was harvested, washed in Dulbecco's PBS 1X (DPBS, Gibco) and resuspended in 190µl of DPBS. Cells were incubated with Hoechst 33342 Ready Flow™ Reagent and incubated for 1h according to the supplier's instructions.

NovoCyt Flow Cytometer with NovoExpress 1.5.6 software (ACEA Biosciences, USA) was used with the appropriate laser and detector for each fluorochrome (Table 2.7). From the total cases for each sample, cells for analysis were selected by the presence of blue fluorescence (DAPI/Hoechst 33342), followed by size and single cell selection. Finally, AcMNPV-infected cells were used as a negative control to establish the positive/negative threshold for EGFP or mCherry fluorescence. For each individual experiment, three replicates for each infection were performed. The data of each infection replicate was determined by three technical replicate reads by the NovoCyt Flow Cytometer.

Table 2.7- Flow cytometry laser and detector settings

Fluorochrome	Laser	Detector
Hoechst 33342/DAPI	405nm	445nm
EGFP	488nm	530nm

2.7 Fluorescence analysis by plate reader

Cells were grown as described above (section 2.6), and at the desired time point transferred to a black-wall 12-well plate and incubated at 28°C for 1h to attach. To measure background fluorescence, ESF-921 media was dispensed in replicate wells. Fluorescence was read at 480nm/530nm for EGFP and 580nm/610nm for mCherry respectively for excitation and emission using the SpectraMax iD3 Microplate reader (Molecular devices, USA). For each individual experiment, three replicates for each infection were performed. The data of each infection replicate was determined by three technical replicate-well reads by the Microplate reader.

2.8 Microscopy techniques

Bright field and fluorescence microscopy was performed using a Revolve-D200 (Echo Laboratories; 2.2.4 software). Fluorescence was read at 470/30nm and 525/50nm for EGFP, 560/40nm and 630/75nm for mCherry, and 380/30nm and 450nm for Hoechst 33342/DAPI, respectively for excitation and emission.

2.9 Statistics

Statistical analysis was performed and graphs produced using Microsoft® Office Excel 2016. F-tests for variance and Students T-test for statistical significance were performed and results considered significant if $p < 0.05$. The coefficient of determination (R^2) value was calculated to determine the relationship between two factors.

Chapter 3:

**Identification of baculovirus genes
required for budded virus production
using RNA Interference**

3.1 Introduction

As described in Chapter 1 (section 1.8), RNA interference (RNAi) is a natural phenomenon present in organisms such as fungi, plants, metazoans, insects and mammals (reviewed by Hammond *et al.*, 2001). It utilises microRNA (miRNA) to induce the degradation of the endogenous cellular mRNA homologue via an RNA-induced silencing complex (RISC), thus preventing translation of the mRNA to protein. This natural process of RNAi can be induced *in vitro* by delivering double-stranded RNA (dsRNA) into cells experimentally (Fire *et al.*, 1998), and is a popular tool for the study of gene expression. The dsRNA is cleaved by Dicer to produce short interfering RNA (siRNA) ~20-25nt in length, which binds to RISC and induces the degradation of the endogenous cellular mRNA homologue (Figure 1.5).

RNAi is a popular tool for the study of gene regulation. Rather than making time consuming gene deletions to silence gene expression, RNAi can be used instead to target the destruction of relevant mRNA. For example, Valdes *et al.* (2003) used the method of RNAi to prevent AcMNPV infection *in vivo* and *in vitro* by targeting *gp64* and *ie1* mRNA. Other research has also employed RNAi to study the various processes involved in virus infection (reviewed by Terenius *et al.*, 2011). RNAi has also been used to improve the production of recombinant proteins from various expression platforms including the BEVS in insect cells (reviewed by Chavez-Pena and Kamen, 2018), often via the production of cell lines with improved performance (Kim *et al.*, 2007; Hebert *et al.*, 2009; Lai *et al.*, 2012; Wang *et al.*, 2016b).

As described previously (section 1.7), the BEVS has been shown to be a valuable system for the expression of VLPs and other recombinant proteins. A significant limitation of the BEVS, however, is the co-production and therefore contamination of VLPs with the baculovirus BV. This represents a particular concern to regulatory bodies as BVs are enriched with the glycoprotein GP64, which is immunogenic in mammals (Luo *et al.*, 2013). Therefore, the presence of BVs would be undesirable in a VLP vaccine or other biopharmaceutical preparations. Furthermore, the cost of downstream processing to remove contaminating BV particles in terms of both time and money is considerable, particularly because BVs are similar in size, architecture, and physical properties to some VLPs (Wagner *et al.*, 2014). Additionally, harsh purification can reduce VLP potency (particularly that of enveloped VLPs) and yield by harming their physical structure (Huhti *et al.*, 2010; Pijlman, 2015).

Consequently, this thesis aimed to develop an upstream solution which inhibited BV production, thus reducing the need for such complex downstream processing. A novel, repressible system was developed which employed components of the *E. coli lac* Operon to repress the expression of essential genes required for BV production during VLP production (the LacI/O Repressor System). This chapter, therefore, focused on first identifying the key, essential genes targeted for knockdown by this system.

There are many genes involved in the production of structural proteins associated with the BVs (Table 3.1) (reviewed by Blissard and Theilmann, 2018; Rohrmann, 2019). RNAi provides a quick and easy tool to silence the expression of specific genes by targeting the degradation of their mRNA transcript. RNAi was, therefore, used in a screening process to silence the expression of a number of different structural genes associated with the formation of BV's (Table 3.2). This allowed for quick assessment of which genes, either individually or in combination, are integral to the successful assembly and release of BVs, and therefore significantly reduced BV production upon silencing. The genes for knockdown included, but were not limited to, *gp64*, *vp39*, *p6.9* and *vp80*. Indeed, previous research already showed potential for reducing BV production by targeting individual genes with RNAi (Marek *et al.*, 2011; Lee *et al.*, 2015). For example, Marek *et al.* (2011) prevented BV production without affecting recombinant protein production by targeting the baculovirus *vp80* gene, whilst Lee *et al.* (2015) reduced BV production by targeting *gp64*. However, in contrast to this previous research, the work of this chapter went a step further by silencing the expression of multiple essential BV structural genes simultaneously, with the aim of achieving a greater or total reduction in BV production than was reported in these papers.

Table 3.1- Structural genes associated with AcMNPV BVs

AcMNPV Gene	Gene Product Name
Ac1	Protein tyrosine phosphatase (ptp)
Ac2	BRO (Baculovirus repeated orf)
Ac9	P78/83; Orf1629
Ac16	BV/ODV-E26
Ac23	F (fusion protein homolog)
Ac35	Ubiquitin-like protein
Ac36	PP31; 39K
Ac49	PCNA

Table 3.1 Continued- Structural genes associated with the AcMNPV BVs

Ac51	J domain
Ac53	Ac53
Ac54	Vp1054 (capsid protein)
Ac58/59	ChaB-like protein
Ac64	GP37; P34.8; spindlin; fusolin
Ac65	DNA polymerase
Ac66	Desmoplakin-like protein
Ac71	lap-2
Ac73	BAG (Bcl-2-associated athanogene) protein
Ac74	Ac74
Ac75	Ac75
Ac76	Ac76
Ac77	VLF-1 (very late factor 1)
Ac80	GP41
Ac82	TLP (telokin-like protein)
Ac83	VP91 (capsid protein); PIF-8
Ac89	VP39 (major capsid protein)
Ac92	P33; sulfhydryl oxidase (sox)
Ac93	P18
Ac94	ODV-E25; p25; 25k
Ac98	38K
Ac100	P6.9
Ac101	BV/ODV-C42
Ac104	Vp80; Vp87
Ac109	Ac109
Ac114	PARG (Poly(ADP-ribose) glycohydrolase)
Ac124	Ac124
Ac126	Chitinase
Ac127	Cathepsin; vcath
Ac128	GP64
Ac129	P24
Ac131	PEP (polyhedron envelope protein)
Ac132	Ac132
Ac139	Me53
Ac141	Exon-0
Ac142	49K; P49
Ac143	ODV-E18
Ac144	ODV-EC27
Ac145	P11
Ac148	ODV-E56, Pif-5
Ac150	Ac150

Table 3.2- dsRNAs targeting specific BV structural genes used during RNAi experiments.

dsRNA ID	AcMNPV Gene Target (orf #/ Name)	Primers (F: forward; R: reverse)	Length of dsRNA (bp)
vp39	Ac89 (<i>vp39</i>)	F: TTAATACGACTCACTATAGGGTTAGACGGCTATTCTCCACCTG R: TTAATACGACTCACTATAGGGATGGCGCTAGTGCCCGTGGG	1086
gp64	Ac128 (<i>gp64</i>)	F: TTAATACGACTCACTATAGGGGAAAACAGTCGTCGCTGTAC R: TTAATACGACTCACTATAGGGGAGTACATGAAGTTGCGCGTG	662
p6.9	Ac100 (<i>p6.9</i>)	F: TTAATACGACTCACTATAGGGTTAATAGTAGCGTGTCTGTAAC R: TTAATACGACTCACTATAGGGATGGTTTATCGTCGCCGTCGCC	210
p24	Ac129 (<i>p24</i>)	F: TTAATACGACTCACTATAGGGATGAACACGGACGCTCAGTC R: TTAATACGACTCACTATAGGGTCCCGGTACACCAAATAACC	723
Ac54(a)	Ac54 (<i>vp1054</i>)	F: TTAATACGACTCACTATAGGGATGTGTTGACCAAGAAACCG R: TTAATACGACTCACTATAGGGCTACACGTTGTGTGCGTG	1140
Ac54(b)	Ac54 (<i>vp1054</i>)	F: TTAATACGACTCACTATAGGGCATCGATGCGGTGTCAGTAC R: TTAATACGACTCACTATAGGGCTACACGTTGTGTGCGTG	751
Ac80	Ac80 (<i>gp41</i>)	F: TTAATACGACTCACTATAGGGCAGACGCAGATAGCGG R: TTAATACGACTCACTATAGGGATTACACGAGCCGCAGC	819
Ac83(a)	Ac83 (<i>vp91; pif-8</i>)	F: TTAATACGACTCACTATAGGGTTCAACACCGTCGACC R: TTAATACGACTCACTATAGGGTTGACGAGCAAATTGGAGG	560
Ac83(b)	Ac83 (<i>vp91; pif-8</i>)	F: TTAATACGACTCACTATAGGGTTCAACACCGTCGACC R: TTAATACGACTCACTATAGGGACACGCCATGTATTGGC	2271
Ac83(c)	Ac83 (<i>vp91; pif-8</i>)	F: TTAATACGACTCACTATAGGGCCGATATCGGCGACAC R: TTAATACGACTCACTATAGGGACACGCCATGTATTGGC	1616
Ac92	Ac92 (<i>p33; sox</i>)	F: TTAATACGACTCACTATAGGGCTGGTACGAATCGACAAACG R: TTAATACGACTCACTATAGGGTGACGAGTTGATCGAATG	446
Ac98	Ac98 (<i>38K</i>)	F: TTAATACGACTCACTATAGGGCATCGTTTGACTTTGACG R: TTAATACGACTCACTATAGGGCACAATATTAACGCGC	674
Ac101	Ac101	F: TTAATACGACTCACTATAGGGAACCTTGCAATTGGACACGGC R: TTAATACGACTCACTATAGGGCCCCAGCTATTTCTTTGC	911
Ac142	Ac142 (<i>49K; p49</i>)	F: TTAATACGACTCACTATAGGGCAGCAGTCGACAAAC R: TTAATACGACTCACTATAGGGTTGGGATGAAAGTCGATCG	1023
Ac144	Ac144 (<i>vp80, vp87</i>)	F: TTAATACGACTCACTATAGGGCGCTCAGCAAATACATGG R: TTAATACGACTCACTATAGGGTAAAAAGCGAATGGTCCG	627
vp80(a)	Ac104 (<i>vp80; vp87</i>)	F: TTAATACGACTCACTATAGGGTAACATCCAGCGACCTG R: TTAATACGACTCACTATAGGGCAATGTTGTACAGCGGCG	1005
vp80(b)	Ac104 (<i>vp80; vp87</i>)	F: TTAATACGACTCACTATAGGGTAACATCCAGCGACCTG R: TTAATACGACTCACTATAGGCATGACCAACACGCTGTG	1810
vp80(c)	Ac104 (<i>vp80; vp87</i>)	F: TTAATACGACTCACTATAGGATGCCGTACGGCGAATAGTC R: TTAATACGACTCACTATAGGCATGACCAACACGCTGTG	918
ORF1629	Ac9 (<i>orf1629</i>)	F: TTAATACGACTCACTATAGGGCTTGTATCGGCGTGCG R: TTAATACGACTCACTATAGGGCGTTTTTGGGCGGGT	800
Ac66	Ac66	F: TTAATACGACTCACTATAGGGCCGAGATATCAACACGTTG R: TTAATACGACTCACTATAGGGCGTCAAGTTGTACCTCTG	1250

Therefore, the overall aim of this chapter was to use RNAi as a tool to identify the key genes required for the inhibition of BV production. Firstly, RNAi was used in a screening process to identify which individual structural genes induced a reduction in BV production following silencing. It was then further assessed which combinations of these key genes, if silenced, afforded the greatest reduction in BV production, thus enabling identification of an optimal knockdown combination. The results of this chapter informed the latter stages of the project, in which the key genes were knocked down by the developed LacI/O Repressor System for the purposes of reducing BV production during VLP/protein production.

3.2 Results

3.2.1 Production of dsRNA *in vitro*

For the transfection of cells, dsRNAs targeting structural protein genes associated with the formation of BVs were synthesised using specific primers (Table 3.2). Pure dsRNA samples were visualised on a 1% agarose gel to confirm successful synthesis (Figure 3.1). Results showed each of the synthesised dsRNAs to be of the expected size.

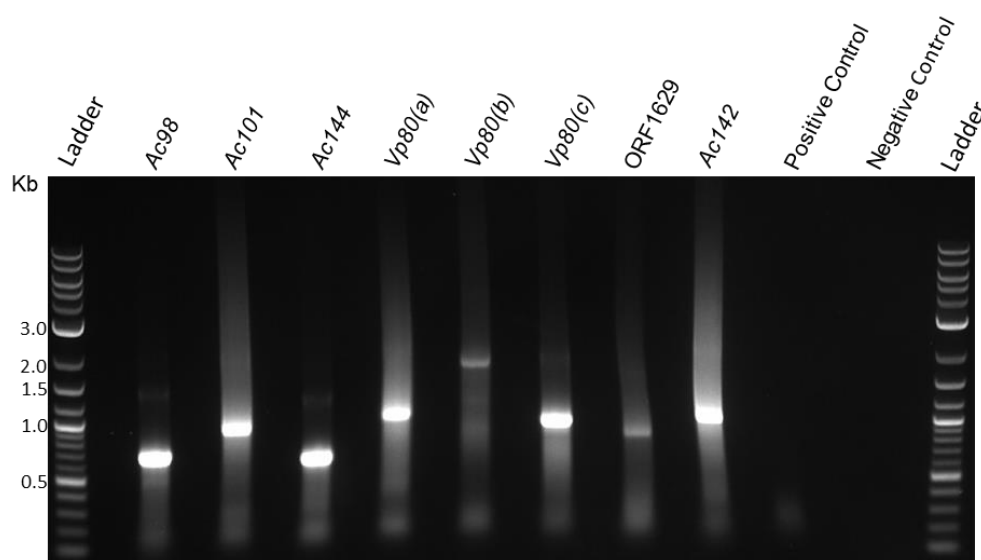


Figure 3.1- Synthesis of dsRNA *in vitro*.

Analysis of some of the synthesised dsRNAs on a 1% agarose gel. Specific gene sequences targeting different structural protein genes associated with the formation of BVs were amplified by PCR from a wild-type AcMNPV DNA template using specific primers. dsRNA was then synthesised using the HiScribe™ T7 High Yield RNA Synthesis Kit (NEB). The kits supplied positive control and a dH₂O negative control were also synthesised. Each dsRNA gene target is labelled above the track. Where the target gene name is followed by a letter in brackets, e.g. (a), (b), (c), this indicates different sequences of dsRNA targeting the same gene.

3.2.2 Assessing the effects of RNAi in different cell lines

To determine which, out of three possible cell lines (Sf9, Sf21 or SfC1B5), was the best to use to assess the effects of RNAi on BV production, the cells were first transfected with *p35* dsRNA. As an anti-apoptotic gene, *p35* knockdown provided a quick and clear phenotype to assess RNAi activity. Successful knockdown would result in significant cellular apoptosis, which could be assessed by simple visual inspection of cells by light microscopy. Control cells, however, would not show signs of apoptosis. Sf21 cells were originally derived from the pupal ovarian cells of the fall army worm, *S. frugiperda* (Vaughn *et al.*, 1977); Sf9 (ATCC® CRL-1711™) and SfC1B5 (personal communication, R. Possee OET Ltd) are clonal cell lines derived from Sf21 cells.

Firstly, dsRNA targeting *p35* was synthesised. Cells from the three insect cell lines were incubated with the *p35* transfection mix (Chapter 2, 2.4.8) for 5 hours, before being infected with AcMNPV. Observations by light microscopy at 24 h.p.i. revealed no signs of apoptosis in the Sf9 or SfC1B5 cells treated with dsRNA (Figure 3.2D, F), suggesting little or no RNAi activity. The Sf21 cells, however, showed significant apoptosis (Figure 3.2E). Consequently, the Sf21 cells were selected for gene knockdown by RNAi for the purposes of assessing BV production.

3.2.3 Titration of dsRNA in Sf21 cells

Previous research using RNAi to induce specific gene silencing has shown significant variation in the amount of dsRNA used to transfect cells. For example, some studies used up to 60µg of dsRNA per 1x10⁶ cells whilst others have used as little as 1.5µg (Caplen *et al.*, 2002; Valdes *et al.*, 2003; Marek *et al.*, 2011; Salem *et al.*, 2013; Hodgson *et al.*, 2019; Lai *et al.*, 2020). Other research has shown the silencing effects of dsRNA to be dose-dependent when directly injected into insect larvae (Flores-Jasso *et al.*, 2004). Therefore, to determine the amount of dsRNA required to optimise gene knockdown *in vitro*, a titration was carried out in Sf21 cells.

Knockdown of *gp64* has been shown to reduce BV production from Sf21 cells (Lee *et al.*, 2015). Therefore, *gp64* dsRNA was titrated in Sf21 cells and the reduction in BV production assessed by both plaque-assay and qPCR. The percentage decrease in BV production (and therefore the average percent of BV remaining) when transfecting cells with *gp64* dsRNA

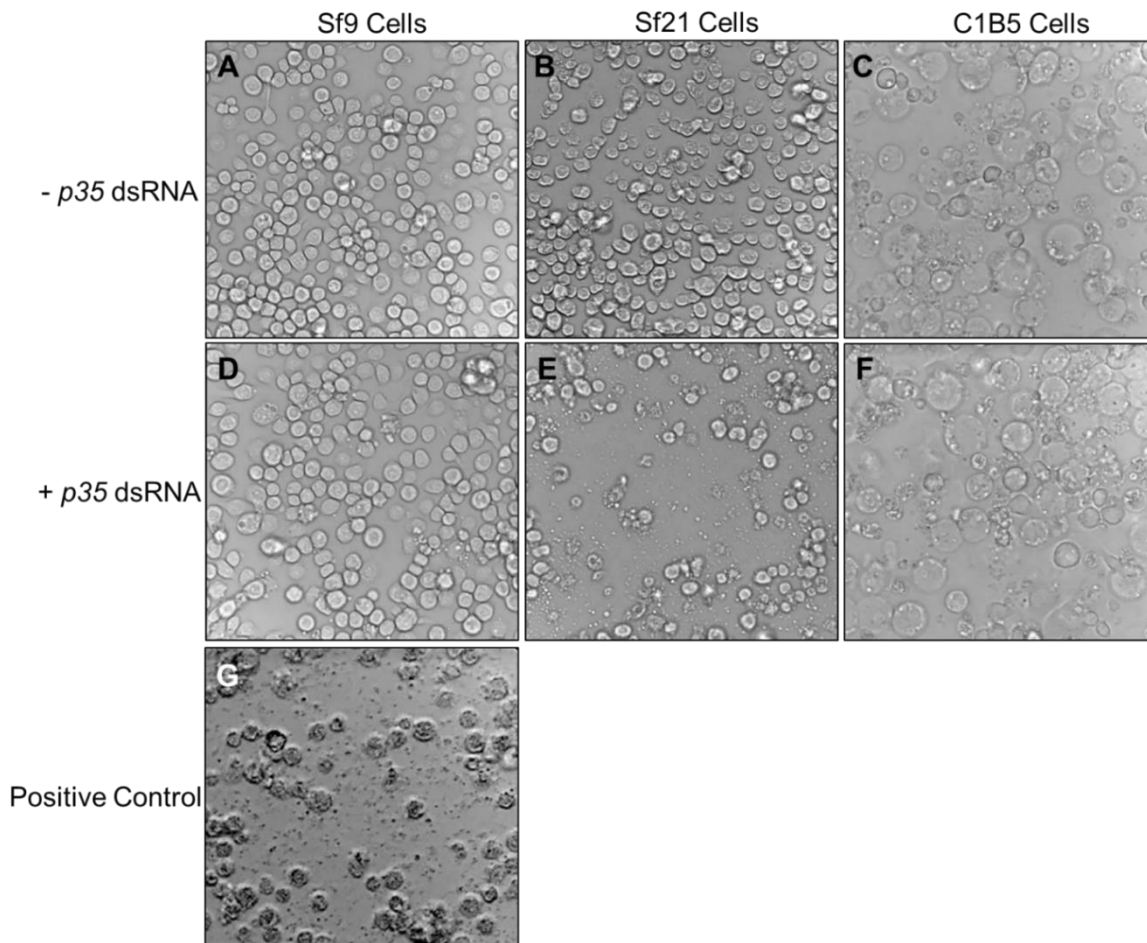


Figure 3.2- P35 RNA Interference in different cell lines.

Sf9, Sf21 and SfC1B5 cells were transfected for 5 hours with 5 μ g *p35* dsRNA (**D, E, F**). Control cells were transfected with no dsRNA (**A, B, C**). The cells were then infected with AcMNPV (MOI 1). Cells were imaged by Revolve bright field microscopy (Echo Laboratories) at 24 h.p.i. (20X Magnification). Transfections were performed in duplicate and a representative image is shown. A general apoptosis positive control was produced by infecting Sf9 cells with AcMNPV lacking *p35* (**G**).

was calculated from the amount of BV produced when transfecting the cells with a non-specific dsRNA as a control for non-specific effects (Figure 3.3).

Each amount of transfected *gp64* dsRNA, except 30ng, induced a significant reduction in BV production ($p < 0.05$). Both the plaque-assay and qPCR results for dsRNA between 2-7 μ g (panels A, B) had a very low coefficient of determination ($R^2 < 0.03$). This indicated that less than 3% of variance in the average percentage of BV remaining was explained by the amount of *gp64* dsRNA used during transfection. Conversely, both the plaque-assay and qPCR results for dsRNA between 3-0.03 μ g (panels C, D) had relatively high coefficients of determination ($R^2 = 0.65$ and 0.69). This indicated that 65-69% of variance in the average

percentage of BV remaining was explained by the amount of *gp64* dsRNA used during transfection. This data, therefore, suggested that using dsRNA between 2-7 μ g during transfection would not yield any significant difference in the levels of gene silencing. However, using less than 2 μ g dsRNA during transfection could result in reduced levels of gene silencing. Consequently, 5 μ g of dsRNA was used for future transfections.

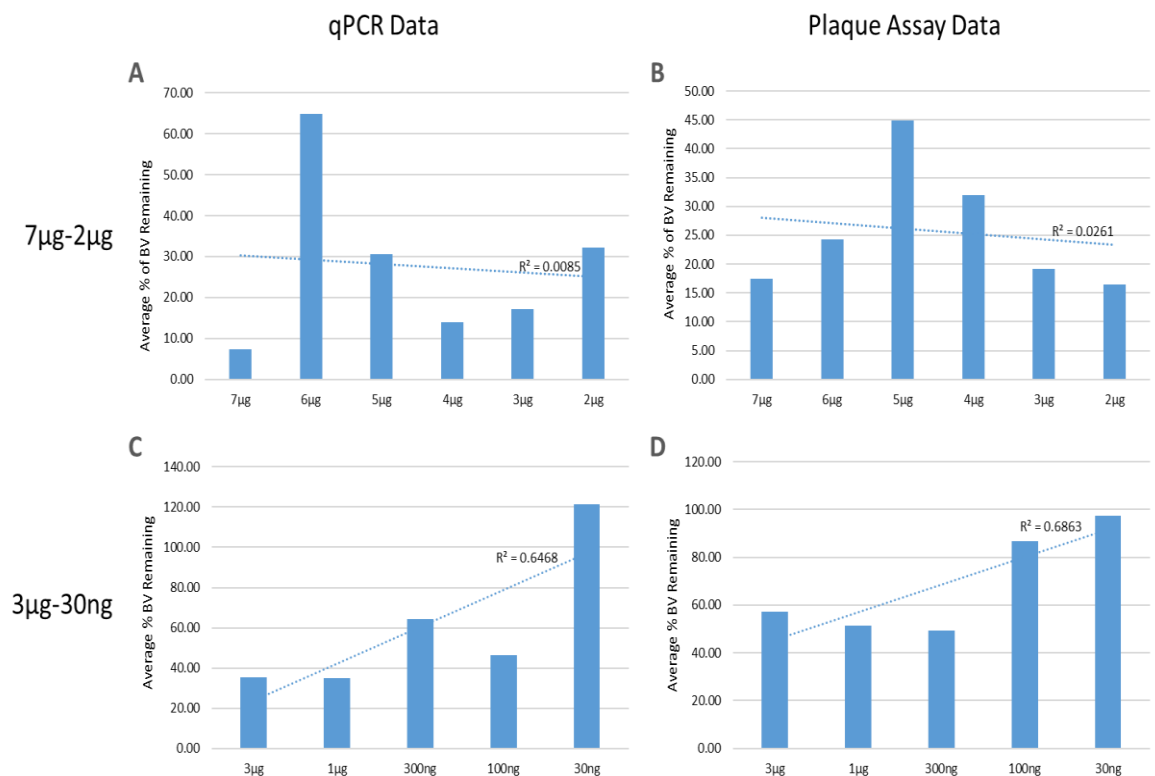


Figure 3.3- Titration of *gp64* dsRNA in Sf21 Cells.

Sf21 cells were transfected with different amounts of *gp64* or a control non-specific luciferase dsRNA, initially ranging between 7-2 μ g (**A, B**) and later between 3-0.03 μ g (**C, D**). The cells were incubated with the transfection mixes for 5 h, before being infected with AcMNPV (MOI 1). At 48 h.p.i. BV production was quantified by plaque-assay (**B, D**) and qPCR (**A, C**). The average percentage of BV remaining was calculated from the amount of BV produced when transfecting cells with the non-specific dsRNA (100%) to a control for non-specific effects of transfection. In each graph, the dashed line represents the regression line of the data points and gives an R^2 value indicating how much the dependent variable is determined by the independent variable. **A**) $R^2=0.0085$. **B**) $R^2=0.0261$. **C**) $R^2=0.6468$. **D**) $R^2=0.6863$.

3.2.4 Inhibition of structural protein gene expression in virus-infected cells

3.2.4.1 *Single gene inhibition*

To identify key structural protein genes integral to the successful production of BV's, and which would therefore inhibit BV production if targeted for knockdown by the LacI/O Repressor System, individual structural protein genes associated with the formation of BVs (Table 3.2) were screened for knockdown by RNAi. Sf21 cells were transfected with either a non-specific dsRNA targeting Luciferase to control for non-specific effects of transfection, or dsRNA targeting a specific structural protein gene. 5 h.p.t. cells were infected with AcMNPV (MOI 1). Culture medium was harvested at 48 h.p.i. for BV quantification by qPCR and the percentage decrease in BV production calculated (Figure 3.4A).

Statistical analysis of the qPCR results revealed 18 of the dsRNA transfections to have caused a significant reduction in BV production ($p < 0.05$); 17 at $p < 0.001$. Of the dsRNAs that resulted in 20% or less BV remaining, infectious BV counts were further assessed by plaque-assay (Figure 3.4B). The plaque-assay data supported the qPCR results except for *Ac54(a)*, *Ac54(b)* and *vp80(b)*, and showed 20% or less BV remaining. Consequently, *gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142* were selected as the targets for paired gene knockdowns by RNAi.

3.2.4.2 *Paired gene inhibition*

Following the identification of key individual genes that induced a significant reduction in BV production when silenced, further RNAi experiments were conducted to assess whether paired knockdown of these key genes would further reduce BV production. Sf21 cells were transfected with a non-specific dsRNA as before, or dsRNAs targeting two of the previously identified key structural protein genes. Further control transfections were also performed in which cells were transfected with just one of the selected dsRNAs. 5 h.p.t. cells were infected with AcMNPV (MOI 1). Culture medium was harvested at 48 h.p.i. for BV quantification by qPCR. The percentage decrease in BV production when transfecting the cells with each of the dsRNA pairs was calculated as before (Figure 3.5). The decrease in BV production when silencing the expression of two of the key genes was compared to the decrease in BV production when just one of the genes was silenced.

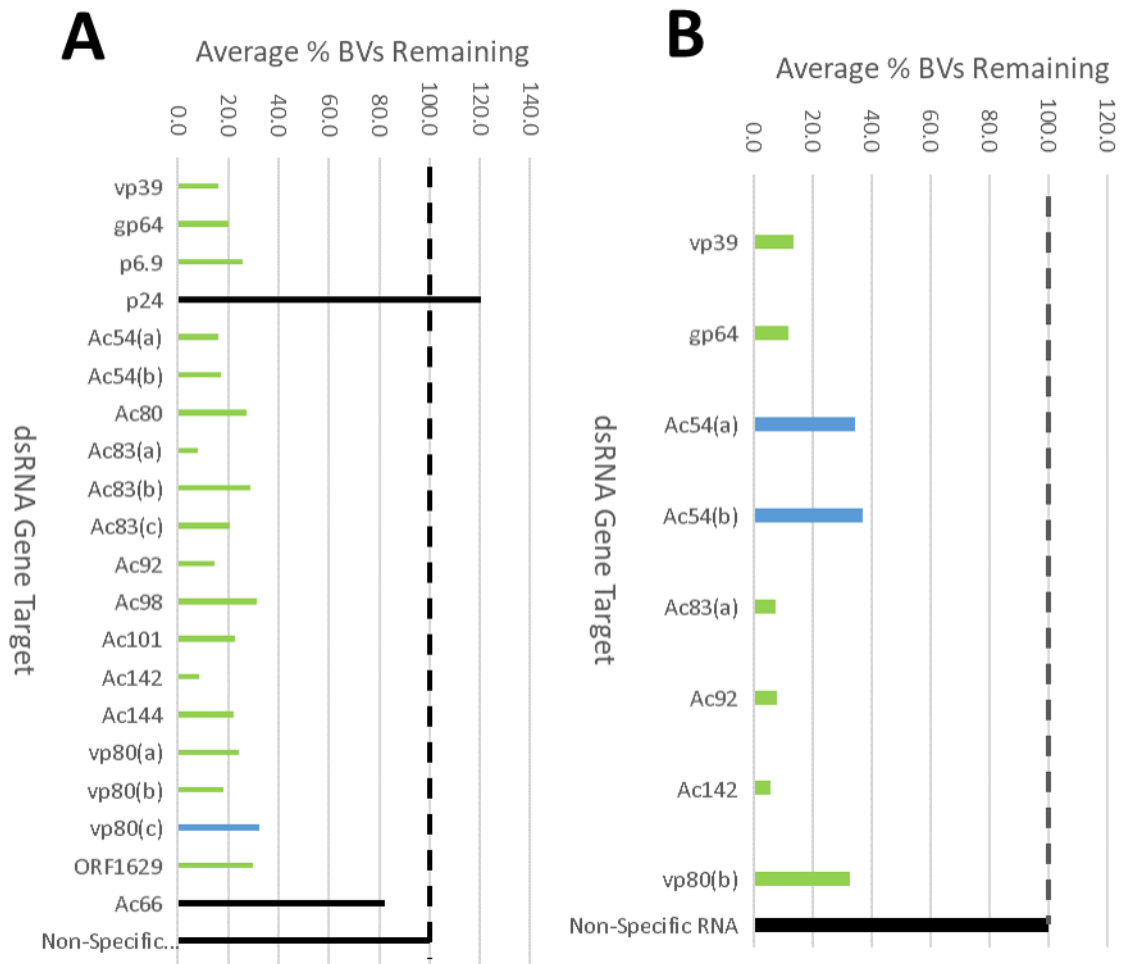


Figure 3.4- BV production from infected Sf21 cells following RNAi knockdown of individual structural protein genes. Sf21 cells were transfected with a non-specific dsRNA targeting Luciferase or dsRNA targeting a specific structural protein gene for 5 h, then infected with AcMNPV (MOI 1). Culture medium was harvested at 48 h.p.i. for BV quantification. Average percentage of BV remaining following gene silencing was determined by qPCR **(A)** and by plaque-assay **(B)**. Where the target gene name is followed by a letter in brackets, e.g. (a), (b), (c), this indicates different sequences of dsRNA targeting the same gene. In each graph, the dashed line indicates the BV production baseline detected for control cells treated with non-specific luciferase dsRNA to control for non-specific effects of transfection. Gene knockdowns that significantly reduced BV production are indicated in blue ($p < 0.05$) or green ($p < 0.001$). Gene knockdowns with no significant reduction in BV production are indicated in black. $n = 2$.

Statistical analysis of the qPCR results revealed the following gene pairs resulted in a significantly greater ($p < 0.05$) reduction of BV production when silenced compared to that observed when just one of the genes in the pair was silenced: *gp64* and *Ac83(a)*, *gp64* and *Ac142*, *vp39* and *Ac83(a)*, *vp39* and *Ac142*, and *Ac83(a)* and *Ac142*. These gene pairs could later be targeted for knockdown by a developed BEVS LacI/O repressor system.

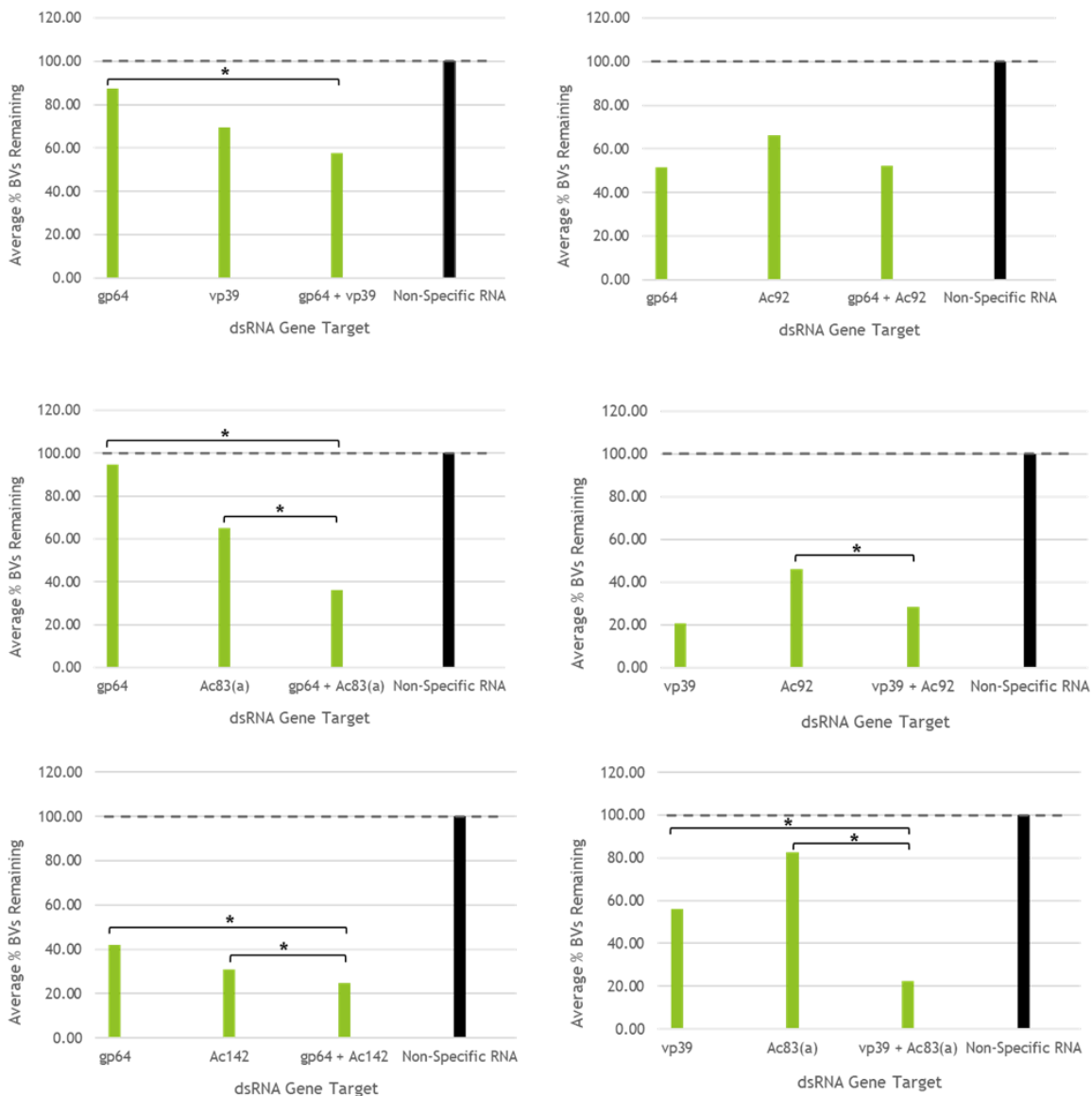


Figure 3.5- BV production from infected Sf21 cells following RNAi knockdowns of pairs of key structural protein genes. Sf21 cells were transfected with a non-specific dsRNA, or dsRNAs targeting one or two key genes. Cells were incubated with the transfection mixes before infection with AcMNPV (MOI 1). Culture medium was harvested at 48 h.p.i. for BV quantification by qPCR. $n=3$. The average percentage of BV remaining calculated from that seen from the non-specific control cells. The dashed line indicates the baseline of BV production detected from control cells treated with non-specific luciferase dsRNA. Where paired gene silencing yielded a significantly greater ($p<0.05$) reduction in BV production than that of the individual gene silencing, this is indicated by an asterisk (*).

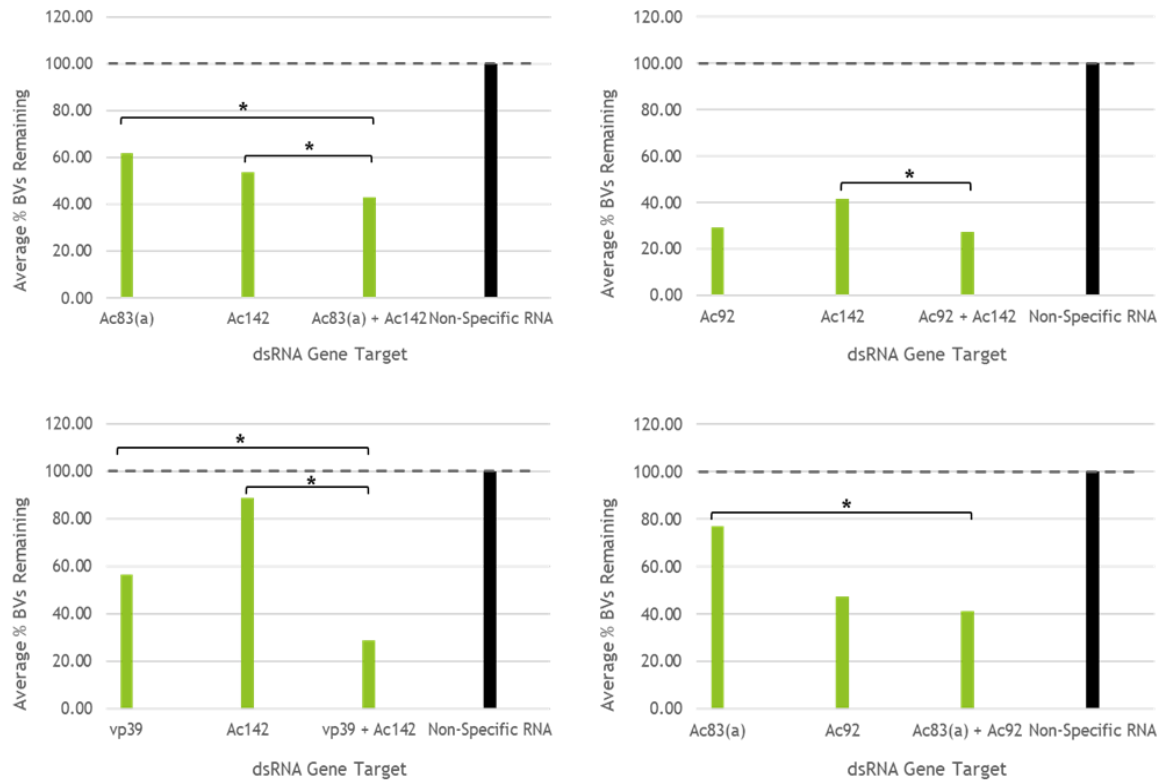


Figure 3.5 Continued- BV production from infected Sf21 cells following RNAi knockdowns of pairs of key structural protein genes. Sf21 cells were transfected with a non-specific dsRNA, or dsRNAs targeting one or two key genes. Cells were incubated with the transfection mixes before infection with AcMNPV (MOI 1). Culture medium was harvested at 48 h.p.i. for BV quantification by qPCR. n=3. The average percentage of BV remaining calculated from that seen from the non-specific control cells. The dashed line indicates the baseline of BV production detected from control cells treated with non-specific luciferase dsRNA. Where paired gene silencing yielded a significantly greater ($p < 0.05$) reduction in BV production than that of the individual gene silencing, this is indicated by an asterisk (*).

3.3 Discussion

The experimental work in this chapter relied on the use of RNAi technology to identify key genes influencing the production of BVs. Therefore, it was first essential to identify which cell line would be best to use to clearly assess the effects of RNAi. Previous research had reported the successful use of both Sf9 and Sf21 cells during RNAi procedures (Marek *et al.*, 2011; Salem *et al.*, 2013; Hodgson *et al.*, 2019; Lai *et al.*, 2020). Currently, there has been no research into the uses/effectiveness of RNAi technology in SfC1B5 cells. These cells were therefore selected for testing as they are rhabdovirus-free. Different approaches have been used by researchers when assessing the effectiveness of RNAi. Miller *et al.* (2012), for

example, measured levels of EGFP expression when using dsRNA targeted to *egfp*. In this case however, the expression of the anti-apoptotic gene, *p35*, was targeted by RNAi as its successful knockdown would result in a quick and clear phenotype (in the form of apoptosis) indicating RNAi activity. The presence of apoptosis was assessed by light microscopy 24h.p.i and, interestingly, whilst the Sf21 cells showed significant apoptosis as expected, the Sf9 cells showed none.

This unexpected lack of apoptosis/RNAi activity could be explained by differences in the transfection method used compared to others. It is possible that a longer transfection time may be required when working with Sf9 cells as compared to Sf21 cells. For example, Mehrabadi *et al.* (2015) observed successful knockdown of *gfp*, *egfp* and *prohibitin* genes by RNAi when Sf9 cells were incubated with the transfection mix for a much longer time of 24 hours. A longer transfection period was also employed by Lai *et al.* (2020), where Sf9 cells were incubated with the transfection mix for 24 hours prior to infection. The fact that Sf9 cells may require a longer transfection time than Sf21 cells to yield the same RNAi response, could be due to reduced Dicer activity/RNA processing in Sf9 cells, whether as a result of less Dicer protein being present in these cells or the Dicer activity being less efficient. Indeed, Flores-Jasso *et al.* (2004) showed that Dicer activity was reduced in Sf21 cells as compared to HEK cells. It is therefore reasonable to assume that, whilst potentially not as severe, a similar reduction in Dicer activity and therefore RNA processing may be observed in Sf9 cells as compared to Sf21 cells.

Alternatively, this need for a longer transfection period for Sf9 cells could be due to a less efficient uptake of dsRNA by these cells. More time would therefore be needed by Sf9 cells than Sf21 cells to achieve sufficient uptake of dsRNA. Whilst Lai *et al.* (2020) may have achieved a successful RNAi response in Sf9 cells by employing a much longer transfection time, they also used a much greater amount of dsRNA during transfection (50µg/1x10⁶ cells). It may be possible that a larger amount of dsRNA is required when transfecting Sf9 cells as compared to Sf21, particularly if a shorter transfection time is used. For example, Marek *et al.* (2011) achieved a successful RNAi response in Sf9 cells when transfecting with the equivalent of 100µg dsRNA per 1x10⁶ cells (20µg/2x10⁵ cells), whilst only transfecting for 4 hours. Again, this need for more dsRNA during transfection could imply poorer dsRNA uptake efficiency in Sf9 cells compared to Sf21.

Although Sf9 cells are derived from Sf21 cells, and so may be assumed to have similar Dicer or dsRNA uptake characteristics, the fact that Sf21 cells are a mixed population whilst Sf9s are a clonal population may explain the differences in response to *p35* inhibition between the two cell lines. As a mixed population, the Sf21 cells likely contained a mix of susceptible cells (due to increased Dicer activity or increased dsRNA uptake) and non-susceptible cells. Apoptosis occurring in the susceptible cells may then also trigger an apoptotic response in the non-susceptible cells. However, because Sf9 cells are a clonal population, all the cells may have been derived from a non-susceptible cell. Consequently, this could explain the difference in apoptotic response between these two closely related cell lines. Overall, whether due to reduced Dicer activity or less efficient dsRNA uptake, it seemed that Sf21 cells were more efficient at yielding an RNAi response (and are therefore more economical). Consequently, these cells were chosen for use in all subsequent RNAi experiments.

As noted above, previous research using RNAi to induce specific gene silencing showed significant variation in the amount of dsRNA used to transfect cells (Caplen *et al.*, 2002; Valdes *et al.*, 2003; Marek *et al.*, 2011; Salem *et al.*, 2013; Hodgson *et al.*, 2019; Lai *et al.*, 2020). In addition to this, previous studies have indicated the silencing effects of dsRNA to be dose-dependent when directly injected into insect larvae (Flores-Jasso *et al.*, 2004). Therefore, a titration experiment was carried out in Sf21 cells to determine the amount of dsRNA required to optimise gene knockdown. Firstly, the coefficient of determination for both the plaque assay and qPCR results ($R^2 < 0.03$) indicated that between 7-2 μ g (Figure 3.3A, B), less than 3% of variation in the average percentage of BV remaining was explained by the amount of *gp64* dsRNA used during transfection. Therefore, these results suggested that using any dsRNA amount between 7-2 μ g during transfection would yield no significant difference in the levels of gene silencing. However, between 3-0.03 μ g (Figure 3.3C, D), the coefficient of determination ($R^2 = 0.65$ and 0.69) indicated that 65-69% of variation in the average percentage of BV remaining was explained by the amount of *gp64* dsRNA used during transfection. Therefore, the effects of RNAi appear to become significantly dsRNA dose-dependent when using less than $\sim 2\mu$ g dsRNA, with smaller dsRNA quantities resulting in reduced levels of gene silencing. Consequently, this data suggested that dsRNA amounts greater than 3 μ g should be used when transfecting Sf21 cells in order to achieve optimal levels of RNAi. 5 μ g of dsRNA was, therefore, used for all future transfections of Sf21 cells.

Interestingly, the results of the dsRNA titration in Sf21 cells suggested that to achieve optimal levels of gene silencing by RNAi, more dsRNA needed to be used when transfecting cultured cells than when it is directly injected in to insect larvae. For example, Flores-Jasso *et al.* (2004) saw protective RNAi effects in 95% of injected insect larvae when using only 0.5µg dsRNA. Furthermore, Miller *et al.* (2012) silenced the expression of *egfp* when as little as 0.0006µg of *egfp* dsRNA was injected into larvae. The results presented in this thesis, however, showed that when transfecting Sf21 cells a dsRNA dose-dependency occurs when using less than ~2µg dsRNA and hence more than 3µg dsRNA should be used when transfecting cells.

It is important to note that the dsRNA titration experiments were carried out in Sf21 cells, as these were the cells chosen to be used when silencing gene expression by RNAi. As discussed above, other insect cells such as Sf9 cells seem to require much greater amounts of dsRNA during transfection to induce a successful RNAi response (Marek *et al.*, 2011; Lai *et al.*, 2020). Therefore, whilst a minimum of 3µg of dsRNA may be sufficient for transfecting Sf21 cells, the exact amount required for a successful induction of RNAi may be different in other insect cell lines. It would, therefore, be interesting to explore whether the effects of RNAi are dsRNA dose-dependent in other insect cell lines and at what range this may occur in.

The main aim of this chapter was to initially identify key genes required for the inhibition of BV production. Therefore, select genes (Table 3.2) underwent a screening process in which RNAi was employed to identify which of the genes have potential for reducing BV production if silenced by a BEVS LacI/O repressor system. Whilst many structural genes could have been selected for screening (Table 3.1), time constraints meant that this tailored list of genes, which research had shown or suggested may have potential for inhibiting BV production if silenced, was chosen. Cells were transfected with gene-specific dsRNA, and the production of BV assessed by qPCR. The percentage reduction in BV production was then calculated from a baseline amount of BV produced when cells were transfected with a non-specific dsRNA. This allowed the results to reflect the true knockdown potential of each gene by accounting for any non-specific effects that may have occurred as a result of the transfection process. Results were displayed as percent of BV remaining for ease of observation. The majority of the genes silenced saw a significant ($p < 0.01$) reduction in BV

production. The individual genes that showed most potential for reducing BV production (genes which resulted in 20% or less BV remaining) were confirmed by plaque assay. These genes were *vp39*, *gp64*, *Ac54(a)*, *Ac54(b)*, *Ac83(a)*, *Ac92*, *Ac142*, and *vp80(b)*. For *vp39*, *gp64*, *Ac83(a)*, *Ac92*, and *Ac142*, the plaque assay data supported the qPCR results and showed 20% or less BV remaining. For *Ac54(a)*, *Ac54(b)* and *vp80(b)*, however, the plaque assay results showed a greater percentage of BV remaining. This therefore, suggested that whilst the total amount of BV remaining may be less than 20%, the infectious count of BV remaining was not reduced as significantly. Therefore, the genes chosen to be taken forward to assess their knockdown potential in combination were *gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*.

Gp64 (*Ac128*) is present in all Group 1 nucleopolyhedroviruses (NPVs), a single granulovirus (GV) and some lineages of orthomyxovirus. It encodes a fatty acid acylated glycoprotein (Roberts and Faulkner, 1989) and a low pH activated envelope fusion protein (Blissard and Wenz, 1992), which is acquired as virions bud from the host cell plasma membrane. It is one of the three most abundant proteins found associated with AcMNPV BVs (Wang *et al.*, 2010). GP64 is probably the most well characterised baculovirus structural protein of Group 1 baculoviruses, with early studies highlighting its importance for AcMNPV BV infectivity (Blissard and Rohrmann, 1989; Oomens *et al.*, 1995; Monsma *et al.*, 1996; Washburn *et al.*, 2003). Deletion of *gp64* has been found to result in viruses that replicate in a single cell, but are unable to bud out and infect surrounding cells (Monsma *et al.*, 1996; Oomens and Blissard, 1999). Lee *et al.* (2015) also showed *gp64* silencing by RNAi to result in a reduction of BV production.

Vp39 (*Ac89*) encodes the major viral capsid protein which is one of the three most abundant proteins found associated with the AcMNPV BV (Wang *et al.*, 2010). It is involved in viral DNA packaging and nucleocapsid assembly (Katsuma and Kokusho, 2017). Ono *et al.* (2012) found deletion of *bm72* (a *vp39* homologue) from BmNPV to result in the inhibition of BV production. VP39 has been shown to directly interact with a conserved domain of Kinesin 1, with suggestions that this interaction is involved in the transport of nucleocapsids destined to become BV to the cell membrane following their assembly (Danquah *et al.*, 2012).

Ac83 (PIF8) encodes a virion-associated protein called VP91 and was identified as a component of the *per os* infectivity factor (PIF) complex (Peng *et al.*, 2012). It contains a *cis*-acting nucleotide sequence known as the nucleocapsid assembly-essential element (NAE) which indeed is essential for the assembly of the virus nucleocapsid (Huang *et al.*, 2017). Research has shown the deletion of *Bn69* (a *Ac83* homologue) from BmNPV, to result in the inhibition of BV production (Ono *et al.*, 2012).

Ac92 (*p49*) encodes a conserved sulfhydryl oxidase protein known as P33, which is associated with the envelope of both budded and occlusion-derived virus (Long *et al.*, 2009; Blissard and Theilmann, 2018), and may be involved in disulphide bond formation during virion assembly (Wu and Passarelli, 2010). A study by Nie *et al.* (2011) found *Ac92* deletion to reduce BV production by over 99.99%, whilst results of a study by Wu and Passarelli (2010) also suggested deletion of *Ac92* inhibited BV production.

Ac142 (*p49*) appears to be involved in the assembly of virion nucleocapsids, as deletion experiments produced nucleocapsids with structural defects (Vanarsdall *et al.*, 2007; Yang *et al.*, 2008). Another study in which less of the *Ac142* gene was deleted showed similar results, except nucleocapsids were fully formed but were not enveloped in the nuclei or occluded (McCarthy *et al.*, 2008). Further to this, work by Yue *et al.* (2018) found *Ac142* to interact with components of the ESCRT-III complex and suggested it may therefore be involved in nucleocapsid release at the nuclear membrane.

It is important to note that all the dsRNAs used during the RNAi gene screenings were different sizes/bp lengths. Previous research has suggested the size of the dsRNA used during transfection does not have an effect on RNAi, with Flores-Jasso *et al.* (2004) finding long (700nt) dsRNA-*gfp* and short (25nt) siRNA-*gfp* to both be equally effective at inhibiting GFP fluorescence in Sf21 cells. However, other research has suggested that chemically synthesised siRNAs at doses as low as 9 nM are more potent than dsRNAs used at doses up to five times higher (Agrawal *et al.*, 2004), with this difference in potency being attributed to the poor processing of the dsRNA by the Dicer enzyme in insect cell lines (Lee *et al.*, 2013). This therefore suggests that the effects of RNAi would be increased if shorter dsRNAs were used during transfection as they would require less processing by Dicer. Indeed, this may explain the difference seen in BV production between the three dsRNAs targeting *Ac83* (*Ac83(a)*, *Ac83(b)* and *Ac83(c)*). *Ac83(b)*, the longest dsRNA of the three (2271bp), saw the

most BV remaining at 28.9% whilst *Ac83(a)*, the shortest dsRNA of the three (560bp), saw the least BV remaining at 8.1%. However, the same pattern was not seen for the three dsRNAs targeting *vp80* (*vp80(a)*, *vp80(b)* and *vp80(c)*) with the shortest dsRNA of the three (*vp80(c)*, 918bp) seeing the greatest amount of BV remaining (32.4%) and the longest dsRNA (*vp80(b)*, 1810bp) seeing the least BV remaining (18.3%). These results therefore, imply that the dsRNA size used during transfection does not have an effect on the observed RNAi response within individual cell lines. However, it would perhaps be advisable to use dsRNAs of the same size when inducing RNAi, as this would ensure any possible effects of differences in dsRNA size are eliminated.

Surprisingly, *vp80* knockdown by RNAi did not reduce BV production as much as other research suggested it would. Marek *et al.* (2011) observed a complete inhibition of BV production when *vp80* expression was silenced by RNAi. The results presented in this thesis, however, showed only an 81.7% reduction in BV production at best when targeting *vp80* with the *vp80(b)* dsRNA. This difference in the results could be contributed to variations in the method used when transfecting cells with dsRNA. For example, Marek *et al.* (2011) transfected Sf9 cells with *vp80* dsRNA, whilst this study used Sf21 cells. It is possible that the differences in the strength of the RNAi response between these two cell lines may be due to a different efficiency of dsRNA uptake and processing. Differences in dsRNA size may have also influenced the outcome of the results. Marek *et al.* (2011) used a much shorter dsRNA sequence of only 616bp to target *vp80* compared to 1810bp of *vp80(b)*. As discussed above, Dicer activity has the potential to be reduced in different cell lines. It is therefore possible that using a shorter dsRNA sequence and reducing the need for RNA processing, resulted in the induction of a stronger RNAi response, and hence a stronger inhibition of BV production. Different transfection reagents may have also resulted in different levels of cytotoxicity on treated cells, aiding the inhibition of BV production. However, whilst the exact amount of BV produced may have differed slightly, both sets of results still highlight *vp80* as a gene with potential to inhibit BV production if knocked down permanently.

The paired gene inhibition experiments (3.2.4.2) found the following key gene pairs to result in a significantly greater ($p < 0.05$) reduction of BV production when silenced, compared to that observed when just one of the genes in the pair was silenced: *gp64* and

Ac83(a), *gp64* and *Ac142*, *vp39* and *Ac83(a)*, *vp39* and *Ac142*, and *Ac83(a)* and *Ac142*. Whether as a result of synergy or simply addition affects, exactly why these gene combinations provided greater inhibition of BV production when silenced together is currently unclear and requires further research. However, this data does suggest that inhibition of multiple key genes by a BEVS LacI/O repressor system could induce a greater reduction in BV production than inhibition of just one key gene.

Interestingly, not all the gene pairs induced a significantly greater reduction in BV production. This could, therefore, suggest that specific synergistic effects were at play between the successful gene pairs noted above. However, it is possible that the reason some gene pairs did not achieve significantly greater inhibition of BV production was due to limitations of the RNAi procedure itself. For example, none of the knockdown pairs in which *Ac92* was one of the targets achieved a significantly greater reduction in BV production than when the two genes were silenced individually. This could be attributed to competitive inhibition between the two dsRNAs resulting in only one of the genes being effectively silenced. Research has shown that oversaturation of the RNAi machinery can occur when multiple ds- or siRNAs are delivered to cells (Barik, 2006; Grimm *et al.*, 2006), resulting in competition between the dsRNAs for the RNAi machinery components. Additionally, competition for cell entry and transport components can further increase the levels of dsRNA competition. Indeed, research has shown the uptake of smaller dsRNAs in insect cells to be less efficient than that of larger dsRNAs (Saleh *et al.*, 2006; Miller *et al.*, 2012). Furthermore, it has been shown that some dsRNAs have greater competition potency than others (Koller *et al.*, 2006; Yoo *et al.*, 2008). Overall, this competitive inhibition can result in an inability to effectively silence multiple genes at the same time (Formstecher *et al.*, 2006; Castanetto *et al.*, 2007; Stierle *et al.*, 2007). Therefore, depending on the combination of dsRNAs used, different levels of competition may occur.

In the case of this research, the *Ac92* dsRNA was the smallest of all the dsRNAs transfected in combination (446bp, Table 3.2). It is likely therefore, that firstly, its uptake would have been worse in comparison to any of the other dsRNAs it was transfected in combination with. This would have given the other dsRNAs a competitive advantage for access to the RNAi machinery, which may have then become saturated with that dsRNA before uptake of the *Ac92* dsRNA, resulting in preferential inhibition by the larger dsRNA. However, the

difference in dsRNA size does not seem to be the only factor contributing to the competitive inhibition that prevents a significant reduction in BV production following RNAi for two genes. Indeed, significant reductions in BV production were observed from the *gp64/Ac142*, *vp39/Ac83(a)* and *Ac83(a)/Ac142* dsRNA pairs, which all also had large differences in their bp lengths (Table 3.2). Therefore, along with its reduced uptake potential, the *Ac92* dsRNA was also likely to have had a lower competition potency than those it was transfected with. Previous research has shown ds/siRNAs to differ in their competitive potency (Formstecher *et al.*, 2006; Koller *et al.*, 2006) and Yoo *et al.* (2008) showed this potency to be strongly influenced by the 5' half of the antisense strand sequence.

3.4 Conclusion

Overall, the results of this chapter showed the effects of RNAi to be significantly dsRNA dose-dependent when using less than ~2µg dsRNA in Sf21 cells. Therefore, dsRNA amounts greater than 3µg should be used when transfecting Sf21 cells. Most importantly however, five key structural genes (*gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*) were identified as having potential to significantly reduce BV production if knocked down by a BEVS LacI/O repressor system. The paired key gene RNAi experiments also demonstrated that silencing of two of the key genes could further reduce BV production. The identified key genes would later be targeted for knock-down by a developed BEVS LacI/O repressor system for the purposes of reducing BV production during VLP/protein production.

Chapter 4:

**Development of a baculovirus gene
promoter with the capacity to inhibit
key gene expression**

4.1 Introduction

The main aim of this thesis was to produce a novel, repressible system (the LacI/O Repressor System) that can block expression of the key baculovirus genes identified in 3.1.4 and, therefore, reduce or significantly inhibit BV production during recombinant protein or VLP production. To achieve this, one or more key genes were placed under the control of a promoter modified to contain the *E. coli lacO* sequence and a recombinant virus produced. An Sf9 cell line constitutively expressing the *E. coli* repressor protein *lacI* was also developed (Chapter 5) so that upon infection of these cells with the recombinant virus, LacI will bind the *lacO* in the modified promoter and block the expression of key genes essential for BV production. In the absence of LacI, the key genes would not be repressed, allowing for normal levels of BV production for the amplification and maintenance of viral stocks.

The late *chiA/cath* promoter (Figure 4.1), which lies between *chitinase* (*chiA*) and *cathepsin* (*cath*), was selected for modification for two reasons. Firstly, the promoter is relatively simple with just a single TAAG motif and a short ~10bp 5'UTR (untranslated region) for *chiA*. The 5'UTRs of other AcMNPV late promoters can vary in length significantly. For example, the 5'UTR of *vp80* is ~32bp and for *vp39* is ~55bp (Morris and Miller, 1994). The other late promoters can also vary in terms of complexity with some (*gp64*, *vp39*, *gp41*) containing more than one TAAG late transcription start site (Blissard and Rohrmann, 1989; Thiem and Miller, 1989; Whitford and Faulkner, 1992). The simple *chiA/cath* promoter had fewer options for *lacO* insertion sites, meaning a position that would yield a significant repression effect without reducing promoter activity was more likely to be found.

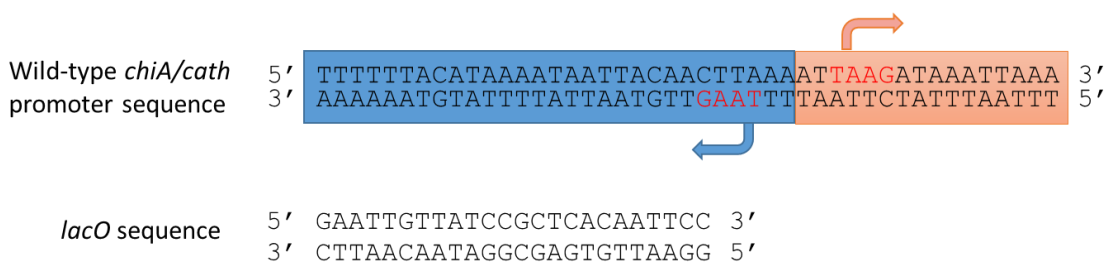


Figure 4.1- DNA sequences of the LacI/O Repressor System promoter components.

The wild type *chiA/cath* promoter (top) acts as a bi-directional transcription unit with late promoter TAAG motifs (shown in red) on each DNA strand to regulate the expression of both the *chitinase* and *cathepsin* genes. Regulation of *cathepsin* expression is from the blue portion of the promoter (arrow) whilst regulation of *chitinase* expression is from the pink portion of the promoter (arrow). The regulatory *lac* operator (*lacO*) sequence of the *E. coli lac* operon is shown (bottom).

The second reason the *chiA/cath* promoter was selected was due to its ability to act as a bi-directional transcription unit with TAAG motifs on either strand to regulate expression of both *chiA* and *cath* (Figure 4.1). This allows for the inhibition of two of the previously identified key genes rather than just one, which previous results (section 3.1.4.2) demonstrated a greater reduction of BV production.

The regulatory elements of the *E. coli lac* operon (*lacO* and *lacI*) were chosen for the production of the repressible promoter required for the LacI/O Repressor system. Firstly, repression from the *lac* operon has been found to be particularly strong in comparison to other negatively regulated bacterial systems. In the presence of inducer, the level of the *lac* enzymes can increase approximately 1000-fold, whereas the *gal* operon is only induced <20-fold (Oehler *et al.*, 1990). The *lac* operon elements should therefore allow for the construction of a promoter construct that yields the strongest levels of repression possible. Further to this, past research employing the *lac* operon regulatory elements in a vaccinia virus late promoter provides data that is promising for the production of a LacI/O regulatory system in the BEVS. Fuerst *et al.* (1989) observed a 97% reduction in β -galactosidase activity when *lacO* was inserted into the F17R (P11) vaccinia virus late promoter. Zhang and Moss (1991) also reported a 95% reduction in gene transcription when *lacO* was inserted into the same promoter. Alongside the work of Fuerst *et al.* (1989) and Zhang and Moss (1991), other research has provided insights into the mechanics (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018) and design of a synthetic *lac* repressor system (Shore *et al.*, 1981; Shore and Baldwin, 1983; Krämer *et al.*, 1987; Oehler *et al.*, 1994; Becker *et al.*, 2013). Consequently, the regulatory elements of the *lac* operon appeared ideal candidates for the production of a repressor system incorporated into the BEVS.

Initially, a synthetic version of the *chiA/cath* promoter (*chiA/cath*^(syn) promoter) was synthesised with the antisense TAAG motif for *cath* mutated to enable repression of a single gene to be assessed. From this modified *chiA/cath* promoter, a number of promoter constructs containing the *lacO* sequence (*chiA/lacO* promoters) at different positions relative to the *chiA* TAAG motif were produced. Previous research employing different repressor systems, including the tet (O'Connel *et al.*, 2020) and *lac* (Fuerst *et al.*, 1989; Zhang and Moss, 1991) systems, all reported significant levels of gene repression when the

relevant operator sequence was inserted downstream of the conserved site of transcription initiation. However, a model of repression for the *lac* repressor system proposed by Lanzer and Bujard (1988) found that following initiation of transcription, a *lacO* upstream of the RNA polymerase (RNAP) binding site is uncovered by the transcribing RNAP, so LacI can bind before a second RNAP can bind to the promoter (as the transcribing RNAP is still covering large portions of the promoter further downstream). This suggests that LacI binding may have a competitive advantage over RNAP if *lacO* is inserted upstream of the TAAG motif.

Consequently, to explore how the positioning of the *lacO* within the *chiA/cath*^(syn) promoter may affect levels of gene repression, *chiA/lacO* promoter constructs with *lacO* inserted either before or after the TAAG motif were designed. In addition to these, promoter constructs were designed with *lacO* inserted both before and after the TAAG motif or with two *lacO* sequences after the TAAG motif to explore whether multiple copies of the *lacO* within the promoter would impact the levels of gene repression.

Later experiments involved the use of the *chiA/cath* promoter with both TAAG motifs functional, allowing for the control/production of two genes simultaneously, which previous results (Chapter 3) suggested could further reduce BV production. The *lacO* insertion sites within this *chiA/cath* promoter were chosen based on the results of the single gene promoter experiments.

Whilst the inhibition of BV production during recombinant protein/VLP production was the main aim of developing a BEVS LacI/O Repressor System, another essential feature was the ability to resume normal levels of BV production in the absence of LacI, allowing for amplification and maintenance of viral stocks. It was therefore essential that the insertion of *lacO* into the *chiA/cath* promoter did not inhibit or significantly reduce its basal activity.

Finally, the use of LacI/O regulatory elements in baculoviruses is currently unexplored. Although research employing vaccinia viruses has provided promising data, it is still unknown whether a similar system will work in baculovirus. The work of this chapter, therefore, focused on first confirming the integrity of each of the *chiA/lacO* promoter constructs and assessing how *lacO* positioning within those constructs affects the basal levels of gene expression from the promoter.

4.2 Results

4.2.1 Construction and activity of a synthetic *chiA/cath* promoter

Two oligonucleotides (RDP1376 and RDP1377; Table 2.4, Chapter 2) were annealed to form the *chiA/cath*^(syn) promoter with *EcoRV* and *BamHI* complementary ends. This synthetic version of the *chiA/cath* promoter had the antisense *cath* TAAG motif mutated by replacing the second A of the TAAG with a T to derive an inactive TATG (Figure 4.2B).

A plasmid with *egfp* under the *chiA/cath*^(syn) promoter was produced (pChiA-EGFP, Figure 4.2) to confirm the integrity of the *chiA/cath*^(syn) promoter with EGFP acting as a reporter protein. Sf9 cells were co-transfected with pChiA-EGFP or pPolh-EGFP, with *egfp* under the *polh* promoter, and *flashBAC* ULTRA™ DNA to produce recombinant viruses with *egfp* under the *chiA/cath*^(syn) (AcChiA-EGFP) and *polh* (AcPolh-EGFP) promoters, respectively (Figure 4.2). Expression of *egfp* from the very late *polh* promoter acted as a positive control in these experiments. Recombinant virus stocks were screened by PCR to confirm the incorporation of *egfp* into the virus genome (Chapter 2).

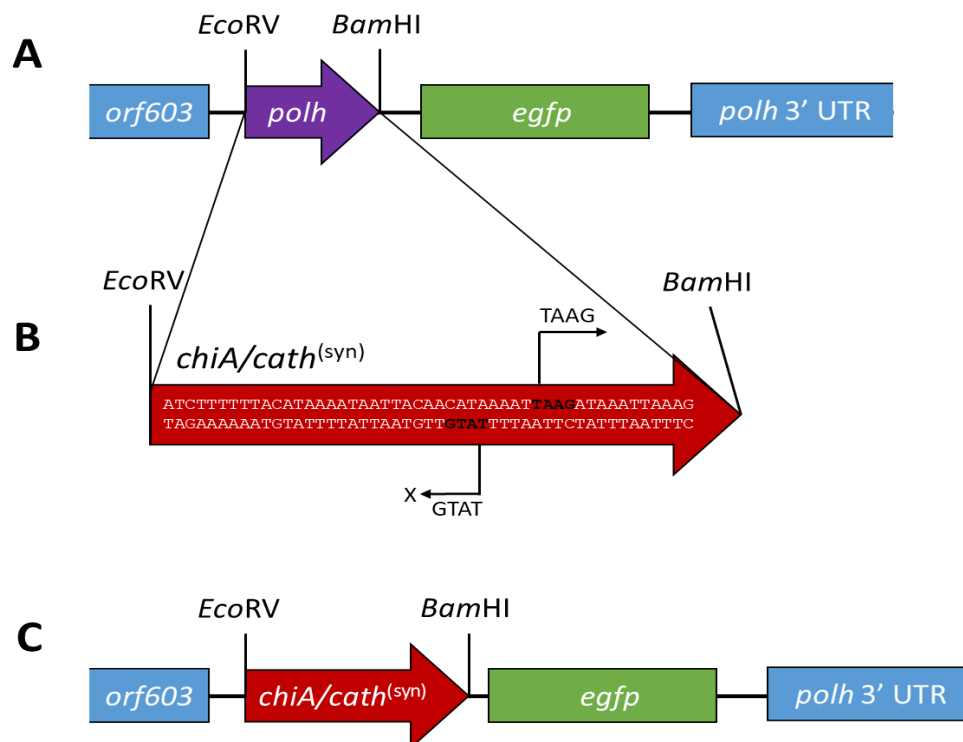


Figure 4.2- Generation of pChiA-EGFP.

Promoters are shown as arrows, indicating direction of transcription. **(A)** pPolh-EGFP (provided by R Arinto Garcia, OET Ltd) with *egfp* under the *polh* promoter was digested with *EcoRV* and *BamHI* to excise the *polh* promoter (Chapter 2). **(B)** The synthetic *chiA/cath*^(syn) promoter (Eurofins; showing the *chiA* TAAG motif and the mutated antisense *cath* TAAG motif) was inserted into the digested pPolh-EGFP (Chapter 2). The pChiA-EGFP plasmid DNA **(C)** was purified and its sequence verified (Source Bioscience, data not shown).

Sf9 cells were infected with AcChiA-EGFP or AcPolh-EGFP (MOI 2) and imaged by fluorescence microscopy at 48 h.p.i. (Figure 4.3). AcMNPV- and mock-infections were also carried out as negative controls. Expression of *egfp* was observed in the AcChiA-EGFP infected cells, although less strongly than that from the AcPolh-EGFP infected cells, as expected. This confirmed the integrity of the synthetic *chiA/cath*^(syn) promoter with results showing it capable of driving *egfp* expression.

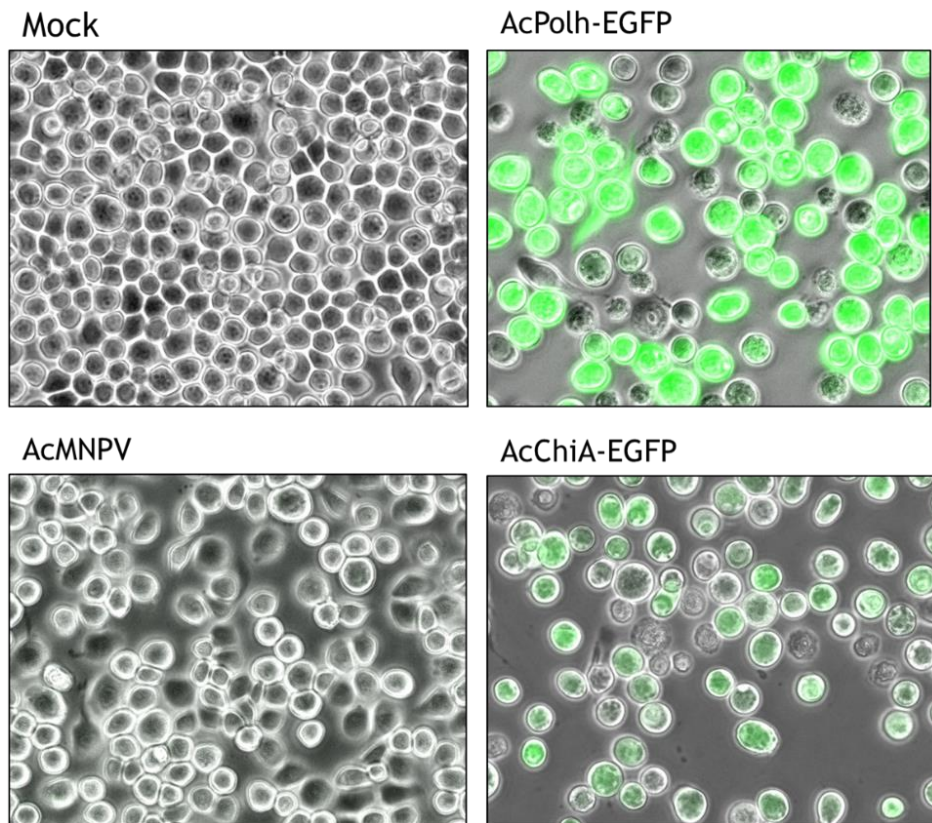


Figure 4.3- Expression of *egfp* by recombinant baculoviruses.

Sf9 cells were infected with AcChiA-EGFP, AcPolh-EGFP, AcMNPV (MOI 2) or were mock-infected. Infected cells were imaged by Revolve fluorescence microscopy (Echo Laboratories) at 48 h.p.i. (20X Magnification). Infections were performed in duplicate and a representative image is shown.

4.2.2 A synthetic *chiA/cath* promoter can drive late gene expression

To assess baculovirus late gene expression using the *chiA/cath*^(syn) promoter, the latter was inserted into a plasmid containing a synthetic copy of *gp64* lacking a promoter (*pgp64*_Twist, Twist Bioscience) to derive pChiA-*gp64* (Figure 4.4). To test for *gp64* expression, the plasmid was used to rescue a *gp64* knockout bacmid (*gp64*Δbacmid) to assess BV production. The *gp64*Δbacmid DNA was purified from *E. coli* DH5α cells by precipitation and digested with *Hind*III to confirm its integrity (data not shown).

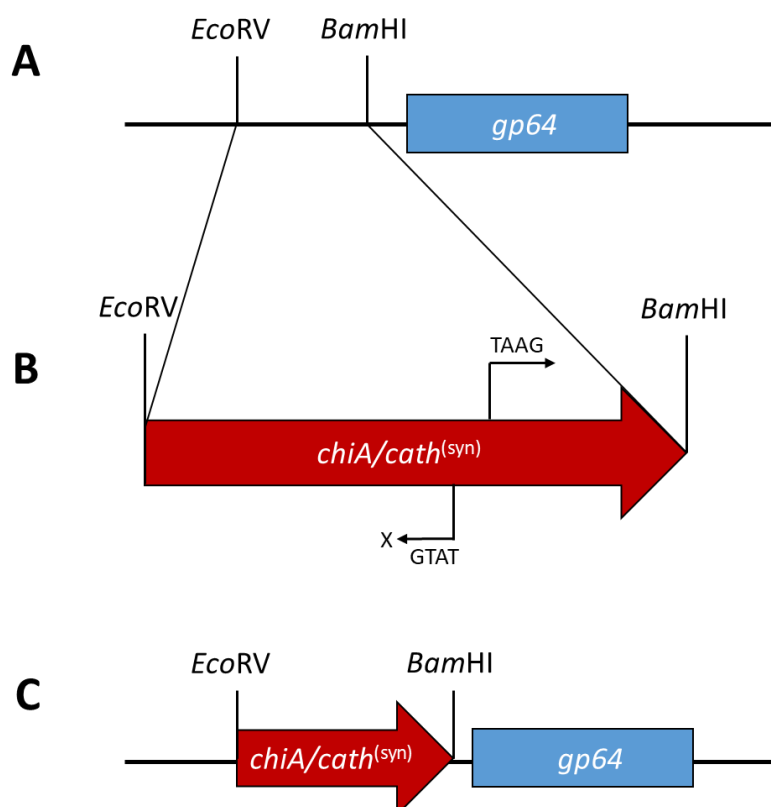


Figure 4.4- Generation of pChiA-gp64.

Promoters are shown as arrows, indicating direction of transcription. **(A)** *pgp64*_Twist, synthesised by Twist Biosciences, contained a synthetic copy of *gp64* without a promoter, which was flanked by *polh* locus sequences to permit homologous recombination. **(B)** The *chiA/cath^(syn)* promoter was inserted into *pgp64*_Twist at the *EcoRV* and *BamHI* sites. **(C)** The pChiA-gp64 DNA was purified and its sequence verified by the Sanger sequencing method (Source Bioscience, data not shown).

Sf21 cells were co-transfected with pChiA-gp64 (Figure 4.4) and the *gp64*Δbacmid DNA. Cells were also co-transfected with the *gp64*Δbacmid DNA and either *pgp64*_Twist or pAcBgIII-E (a plasmid containing the AcMNPV BgIII-E fragment in which *gp64* is in its native setting) as controls. Further controls included transfections with AcMNPV DNA, *gp64*Δbacmid DNA, pAcBgIII-E or mock-transfections. Cell medium containing BV was harvested at 120 h.p.t and quantified by plaque-assay (Table 4.1). Results showed a substantial production of BV from the cells co-transfected with pChiA-gp64 and *gp64*Δbacmid, as well as the positive controls. Whilst a very small virus titre was produced from the *pgp64*_Twist and *gp64*Δbacmid co-transfected cells, this was most likely due to spontaneous re-insertion of *gp64* at its native position or DNA around the *polh* locus acting as a weak driver/promoter of expression. These results confirmed the ability of the *chiA/cath^(syn)* promoter to express *gp64*.

Table 4.1- Activity of the *chiA/cath*^(syn) promoter: budded virus production from Sf21 cells co-transfected with baculovirus DNA

Co-transfection Sample	Budded Virus Titre* (PFU/mL)
Mock	0
AcMNPV	4.6 x 10 ⁶
<i>gp64</i> ΔBacmid ¹ only	0
pAcBgIII-E ² only	0
<i>pgp64</i> _Twist ³	0
<i>gp64</i> ΔBacmid ¹ + pAcBgIII-E ²	4.0 x 10 ⁴
<i>gp64</i> ΔBacmid ¹ + <i>pgp64</i> _Twist ³	57.0
<i>gp64</i> ΔBacmid ¹ + pChiA- <i>gp64</i> ⁴	4.2 x 10 ⁴

*At 120h.p.t.; ¹*gp64* knockout bacmid DNA; ²Plasmid DNA containing *gp64* in its native setting; ³Plasmid DNA containing a synthetic copy of *gp64* without a promoter; ⁴Plasmid DNA containing a synthetic copy of *gp64* under the *chiA/cath*^(syn) promoter

4.2.3 Activity of the synthetic *chiA/cath* promoter modified to contain the *lac* operator

Four promoters based on the *chiA/cath*^(syn) promoter, each containing *lacO* inserted at different positions were designed (LacO77, LacO79, LacO81 and LacO83). LacO77 had one *lacO* inserted 5bp upstream of the TAAG motif, whilst LacO79 contained one *lacO* 11bp after the TAAG motif. Both LacO81 and LacO83 contained two copies of the *lacO*, one located 5bp before and one 11bp after the TAAG motif for LacO81, or both directly next to each other 11bp after the TAAG motif for LacO83. Pairs of oligonucleotides synthesised by Eurofins MWG Operon (Table 2.4, Chapter 2), were annealed to form each promoter (Figure 4.5).

To assess the continued integrity of the *chiA/cath*^(syn) promoter following insertion of *lacO* at various positions, four plasmids were produced each with *egfp* under the control of one of the *chiA/lacO* promoters. The *chiA/lacO* promoter constructs were cloned into the previously digested pPolh-EGFP (*polh* promoter removed) to derive the plasmids pLacO77-EGFP, pLacO79-EGFP, pLacO81-EGFP and pLacO83-EGFP (Figure 4.6). Sf9 cells were co-transfected with these plasmids and *flashBAC* ULTRA™ DNA to produce recombinant viruses with *egfp* under each promoter construct (AcLacO77-EGFP, AcLacO79-EGFP,

AcLacO81-EGFP and AcLacO83-EGFP). Recombinant virus genomes were screened by PCR to confirm the incorporation of *egfp* (data not shown).

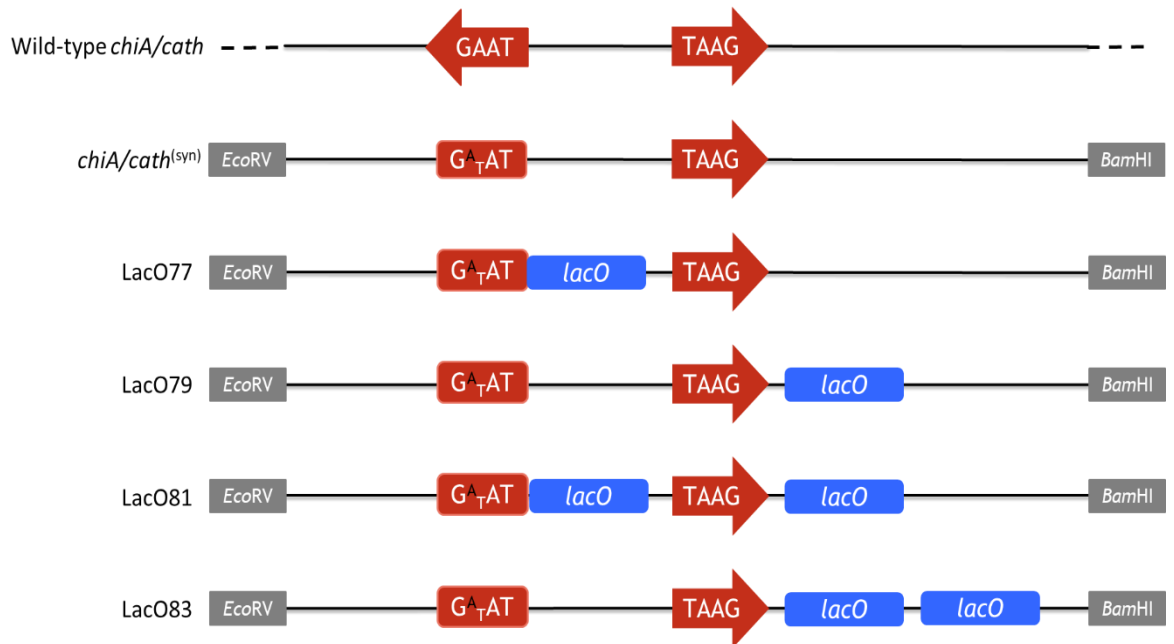


Figure 4.5- Structural representation of the single expression *chiA/lacO* promoter constructs.

The wild-type *chiA/cath* promoter possesses two transcription initiation TAAG motifs in opposite orientations (indicated by red arrows) for the regulation of *chiA* and *cath*. A synthetic promoter (*chiA/cath*^(syn)) had the antisense *cath* TAAG motif mutated to TATG (as indicated) to allow for regulation of one gene. Four *chiA/lacO* single expression promoter constructs were designed using the *chiA/cath*^(syn) promoter, each containing the *lacO* sequence (indicated by blue box) at different positions relative to the functional TAAG motif. Terminal restriction enzyme sequences facilitated cloning into the expression plasmid.

Sf9 cells were infected with AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP or AcLacO83-EGFP (MOI 2) and imaged by fluorescence microscopy at 48 h.p.i. (Figure 4.7). AcPolh-EGFP and AcChiA-EGFP infections were carried out as positive and comparative controls for *egfp* expression. Results showed *egfp* expression from all the *chiA/lacO* promoter constructs, with expression strongest from LacO79 and LacO83. The LacO79, LacO81 and LacO83 promoter constructs also appeared to yield stronger *egfp* expression than that observed from the original *chiA/cath*^(syn) promoter. Firstly, these results confirmed the integrity of the *chiA/lacO* promoters. They also suggested that the position of *lacO* within the *chiA/cath*^(syn) promoter did affect basal gene expression and that insertion after the TAAG motif may have increased promoter activity.

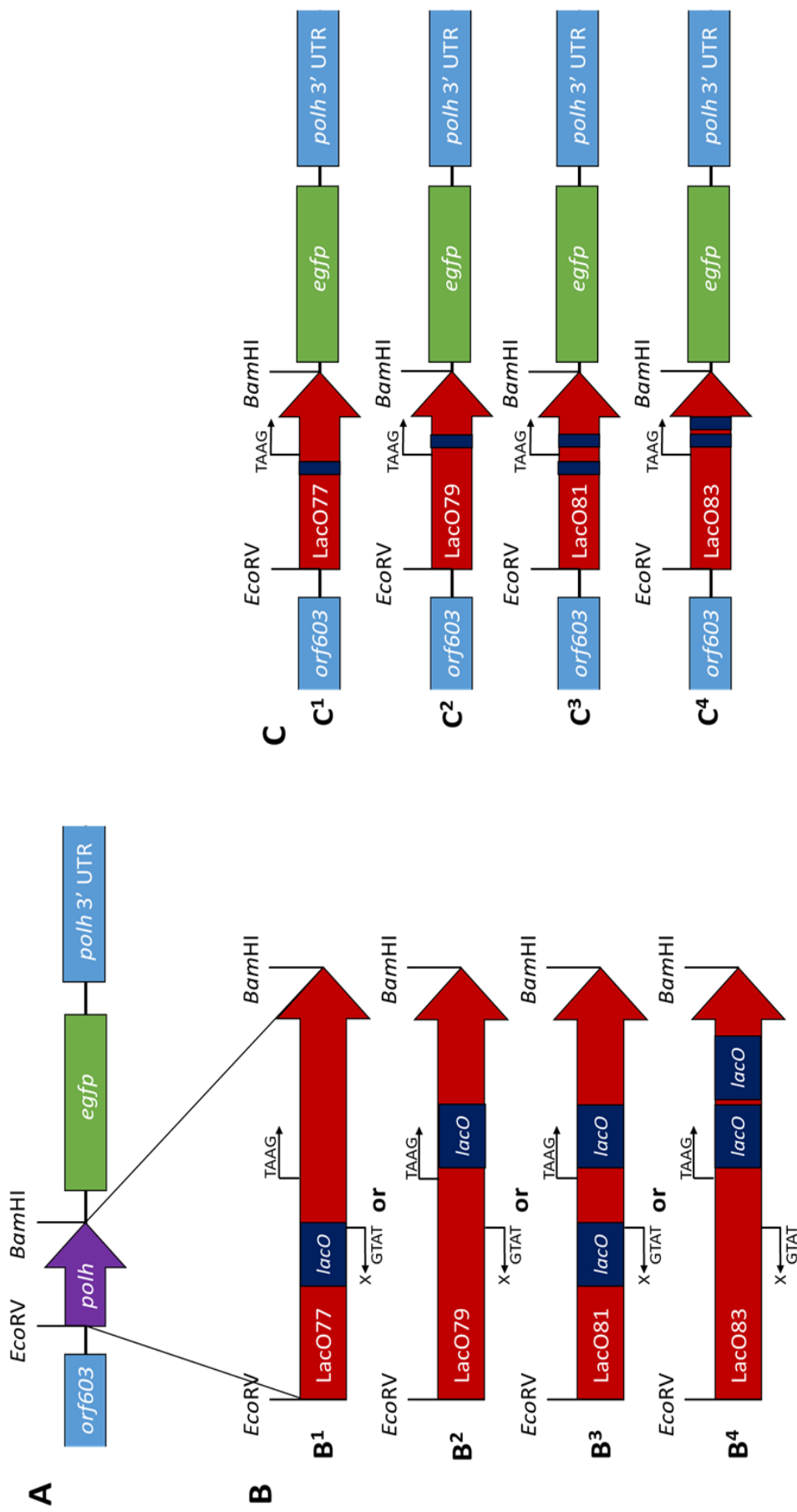


Figure 4.6- Generation of the *lacO/egfp* plasmids and their construction steps. A ligase reaction was carried out pPolh-EGF (provided by R Arinto Garcia, OET Ltd) (A), was digested with *EcoRV* and *BamHI* to remove the *polh* promoter. A ligation reaction was carried out to insert the *LacO77* (B¹), *LacO79* (B²), *LacO81* (B³) or *LacO83* (B⁴) promoter construct into the digested pPolh-EGFP. Ligation products, pLacO77-EGFP (C¹), pLacO79-EGFP (C²), pLacO81-EGFP (C³) or pLacO83-EGFP (C⁴) plasmid DNA were purified and sequenced by the Sanger sequencing method (data not shown).

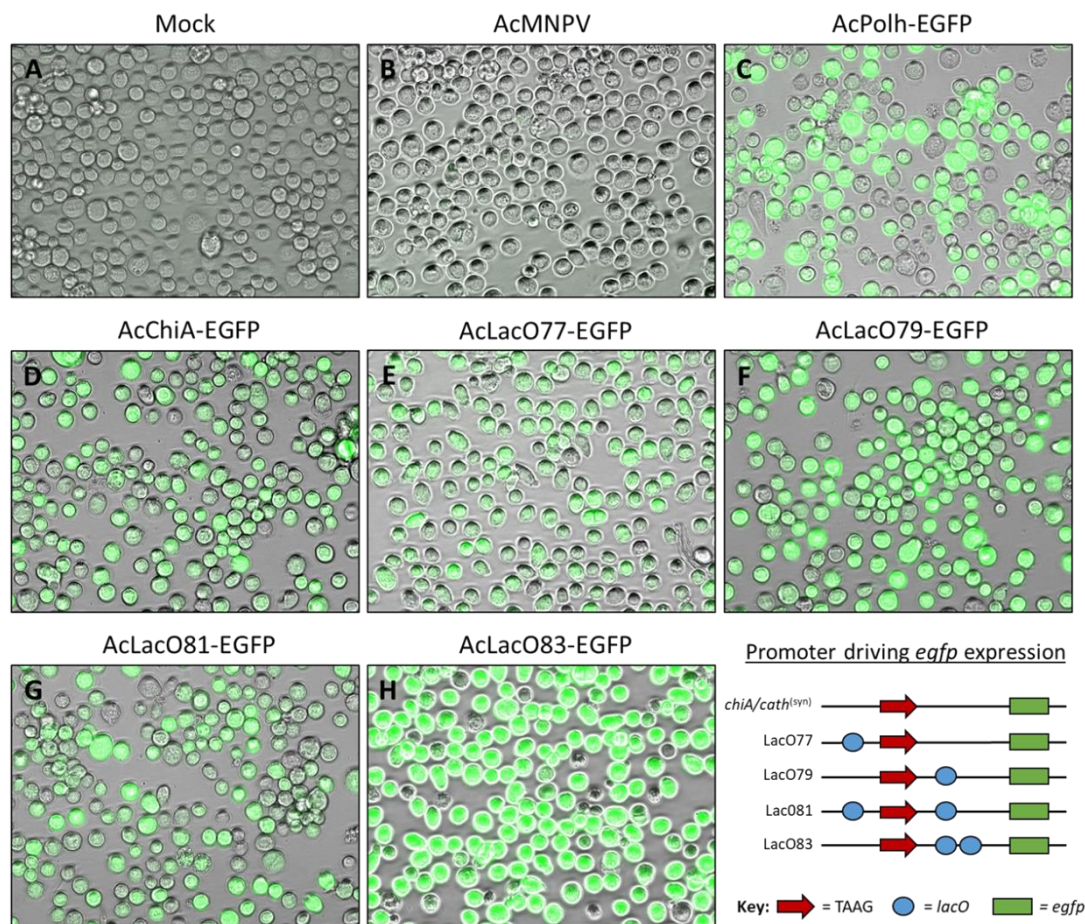


Figure 4.7- Activity of the *chiA/lacO* promoters: expression of *egfp* by recombinant baculoviruses. Sf9 cells were infected with AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP, AcLacO81-EGFP, AcPolh-EGFP, AcChiA-EGFP, AcMNPV (MOI 2) or were mock-infected and imaged by Revolve fluorescence microscopy (Echo Laboratories) at 48 h.p.i. (20X magnification) Infections were performed in duplicate and a representative image is shown.

4.2.4 The synthetic *chiA/cath* promoter can drive baculovirus late gene (*gp64*) expression following insertion of *lacO*

To assess baculovirus late gene (*gp64*) expression from the *chiA/cath*^(syn) promoter following the insertion of *lacO*, four plasmids were produced with *gp64* under the control of each of the four *chiA/lacO* promoters (LacO79, LacO81, LacO77 and LacO83). A plasmid with *gp64* under the *polh* promoter was also produced to act as a positive control for *gp64* expression. To achieve this, *pgp64*_Twist plasmid (Figure 4.4A) was digested to retrieve *gp64*, which was then inserted into pLacO79-EGFP, pLacO81-EGFP, pLacO77-EGFP, pLacO83-EGFP and pPolh-EGFP in place of *egfp* to produce pLacO79-*gp64*, pLacO81-*gp64*, pLacO77-*gp64*, pLacO83-*gp64* and pPolh-*gp64* (Figure 4.8). These plasmids were then used

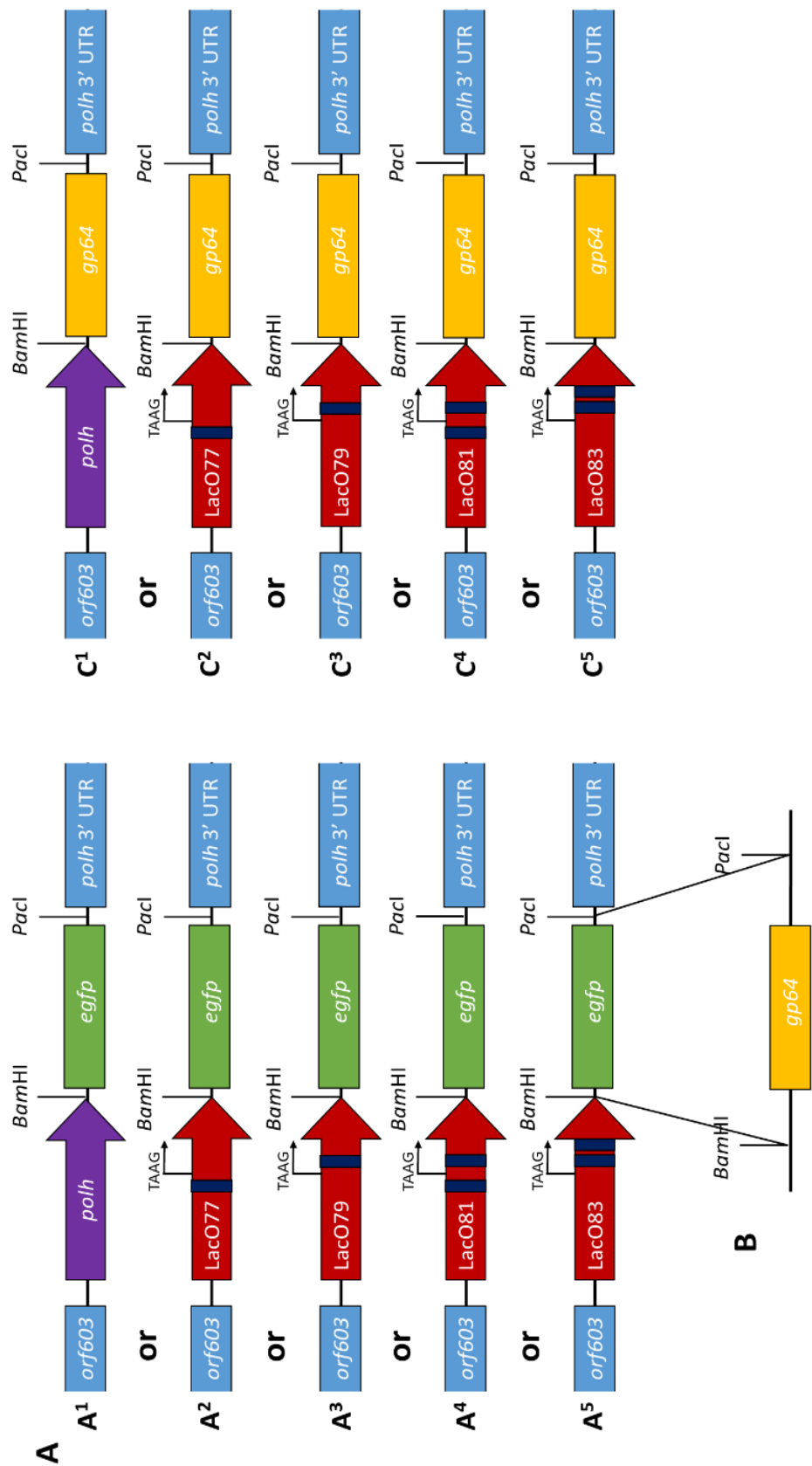


Figure 4.8- Construction of pPolh-gp64 and the lacO/gp64 plasmids. pPolh-EGFP (provided by R Arinto Garcia, OET Ltd) (A¹), pLacO77-EGFP (A²), pLacO79-EGFP (A³), pLacO81-EGFP (A⁴) or pLacO83-EGFP (A⁵) was digested with *Bam*HI and *Pac*I to remove *egfp*. A ligation reaction was carried out to insert *gp64* retrieved from the *pgp64* _Twist plasmid by digestion with *Bam*HI and *Pac*I (B). Ligation products, pPolh-gp64 (C¹), pLacO77-gp64 (C²), pLacO79-gp64 (C³), pLacO81-gp64 (C⁴) or pLacO83-gp64 (C⁵) plasmid DNA were purified and sequenced by the Sanger sequencing method.

to rescue a *gp64* knockout bacmid (*gp64*ΔFBG, native *gp64* removed from *flashBAC* GOLD™ DNA by Red E/T Recombination; Chapter 2) and BV production assessed.

Sf9 cells were co-transfected with pLacO79-*gp64*, pLacO81-*gp64*, pLacO77-*gp64* or pLacO83-*gp64* and *gp64*ΔFBG DNA as described (Chapter 2). Cells were also co-transfected with *gp64*ΔFBG and either pPolh-*gp64*, pChiA-*gp64*, *pgp64*_Twist or pAcBgIII-E as controls for *gp64* expression and BV production. Further controls included transfections with AcMNPV DNA, *gp64*ΔFBG, pAcBgIII-E DNA or mock-transfected. Culture medium was harvested at 120 h.p.t and quantified by plaque-assay (Table 4.2), which showed significant BV production from cells co-transfected with pLacO79-*gp64*, pLacO81-*gp64*, pLacO77-*gp64* or pLacO83-*gp64*. Therefore, it was evident that the *chiA/cath*^(syn) promoter was still capable of driving *gp64* expression following modification to insert the *lacO* at a range of positions.

Table 4.2- Activity of the *chiA/lacO* promoters: budded virus production in Sf9 cells co-transfected with baculovirus DNA

Co-transfection Sample	Budded Virus Titre * (PFU/mL)
Mock	0
AcMNPV	9.7 x 10 ⁷
<i>gp64</i> ΔFBG ¹ only	0
pAcBgIII-E ² only	0
<i>pgp64</i> _Twist ³	0
<i>gp64</i> ΔFBG ¹ + pAcBgIII-E ²	0
<i>gp64</i> ΔFBG ¹ + <i>pgp64</i> _Twist ³	277.0
<i>gp64</i> ΔFBG ¹ + pPolh- <i>gp64</i> ⁴	1.1 x 10 ⁴
<i>gp64</i> ΔFBG ¹ + pChiA- <i>gp64</i> ⁵	1.2 x 10 ³
<i>gp64</i> ΔFBG ¹ + pLacO77- <i>gp64</i> ⁶	6.2 x 10 ⁶
<i>gp64</i> ΔFBG ¹ + pLacO79- <i>gp64</i> ⁷	2.4 x 10 ⁷
<i>gp64</i> ΔFBG ¹ + pLacO81- <i>gp64</i> ⁸	1.7 x 10 ⁷
<i>gp64</i> ΔFBG ¹ + pLacO83- <i>gp64</i> ⁹	2.0 x 10 ⁷

*At 120h.p.t.; ¹*gp64* knockout *flashBAC* GOLD™ DNA; ²*gp64* in its native setting; ³synthetic copy of *gp64* without a promoter; ⁴synthetic copy of *gp64* under *polh* promoter; ⁵synthetic copy of *gp64* under *chiA/cath*^(syn) promoter; ⁶synthetic copy of *gp64* under LacO77; ⁷LacO79; ⁸LacO81; or ⁹LacO83 promoter

4.2.5 Insertion of *lacO* into the synthetic *chiA/cath* promoter alters promoter activity

4.2.5.1 Expression of *egfp* from the *chiA/lacO* promoter constructs

Previous results (section 4.2.3) suggested that following insertion of *lacO* after the TAAG motif, the activity of the *chiA/cath*^(syn) promoter was increased. To explore this further, Sf9 cells were infected with AcChiA-EGFP, AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP or AcLacO83-EGFP (MOI 5). AcPolh-EGFP, AcMNPV and mock-infections were also carried out as controls. At 48 h.p.i., the EGFP fluorescence intensity of the cells was analysed by flow cytometry. The results (Figure 4.9) showed that the LacO83 promoter yielded the greatest EGFP fluorescence intensity. This was followed by the LacO79 promoter, then LacO81 and LacO77.

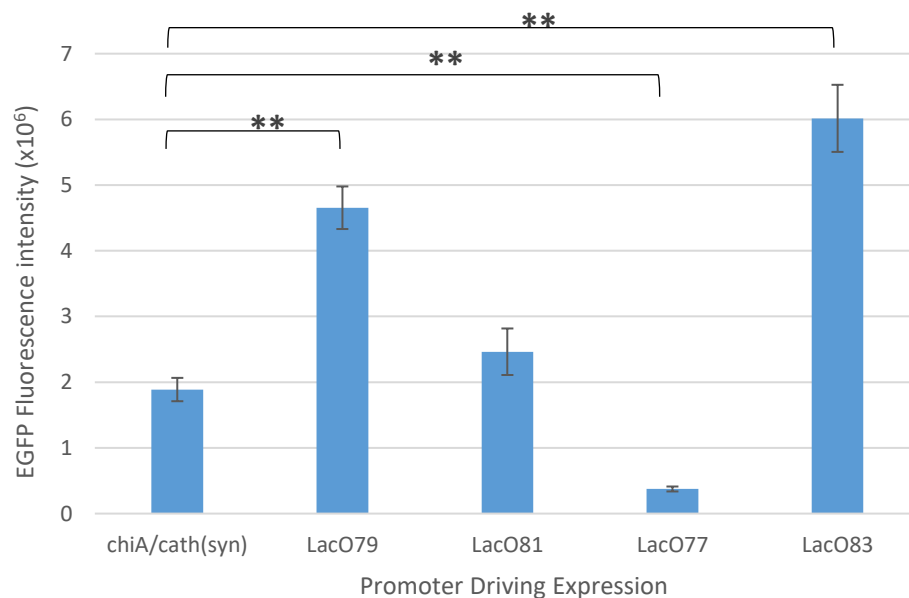


Figure 4.9- Activity of the *chiA/lacO* promoters: intensity of EGFP fluorescence from recombinant baculovirus-infected cells. Sf9 cells were infected with AcChiA-EGFP, AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP, AcLacO81-EGFP, AcMNPV* or AcPolh-EGFP* (MOI 5) or mock-infected*. *data not shown. At 48 h.p.i., the EGFP fluorescence intensity of the cells was analysed by flow cytometry as described (Chapter 2). n = 5. Error bars indicate the standard error of the mean (SEM). AcMNPV-infected cells defined the negative/positive threshold for EGFP fluorescence, from which the fluorescence intensity of the other infected cell samples was calculated. F-tests for variance and Student's t-tests for statistical significance were performed as indicated; **= p<0.01.

The LacO83 (218.7%), LacO79 (146.6%) and LacO81 (30.5%) promoters all yielded a greater fluorescence intensity than that observed from the original *chiA/cath*^(syn) promoter, whilst the LacO77 promoter was less strong (reduced fluorescence by 80.2%). These results

continued to support the idea that the insertion of *lacO* into the *chiA/cath*^(syn) promoter increased its activity. The data also indicated that it was the addition of the *lacO* after the promoter TAAG motif that drove this increased activity. EGFP fluorescence intensity was significantly increased ($p < 0.01$) from the LacO83 and LacO79 promoters. Addition of the *lacO* before the TAAG motif in LacO77, however, appeared to significantly hinder promoter activity ($p < 0.01$). This data also suggested that the increase in promoter activity was further enhanced when multiple *lacO* sequences were inserted after the TAAG motif, although statistical analysis found this was not significant.

Production of *egfp* in the cells was further assessed by western blot in replicate experiments. Typical results shown in Figure 4.10 support the pattern of promoter activity observed from the flow cytometry analysis.

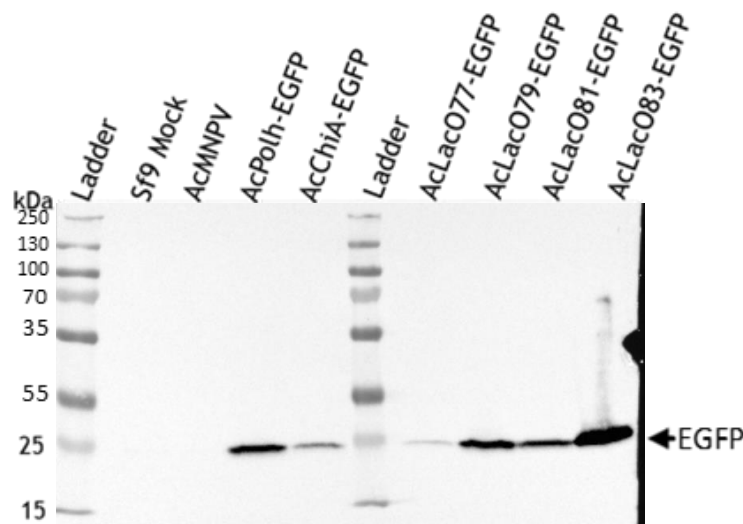


Figure 4.10- Activity of the *chiA/lacO* promoters: production of EGFP in recombinant baculovirus-infected cells. Sf9 cells were infected with AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP, AcLacO83-EGFP, AcPolh-EGFP, AcChiA-EGFP or AcMNPV (MOI 5) or mock-infected. At 48 h.p.i., EGFP (24.6kDa, arrowed) production from the cells was analysed by western blot. Mouse monoclonal to EGFP (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder shown left and centre.

To further analyse the effects of inserting *lacO* into the *chiA/cath*^(syn) promoter, *egfp* expression from each of the *chiA/lacO* promoters was assessed over time. Sf9 cells were infected with AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP or AcLacO83-EGFP (MOI 5). Controls were carried out as previously described. The EGFP fluorescence intensity of the cells was analysed by flow cytometry at 24, 48, and 72 h.p.i. Figure 4.11 shows that

insertion of *lacO* into the *chiA/cath*^(syn) promoter did not just simply increase or decrease promoter activity, but also changes the time at which expression occurs.

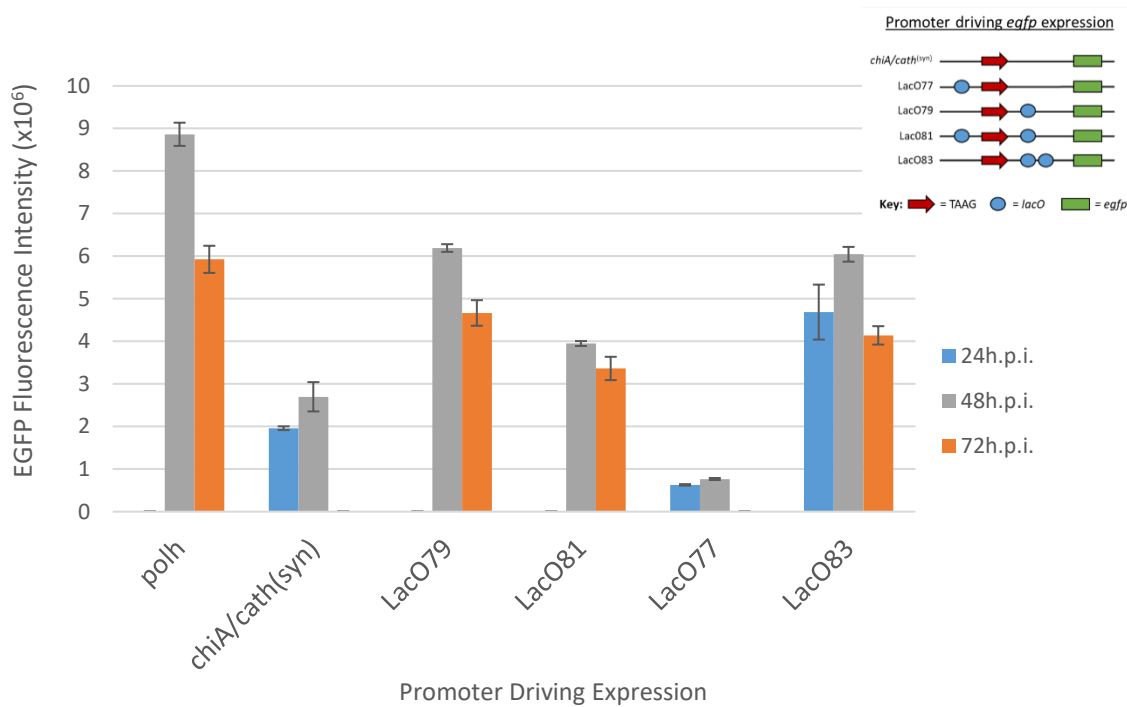


Figure 4.11- Activity of the *chiA/lacO* promoters: intensity of EGFP fluorescence from recombinant baculovirus-infected cells over time. Sf9 cells were infected with AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP or AcLacO83-EGFP (MOI 5). AcPolh-EGFP, AcChiA-EGFP, AcMNPV and mock-infections (data not shown) were carried out as controls. At 24 h.p.i. (blue bars), 48 h.p.i. (grey bars) and 72 h.p.i. (orange bars), the EGFP fluorescence intensity of the cells was analysed by flow cytometry (Chapter 2). $n = 3$. Error bars indicate SEM. AcMNPV-infected cells defined the negative/positive threshold for EGFP fluorescence, from which the fluorescence intensity of the other infected cell samples was calculated.

EGFP fluorescence from the LacO79 promoter (*lacO* after TAAG) was observed from 48-72h.p.i., which is similar to the very late *polh* promoter, rather than from 24-48 h.p.i. like the original late *chiA/cath*^(syn) promoter. EGFP fluorescence from the LacO83 (two *lacO* after TAAG) construct occurred from 24-72 h.p.i. rather than just 48-72 h.p.i., suggesting that addition of a second *lacO* after the TAAG motif not only further increased promoter activity, but also enabled gene expression from the promoter at both early (at 24 h.p.i.) and late (at 72 h.p.i.) times rather than just early or late as observed from all of the other promoters. Using the LacO77 (*lacO* before TAAG) promoter construct, fluorescence was observed from 24-48 h.p.i., which was similar to that of the original *chiA/cath*^(syn) promoter. However, EGFP fluorescence was reduced at both 24 and 48 h.p.i. in comparison.

Production of EGFP in the cells at each time point was further assessed by western blot (Figure 4.12), with results supporting the pattern described above for the flow cytometer analysis. The blot data also showed the cumulative production of EGFP by 72 h.p.i., which suggests that the LacO83 promoter construct can yield greater overall EGFP production than that from the very late *polh* promoter by this time point.

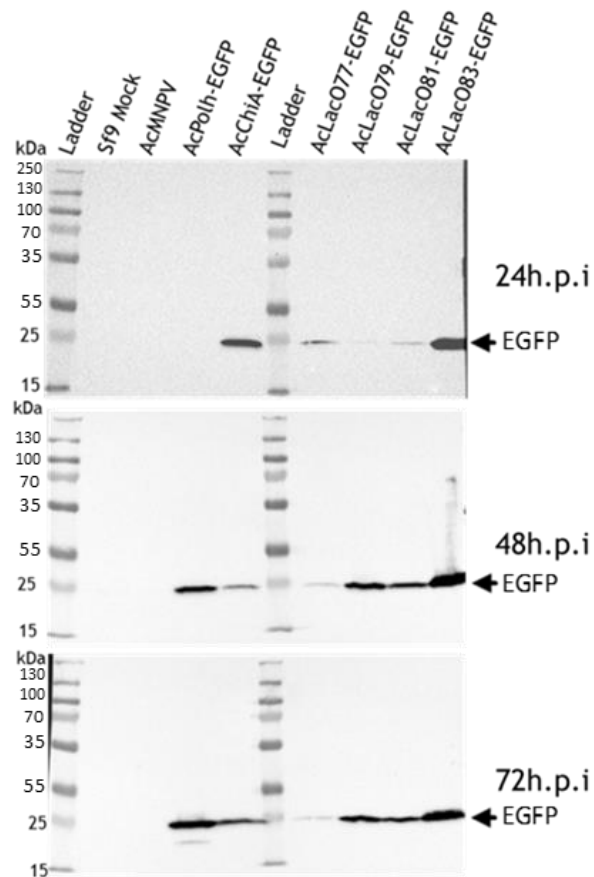


Figure 4.12- Activity of the *chiA/lacO* promoters: production of EGFP in recombinant baculovirus-infected cells over time. Sf9 cells were infected with AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP or AcLacO83-EGFP (MOI 5). AcPolh-EGFP, AcChiA-EGFP, AcMNPV and mock-infections were also carried out as controls for EGFP production. At 24 h.p.i., 48 h.p.i. and 72 h.p.i. EGFP (24.6kDa, arrowed) production in cells was analysed by western blot. Infections were carried out in triplicate and a representative image is shown. Mouse monoclonal to EGFP (1:1000) and anti-mouse HRP were used as primary and secondary antibodies respectively. Molecular mass ladder shown left and centre.

4.2.5.2 Expression of *gp64* from the *chiA/lacO* promoter constructs

To further explore the results of the EGFP production experiments above and the data suggesting that insertion of *lacO* after the TAAG of the *chiA/cath*^(syn) promoter increases its activity, expression of *gp64* from each of the promoter constructs was also assessed. Sf9

cells were co-transfected with the *lacO/gp64* plasmids and *gp64* Δ FBG DNA described previously (section 4.2.4) to produce recombinant virus with *gp64* under each promoter construct (AcLacO77-gp64, AcLacO79-gp64, AcLacO81-gp64 and AcLacO83-gp64). Recombinant viruses were screened by PCR to confirm incorporation of *gp64* into the virus genome and secondly to confirm *gp64* had not spontaneously reinserted itself at its native locus. Sf9 cells were then infected with these viruses (MOI 5) and positive and negative controls. At 48 h.p.i., GP64 production from the cells was analysed by western blot and the results (Figure 4.13) showed the same pattern of promoter activity as those observed from the EGFP production experiments described above (section 4.2.5.1). The LacO83 promoter yielded the greatest production of GP64, followed by LacO79, then LacO81 and LacO77.

The LacO83, LacO79 and LacO81 promoters all drove a greater production of GP64 than that observed from the original *chiA/cath*^(syn) promoter, whilst LacO77 produced less. These results further supported the idea that insertion of the *lacO* after the TAAG motif of the *chiA/cath*^(syn) promoter increases promoter activity. Addition of *lacO* before the TAAG motif, however, again appeared to hinder promoter activity.

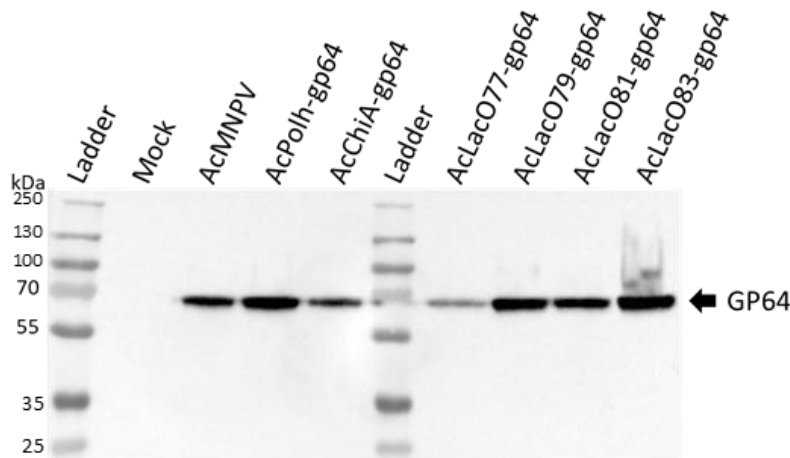


Figure 4.13- Activity of the *chiA/lacO* promoters: production of GP64 in recombinant baculovirus-infected cells. AcLacO77-gp64, AcLacO79-gp64, AcLacO81-gp64 and AcLacO83-gp64 (MOI 5). AcChiA-gp64, AcPolh-gp64, AcMNPV or mock-infections were carried out as controls for GP64 production. At 48 h.p.i., GP64 production (64kDa, arrowed) from the cells was analysed by western blot. Mouse monoclonal to GP64 (1:2800) and anti-mouse HRP were used as primary and secondary antibodies respectively. Molecular mass ladder shown left and centre.

4.2.6 Activity of the dual expression *chiA/cath* promoter

Two oligonucleotides (RDP1413 and RDP1414; Table 2.4, Chapter 2), were annealed to form the dual expression *chiA/cath* promoter with *Bgl*III and *Bam*HI complementary ends.

This promoter mimicked the natural AcMNPV *chiA/cath* promoter with functional TAAG motifs for both *chiA* and *cath*.

A plasmid with *egfp* and *mCherry* under the dual *chiA/cath* promoter was produced (pChiA_TAAG-EGFP/mCherry, Figure 4.14) to confirm the integrity of the *chiA/cath* dual promoter, with EGFP and mCherry acting as reporter proteins. A plasmid with *egfp* and *mCherry* under the *polh* and *p10* promoters of the pOET5 dual expression plasmid was also produced as a positive control for *egfp* and *mCherry* expression (pOET5-EGFP/mCherry, Figure 4.15). Sf9 cells were co-transfected with pChiA_TAAG-EGFP/mCherry or pOET5-EGFP/mCherry and *flashBAC* ULTRA™ DNA to produce recombinant viruses AcChiA_TAAG-EGFP/mCherry and AcpOET5-EGFP/mCherry, respectively. Recombinant virus genomes were screened by PCR to confirm the incorporation of *egfp* and *mCherry*.

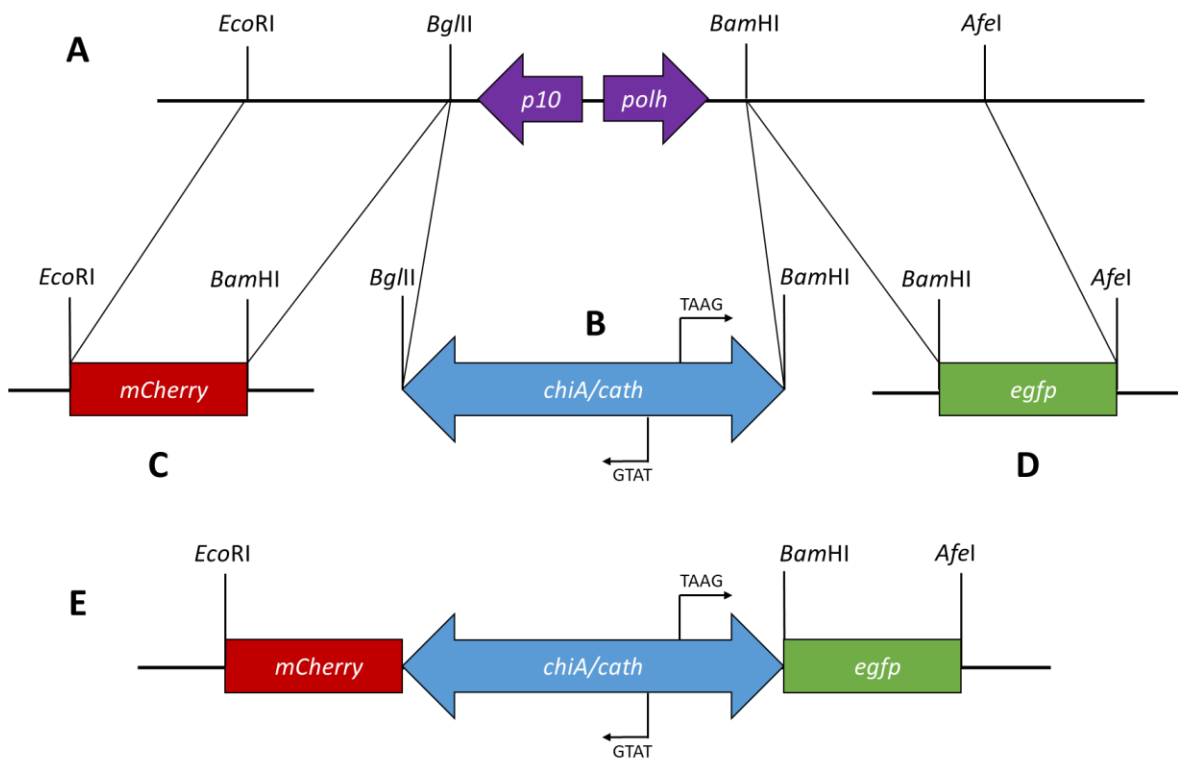


Figure 4.14- Generation of pChiA_TAAG-EGFP/mCherry.

Promoters are shown as arrows, indicating direction of transcription. The pOET5 dual expression plasmid (OET Ltd) (A) was digested with *BglIII* and *BamHI* to facilitate insertion of the *chiA/cath* promoter (with both TAAG motifs functional) (B). The plasmid DNA was then digested with *EcoRI* and *BglIII* to receive the *mCherry* gene, retrieved from pmCherry-Actin (C Bannach, OET Ltd) by digestion with *EcoRI* and *BamHI* (C). The plasmid DNA was then digested with *BamHI* and *AfeI* to receive the *egfp* gene, retrieved from the pPolh-EGFP plasmid (R Arinto Garcia, OET Ltd) by digestion with *BamHI* and *AfeI* (D) to produce the pChiA_TAAG-EGFP/mCherry plasmid (E). The final and intermediate construction steps were verified by DNA sequencing using the Sanger sequencing method.

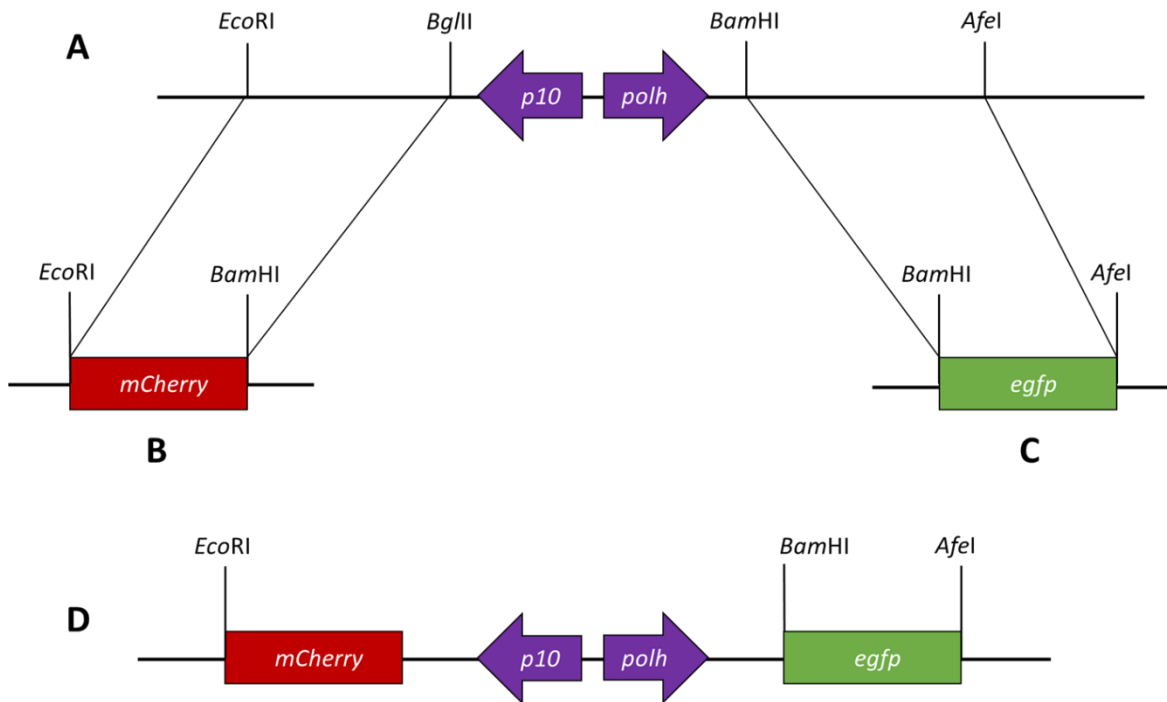


Figure 4.15- Generation of pOET5-EGFP/mCherry.

Promoters are shown as arrows, indicating direction of transcription. The pOET5 dual expression plasmid **(A)** was digested with *EcoRI* and *BglII* to facilitate insertion of the *mCherry* gene, retrieved from pmCherry-Actin (C Bannach, OET Ltd) by digestion with *EcoRI* and *BamHI* **(B)**. The plasmid was then digested with *BamHI* and *AfeI* to receive the *egfp* gene, retrieved from pPolh-EGFP (R Arinto Garcia, OET Ltd) by digestion with *BamHI* and *AfeI* **(C)** to produce pOET5 - EGFP/mCherry plasmid **(D)**. The final and intermediate construction steps were verified by DNA sequencing using the Sanger sequencing method.

Sf9 cells were infected with both dual expressing viruses (MOI 5) and imaged by fluorescence microscopy at 48 h.p.i. AcMNPV- and mock-infections were also carried out as controls. Results (Figure 4.16) showed strong *egfp* and *mCherry* expression from the AcpOET5-EGFP/mCherry infections (panels E and F). Expression of *egfp* and *mCherry* was also observed in the AcChiA_TAAG-EGFP/mCherry infected cells (panels G and H), although not as strongly as that observed from the AcpOET5-EGFP/mCherry infected cells (as expected). This confirmed the integrity of the *chiA/cath* dual expression promoter.

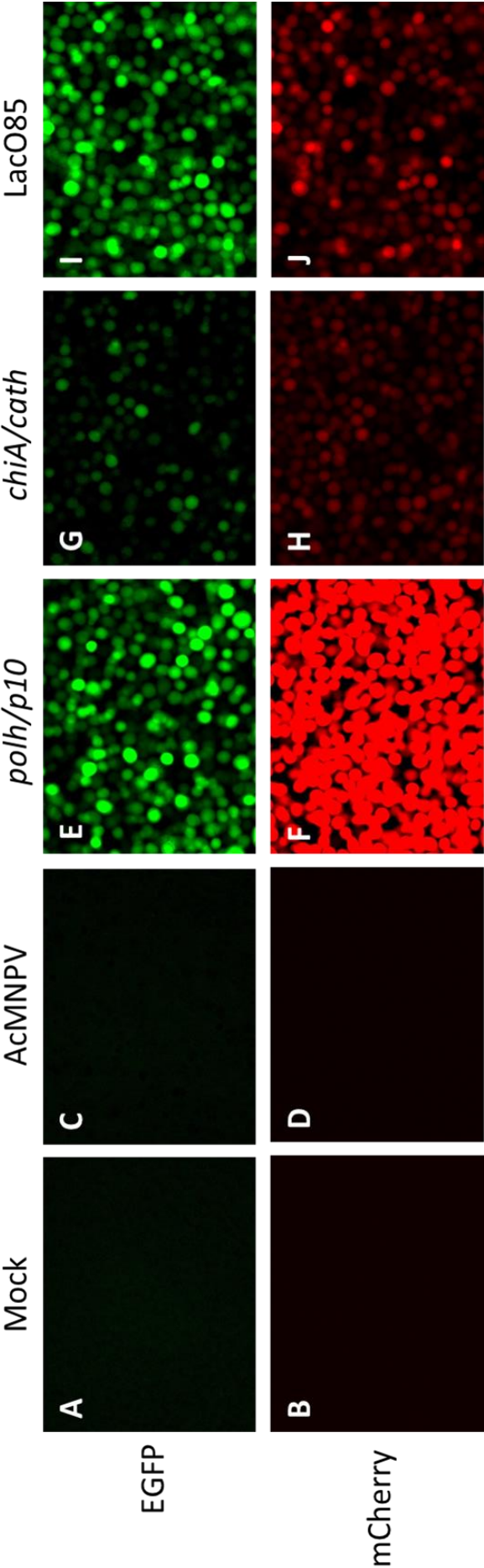


Figure 4.16- Expression of *egfp* and *mCherry* by recombinant baculoviruses. Sf9 cells were infected with AcChiA_TAAG-EGFP/mCherry, AcLacO85-EGFP/mCherry, or AcpOET5-EGFP/mCherry (MOI 5) and imaged by Revolve fluorescence microscopy (Echo Laboratories) for EGFP (top) and mCherry (bottom) at 48 h.p.i. (20X magnification). AcMNPV- or mock infections were used as controls. The promoter(s) driving expression are indicated at the top of each column. Infections were performed in duplicate and a representative image is shown.

4.2.7 Activity of the dual expression *chiA/cath* promoter when modified to contain the *lac* operator

Two oligonucleotides (RDP1415 and RDP1416; Table 2.4, Chapter 2), were annealed to form the dual expression *chiA/cath* promoter with *lacO* inserted once after both the *chiA* and *cath* TAAG motifs (LacO85; Figure 4.17).

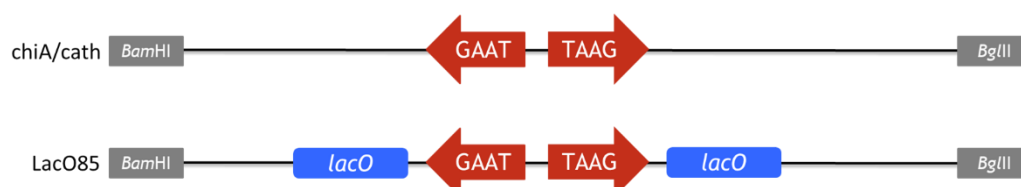


Figure 4.17- Structural representation of the dual expression *chiA/lacO* promoter constructs.

A *chiA/cath* promoter was synthesised with *Bam*HI and *Bgl*II restriction ends to allow for insertion into plasmid DNA. This dual expression promoter possessed two transcription initiation TAAG motifs (indicated by red arrows) for the regulation of two genes. Based on this promoter, the LacO85 promoter was designed with the *lacO* sequence (blue box) inserted after each TAAG motif.

To assess the integrity of the *chiA/cath* dual expression promoter following insertion of *lacO* after each conserved TAAG motif, a plasmid was produced with *egfp* and *mCherry* under the control of the LacO85 promoter. The LacO85 promoter construct was cloned into the previously digested pOET5 plasmid (*polh/p10* dual promoter removed; Figure 4.18). Sf9 cells were co-transfected with the final pLacO85-EGFP/*mCherry* (Figure 4.18E) and *flashBAC* ULTRA™ DNA to produce a recombinant virus AcLacO85-EGFP/*mCherry*. The recombinant virus genome was screened by PCR to confirm the incorporation of *egfp* and *mCherry*.

Sf9 cells were infected with AcLacO85-EGFP/*mCherry* or AcChiA_TAAG-EGFP/*mCherry* (MOI 5), alongside controls as previously described, and imaged by fluorescence microscopy at 48 h.p.i. Figure 4.16 (panels I and J) shows clear *egfp* and *mCherry* expression from the LacO85 promoter construct confirming the integrity of the dual expression *chiA/cath* promoter following insertion of the *lacO* sequence after each TAAG motif. The LacO85 promoter also appeared to yield stronger *egfp* and *mCherry* expression than that observed from the original *chiA/cath* dual promoter. As with the *egfp* expression results above (sections 4.2.3 and 4.2.5.1), these results again suggested that the insertion of *lacO* into the *chiA/cath* promoter increased its activity.

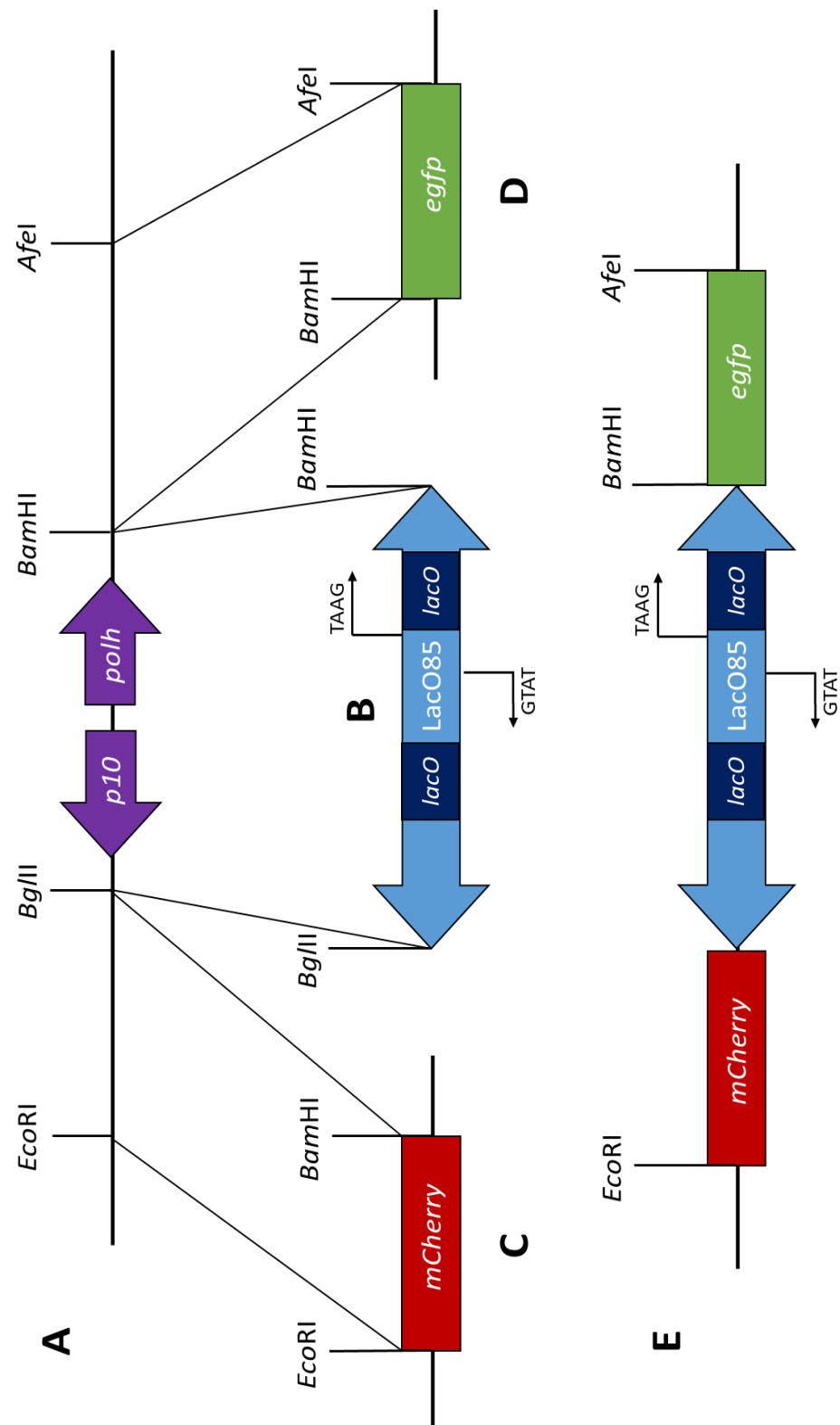


Figure 4.18- Construction of pLacO85-EGFP/mCherry. Promoters are shown as arrows, indicating direction of transcription. The pOET5 dual expression plasmid (A) was digested with *Bgl/II* and *BamHI* to facilitate insertion of the LacO85 promoter (B). The plasmid was then digested with *EcoRI* and *Bgl/II* to receive the *mCherry* gene, retrieved from the pmCherry-Actin plasmid DNA (C Bannach, OET Ltd) by digestion with *EcoRI* and *BamHI* (C). The plasmid was then digested with *BamHI* and *AfeI* to receive the *egfp* gene, retrieved from the pPolh-EGFP plasmid (R Arinto Garcia, OET Ltd) by digestion with *BamHI* and *AfeI* (D), to produce the pLacO85-EGFP/mCherry plasmid (E). The final and intermediate construction steps were verified by the Sanger DNA sequencing method.

4.2.8 Insertion of *lacO* into the dual expression *chiA/cath* promoter alters promoter activity

Previous results (section 4.2.7) suggested that following insertion of the *lacO* after each TAAG motif, the activity of the dual expression *chiA/cath* promoter increased. To explore this further, Sf9 cells were infected with AcChiA_TAAG-EGFP/mCherry and AcLacO85-EGFP/mCherry (MOI 5). At 48 h.p.i., the EGFP and mCherry fluorescence intensity of the cells was analysed using a plate reader (Figure 4.19). The results showed that the LacO85 promoter significantly ($p < 0.001$) increased expression of both *egfp* (~10-fold) and *mCherry* (~3-fold) compared with the original *chiA/cath* promoter.

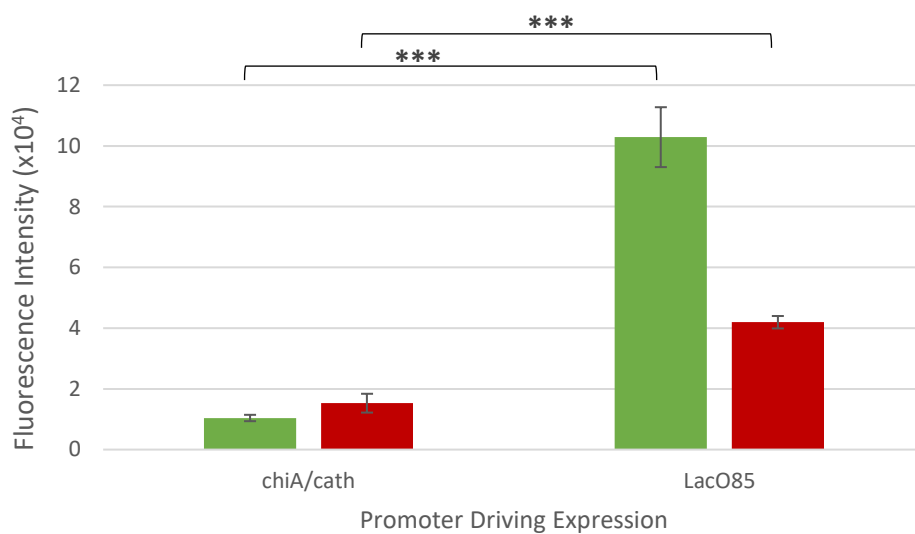


Figure 4.19- Activity of the dual *chiA/lacO* promoter: intensity of EGFP and mCherry fluorescence from recombinant baculovirus-infected cells. Sf9 cells were infected with AcChiA_TAAG-EGFP/mCherry or AcLacO85-EGFP/mCherry (MOI 5). Mock-, AcMNPV- and AcpOET5-EGFP/mCherry-infections were carried out as controls (data not shown). At 48 h.p.i., EGFP and mCherry fluorescence intensity of the cells was analysed by plate reader. $n = 3$. Error bars indicate SEM. AcMNPV-infected cells defined the negative/positive threshold for EGFP fluorescence, from which the fluorescence intensity of the other infected cell samples was calculated. F-tests for variance and Students t-tests for statistical significance were performed and significance is shown (***) = $p < 0.001$).

4.3 Discussion

The first step in the production of the BEVS LacI/O Repressor System was to produce a promoter construct modified to contain *lacO*. As discussed above (section 4.1), the *chiA/cath* late promoter was selected due to its relative simplicity compared to other late promoters (a short 5'UTR and only one transcription initiation site for *chiA* or *cath*) and it

ability to act as a bi-directional transcription unit with late promoter TAAG motifs on either strand to regulate expression of both *chiA* and *cath*, which could potentially allow for the inhibition of two key genes rather than just one.

Initially, a synthetic version of the *chiA/cath* promoter was generated with the antisense TAAG motif for *cath* mutated (*chiA/cath*^(syn)) to assess regulation of one gene. As it was not known how *lacO* positioning within the promoter would affect gene repression in the presence of LacI, or how it would affect basal gene expression in the absence of LacI due to previous research giving conflicting advice on the best insertion site (as discussed in section 4.1; Lanzer and Bujard, 1988; Fuerst *et al.*, 1989; Zhang and Moss, 1991; O’Connell *et al.*, 2020), four single expression promoter constructs were designed. These were based on the *chiA/cath*^(syn) promoter, and each contained the *lacO* at different positions relative to the conserved TAAG motif (LacO77, LacO79, LacO81 and LacO83; Figure 4.5). LacO77 had one *lacO* inserted 5bp upstream of the TAAG motif, whilst LacO79 contained one *lacO* 11bp after the TAAG motif. Both LacO81 and LacO83 contained two copies of the *lacO*, one located 5bp before and one 11bp after the TAAG motif for LacO81, or both directly next to each other 11bp after the TAAG motif for LacO83. For the upstream *lacO* insertion site, a position 5bp before the conserved TAAG motif was chosen. This location ensured that the *lacO* was upstream of the TAAG for *chitinase* but not preceding the inhibited TAAG motif for *cathepsin*, which could have presented positioning problems for the design of the dual promoter construct if insertion before the TAAG motif was identified as the preferential position. For the downstream *lacO* insertion site, a position 11bp after the TAAG motif was chosen as this avoided alteration of the 6bp immediately following TAAG, which research by others has shown to influence promoter activity (Morris and Miller, 1994). This position also reflected the natural positioning of *lacO* in *E. coli* (+11bp from the RNAP binding site) (Reviewed by Lewis, 2005) which may prove beneficial.

Later experiments involved the use of the *chiA/cath* promoter with both TAAG motifs functional to allow for the regulation of two key genes. Based on the results of the single expression *chiA/lacO* promoter experiments, this dual expression promoter was then modified to insert *lacO* after each of the TAAG motifs (LacO85; Figure 4.17).

Whilst the inhibition of BV synthesis during recombinant protein/VLP production was the main aim of the LacI/O Repressor System, another essential feature was its ability to yield

normal levels of BV production in the absence of LacI. It was therefore essential that the insertion of *lacO* into the *chiA/cath* promoter did not inhibit its basal activity. Consequently, the main focus of this chapter was first to confirm the integrity of each of the modified *chiA/cath* promoter constructs and to assess how *lacO* positioning within those constructs affected the basal levels of gene expression.

Results of the *chiA/cath*^(syn) promoter studies (section 4.2.1) found the *chiA/cath*^(syn) promoter was capable of driving *egfp* expression in Sf9 cells thus confirming its integrity following the inhibition of the *cath* TAAG motif. It was also capable of driving late gene expression (specifically *gp64* expression) in Sf21 cells (section 4.2.2).

To confirm the integrity of each of the four *chiA/lacO* single expression promoter constructs (LacO77, LacO79, LacO81 and LacO83), multiple plasmids were produced, each with one of the promoter constructs driving *egfp* expression, which were subsequently used to produce recombinant virus. EGFP fluorescence in infected Sf9 cells (section 4.2.3) confirmed the integrity of all the *chiA/lacO* single expression promoter constructs. Most interestingly, however, EGFP fluorescence seemed stronger from the LacO79, LacO81 and LacO83 promoter constructs, and weaker from LacO77, than that observed from the original *chiA/cath*^(syn) promoter. Furthermore, EGFP fluorescence seemed stronger from LacO79 and LacO83 than from LacO77 or LacO81. This suggested that the position of *lacO* within the *chiA/cath*^(syn) promoter relative to the TAAG motif did affect basal gene expression, with insertion after the TAAG motif causing increased promoter activity whilst insertion before decreased activity.

Further analysis (section 4.2.5.1) of infected cells by flow cytometry and western blot confirmed the fluorescence microscopy observations, with results showing the same pattern of fluorescence from the promoter constructs. Data analysis of the flow cytometry results revealed that insertion of *lacO* prior to the TAAG motif in LacO77 significantly decreased (80.2%; $p < 0.05$) the *chiA/cath*^(syn) promoters activity. Insertion of *lacO* after the TAAG motif in LacO79 (146.6%) and LacO83 (218.7%), however, significantly increased ($p < 0.05$) the *chiA/cath*^(syn) promoters activity. Whilst EGFP fluorescence from LacO81 (*lacO* before and after TAAG) was increased (30.5%) compared to that from the original *chiA/cath*^(syn) promoter, this increase was not significant. The increase was also lesser than that observed from LacO79 (*lacO* only after TAAG). This indicated that the increase in

promoter activity induced by insertion of *lacO* after the TAAG motif was partially off-set by the decreasing effects on activity induced by insertion of *lacO* before the TAAG motif. However, as an increase in EGFP fluorescence was still observed from the LacO81 construct compared to the original *chiA/cath*^(syn) promoter, it appeared that the increasing/decreasing effects of *lacO* before or after the TAAG motif were not equal. The change in promoter activity induced by *lacO* presence before the TAAG motif was less than the change in activity induced by *lacO* after the TAAG motif. Data analysis revealed that insertion of *lacO* after the TAAG motif increased promoter basal activity by 146.6%, whilst insertion before the TAAG motif only decreased basal activity by 80.2%. Consequently, it could be predicted that insertion of *lacO* before and after the TAAG motif would result in an overall 66.4% increase in promoter basal activity. In practice, an overall increase of 30.5% was observed suggesting the mechanisms of repression and enhancement acting on the promoter are not equal in affect.

Fluorescence microscopy (Figure 4.7), western blot (Figure 4.10) and flow cytometry (Figure 4.9) all showed that LacO83 (*lacO* twice after TAAG) yielded greater EGFP fluorescence than that from LacO79 (*lacO* once after TAAG) suggesting that the insertion of additional *lacO* sequences after the TAAG motif further increased promoter activity. However, data analysis of the flow cytometry results found that this increase in activity was not significant.

BV production from Sf9 cells co-transfected with *gp64*ΔFBG DNA and one of the *lacO/gp64* plasmids to rescue *gp64* expression (section 4.2.4) confirmed the ability of all the *chiA/lacO* single expression promoters to drive baculovirus late gene expression. Western blot analysis of GP64 production from each of the *chiA/lacO* promoters (Figure 4.13) showed the same pattern of promoter activity as that observed when the *chiA/lacO* promoters were driving *egfp* expression. The LacO83 promoter drove the greatest production of GP64, followed by LacO79, LacO81 and LacO77. The LacO83, LacO79 and LacO81 promoters all drove a greater production of GP64 than that observed from the original *chiA/cath*^(syn) promoter, whilst LacO77 produced less. These results confirmed that insertion of *lacO* after the TAAG motif of the *chiA/cath*^(syn) promoter increased promoter activity whilst insertion before the TAAG motif hindered promoter activity. It is worth noting, however, that when comparing the western blot results for EGFP production (Figure 4.10) to those for GP64

production (Figure 4.13) from the *chiA/lacO* promoters, there was less variation in GP64 production between each of the promoters compared to that observed for EGFP. Whether this is due to differences in post-translational events for each gene, or due to inherent differences in expression of each gene from the *chiA/lacO* promoters requires further analysis.

Interestingly, when comparing the western blot results for GP64 production (Figure 4.13) to the BV production results in section 4.2.4, the *chiA/lacO* promoters driving increased GP64 production in comparison to the original *chiA/cath^(syn)* promoter also drive a greater production of BV. Indeed, this might have been expected as assuming it was the limiting factor, a greater production of the key structural protein responsible for BV cell exit and infectivity would result in an increase in BV production. However, more research is needed to determine whether the enhanced production of GP64 increased BV titre, determined by plaque assay, by enabling more nucleocapsid exit from cells or by increasing BV infectivity (or a combination of the two).

The levels of basal gene expression observed from each of the *chiA/lacO* single expression promoters appeared to correlate with the conclusions of Fuerst *et al.* (1989), who observed the best levels of gene expression when *lacO* was inserted downstream of the transcription initiation site (TAAAT sequence) in a vaccinia virus promoter, and worst levels when *lacO* was inserted before this sequence. However, whilst both Fuerst *et al.* and the results of this chapter found the best levels of gene expression when *lacO* was inserted downstream of the transcription start site, Fuerst *et al.* still observed a 34% reduction in basal activity from the promoter in contrast to the 146.6% increase observed in this chapter. Fuerst *et al.* also observed a total inhibition of downstream expression when *lacO* was inserted upstream of TAAAT, whereas the results of this chapter saw an 80.2% reduction.

These differences are likely due to the structural/sequence differences between vaccinia virus and baculovirus late promoters and differences in the *lacO* insertion sites. Unlike AcMNPV late promoters, research has shown an A and T rich region of approximately 20bp upstream of the conserved vaccinia virus TAAAT sequence to contribute to promoter strength (Davidson and Moss, 1989). Sequences 15bp upstream of the TAAAT are also thought to be involved in the binding of virus-encoded proteins, which stimulate transcription from the promoter (Zhu *et al.*, 1998). Furthermore, a core element at

positions -14 to -8 relative to the second A of the TAAAT motif has been found essential for full promoter activity (Knutson *et al.*, 2006). Whilst Fuerst *et al.* (1989) did not delete these important regions upstream of the TAAAT, insertion of the *lacO* sequence immediately upstream of the TAAAT sequence may have impacted their influence on transcription by changing their positions relative to the TAAAT motif. Indeed, Knutson *et al.* (2006), showed that the addition of base pairs to the spacer sequence between the core element and the TAAAT motif reduced promoter activity. Consequently, disruption to these numerous important elements upstream of the vaccinia virus promoter could partially explain the greater reduction in promoter activity compared to that observed from the *chiA/cath*^(syn) promoter.

With regards to the decrease in promoter activity observed by Fuerst *et al.* (1989) following insertion of *lacO* downstream of the transcription initiation start site, compared to the increase in activity seen in this chapter, Fuerst *et al.* inserted *lacO* only 1bp downstream of TAAAT and also changed the G immediately following the TAAAT to A. Research has shown the two base pairs immediately following the TAAAT sequence to be important for promoter function, with their alteration resulting in a decline in promoter activity (Hänggi *et al.*, 1986; Knutson *et al.*, 2006). In LacO79, however, the *lacO* was inserted 11bp downstream of the TAAG motif thus avoiding altering the 6bp downstream of TAAG, which have been shown to influence promoter activity (Morris and Miller, 1994). This difference in *lacO* positioning is likely therefore, in part, to explain the difference in influence on promoter activity observed following insertion of *lacO* downstream of the transcription start site.

The results from the analysis of *egfp* expression from each of the *chiA/lacO* promoters over time (Figure 4.11) revealed that insertion of *lacO* into the *chiA/cath*^(syn) promoter did not just simply increase or decrease promoter activity, but also changed the time at which expression occurred, providing insights into the underlying mechanisms causing the observed change in promoter activity. EGFP fluorescence was observed earlier from the late *chiA/cath*^(syn) promoter (from 24-48 h.p.i.) than from the very late *polh* promoter (from 48-72 h.p.i.), as expected. Interestingly, EGFP fluorescence from LacO79 (*lacO* after TAAG) was observed from 48-72 h.p.i. similarly to the *polh* promoter, rather than from 24-48 h.p.i. like the original *chiA/cath*^(syn) promoter. This suggests that insertion of *lacO* after the TAAG

motif not only increased promoter activity (as discussed above), but also delayed the time that gene expression occurred with the promoter functioning more like a very late promoter than a late promoter. This means that the increase in promoter activity observed from LacO79 was not simply a cumulative effect due to the same amount of promoter activity over a longer temporal period, but due to an increase in promoter strength. The promoter activity was delayed and a greater level of gene expression was achieved in the same amount of time (both the *chiA/cath*^(syn) and the LacO79 promoters only induced 2 days of *egfp* expression).

Observation of EGFP fluorescence over time from LacO83 also yielded some interesting results. The fluorescence microscopy (Figure 4.7), western blot (Figure 4.10) and flow cytometry results (Figure 4.9) all showed that LacO83 (two *lacO*s after TAAG) had increased activity compared to LacO79 (one *lacO* after TAAG), although this difference was not statistically significant. The results of the *egfp* temporal expression analysis found that unlike LacO79, EGFP fluorescence from LacO83 occurred from 24-72 h.p.i. rather than 48-72 h.p.i. This suggests that addition of a second *lacO* after the TAAG motif not only further increased promoter activity, but also enabled gene expression from the promoter both early (at 24h.p.i.) and late (at 72h.p.i.) rather than just early or late as observed with the other promoters. Further to this, the EGFP fluorescence from LacO83 at 48 and 72 h.p.i. was comparable (but slightly less) to that observed from LacO79 at the same time points. Consequently, the overall increase in *egfp* expression from LacO83 seems to be the result of cumulative gene expression over a longer period of time rather than additional increases in promoter strength.

Temporal EGFP fluorescence from LacO77 was observed from 24-48 h.p.i. and was similar to that observed for the original *chiA/cath*^(syn) promoter. Insertion of *lacO* prior to the TAAG motif did not, therefore, appear to affect the temporal control of promoter activity (although earlier time points could be assessed). However, EGFP fluorescence was reduced at both 24 and 48 h.p.i. in comparison to the original *chiA/cath*^(syn) promoter further confirming that insertion of *lacO* before the TAAG motif inhibited promoter activity by reducing promoter strength rather than its temporal activity.

Whilst the results of this chapter have shown the insertion of *lacO* into the *chiA/cath* and *chiA/cath*^(syn) promoters to affect promoter activity, it is still unclear as to the molecular

mechanisms behind this. Indeed, there are many ways in which the presence and positioning of the *lacO* sequence could be influencing activity. Firstly, the *lacO* sequence could be acting as an enhancer. Enhancers are specific DNA sequences that can be positioned close to or far from the site of transcriptional initiation and can recruit and concentrate transcription factors (TFs) delivering them to the regulatory region of the gene, thus facilitating transcriptional initiation. Enhancers can also bind transcriptional activators, which elevate the levels of specific RNA synthesis by bringing the enhancer and its associated TFs into proximity to the RNA polymerase complex. Overall, enhancers affect promoter activity and regulate transcription from said promoter by influencing the rate at which RNA polymerase is recruited to, or stabilised, at the promoter and its subsequent ability to efficiently transcribe RNA (reviewed by Rohrmann, 2019).

For example, the *p10* and *polh* promoters were found to contain an A/T rich “burst sequence” responsible for the burst in transcription from very late promoters via interaction with the very late factor 1 (VLF-1) (Ooi *et al.*, 1989; Yang and Miller, 1999). Homologous regions (*hrs*) have also been identified and used as transcriptional enhancers (Guarino and Summers, 1986; Ishiyama and Ikeda, 2010; Rohrmann, 2019). In AcMNPV, *hrs* are found at nine locations and are composed of repeated units of approximately 70bp with an imperfect approximately 30bp palindrome near their centre (Rohrman, 2019). They also contain cAMP and 12-O-tetradecanoylphorbol 13-acetate (TPA) response elements (CRE and TRE)-like sequences, which have been shown to bind to host cell proteins and activate transcription in transient assays (Landais *et al.*, 2006).

Hrs also bind the transcriptional activator IE1. It is possible that IE1 could recruit the transcription factors bound to the homologous region to the RNA polymerase II complex, influencing the rate at which it is recruited to or stabilised at a specific promoter. Whilst the *lacO* sequence lacks a CRE or TRE sequence, it is an imperfect palindrome of similar length to that found in *hrs* (Gilbert and Maxam, 1973). It is therefore possible that when inserted after the TAAG motif, the *lacO* sequence can act in a similar, although weaker, manner to a *hrs*, and through transcription factor/ activator binding, enhance the rate at which RNA polymerase is recruited to or stabilised at the *chiA/cath* promoter.

The *lacO* sequence could further have increased promoter activity by containing an initiator sequence. Two major sequences have been identified in baculovirus early genes; the TATA

promoter motif and a transcriptional initiation consensus sequence, CAGT (Blissard *et al.*, 1992). The consensus sequence can act independently as an initiator, which facilitates the assembly of the RNA polymerase II preinitiation complex and can promote transcription in the absence of a TATA sequence. When studying the transcriptome from AcMNPV infected *Trichoplusia ni* (*T. ni*) cells, Chen *et al.* (2013) found sequences near the early transcription start site at the following frequency: CAGT, 15%; CATT, 9%; and CAAT, 8.7%. The *lacO* inserted into the *chiA/cath* and *chiA/cath*^(syn) promoters contained a CAAT sequence at positions 19-22. Whilst not the most prevalent version of the consensus motif, it is possible that the CAAT sequence was acting in a similar manner and facilitated the assembly of the RNA polymerase II preinitiation complex and promoted transcription in the absence of a TATA sequence. This could perhaps explain the production of EGFP at both early and late time points from LacO83. It is possible that CAAT positioning was important and insertion of a second CAAT further away from the transcription initiation site than the CAAT sequence in LacO79 and LacO81 facilitated its functioning. Future work could explore this possibility further by employing transcription mapping techniques to assess whether the *lacO*, when inserted at different positions within the AcMNPV genome, is capable of initiating transcription.

The *lacO* sequence may also be increasing promoter activity by changing the DNA structure. Indeed, multiple studies have demonstrated the importance of DNA shape readout in conferring both binding affinity and DNA binding specificity to TFs (reviewed by Rohs *et al.*, 2010 and by Slattery *et al.*, 2014). The *lacO* sequence may also be facilitating DNA unwinding, thus increasing access to the DNA by RNA polymerase and enhancing transcription. However, further research into the geometric and physical properties of the *chiA/cath* promoter DNA following insertion of the *lacO* are needed.

However, when the *lacO* sequence was inserted upstream of the TAAG motif, an inhibition of promoter activity was observed. It is most likely that whatever enhancing activity the *lacO* conferred to the *chiA/cath*^(syn) promoter, this was negligible when inserted upstream because of the precise position chosen in this study. For both LacO77 and LacO81, *lacO* was inserted 5bp upstream of the conserved TAAG sequence. This position ensured that the *lacO* was upstream of the TAAG for *chitinase* but not preceding the inhibited TAAG motif for *cathepsin*, which could have presented positioning problems for the design of the dual

promoter construct if insertion before the TAAG motif was identified as the preferential position. However, research has found the 8bp prior to the TAAG motif of late promoters to influence promoter activity (Morris and Miller, 1994). Consequently, interruption of these important bases by the *lacO* sequence could be responsible for the decline in promoter activity observed. It would be interesting for future experiments to explore whether this decline in promoter activity is reduced by inserting the *lacO* further upstream from the TAAG motif for *chitinase* in the *chiA/cath*^(syn) promoter.

Results of the dual expression promoter study (section 4.2.6) found that with both TAAG motifs functional, the *chiA/cath* promoter was capable of driving *egfp* and *mCherry* expression in Sf9 cells, thus confirming its integrity. The results of the single expression *chiA/lacO* promoter studies had found the best levels of basal gene expression from the *chiA/cath*^(syn) promoter were achieved when *lacO* was inserted after the TAAG motif (LacO79 and LacO83). Whilst two copies of *lacO* after TAAG (LacO83) yielded a greater level of basal expression than one copy (LacO79), this increase was not found to be statistically significant.

Consequently, when designing the dual *chiA/lacO* promoter, one copy of *lacO* was inserted after each TAAG motif. EGFP and mCherry fluorescence in infected Sf9 cells (Figure 4.16) confirmed the integrity of the dual *chiA/lacO* promoter, LacO85. Mirroring the activity of the single expression *chiA/lacO* promoters, gene expression from the dual *chiA/cath* promoter seemed stronger following insertion of *lacO* after the TAAG motifs; both EGFP and mCherry fluorescence appeared stronger from LacO85 than the dual *chiA/cath* promoter (Figure 4.16). Further analysis of fluorescence from infected Sf9 cells using a plate reader (Figure 4.19) confirmed this result was significant ($p < 0.001$). Results also showed that gene expression was slightly stronger from the sense TAAG than the antisense motif as EGFP was greater than mCherry fluorescence. However, statistically there was no significant difference. Interestingly, the increase in EGFP fluorescence from LacO85, compared with the dual *chiA/cath* promoter, was much greater (10-fold) than the increase in mCherry fluorescence (3-fold).

This difference may be attributed to the distance between each *lacO* sequence and its corresponding TAAG motif. Whilst the length of the 5'UTR after each TAAG motif was the same (53bp), the *lacO* sequence was 11bp away from the sense TAAG motif but 24bp away

from the antisense motif. If, as theorised above, the insertion of *lacO* enhances DNA unwinding, then unwinding at a position closer to the TAAG motif may further facilitate RNA polymerase binding at the TAAG motif. More research, however, into the mechanisms behind the influence of the *lacO* sequence is needed to better understand the effects insertion of *lacO* has on individual late promoters including dual promoters.

Currently, foreign gene expression from the BEVS is usually driven by the strong *polh* or *p10* very late promoters to produce the largest possible quantities of recombinant protein. The construction of multi/dual-expression BEVS that contain multiple copies of the *p10* and *polh* promoters (Weyer and Possee, 1991; Belyaev *et al.*, 1995; Berger *et al.*, 2004) has also facilitated co-expression of multiple genes in baculovirus infected cells for production of multi-protein complexes. Consequently, the BEVS is increasingly being used to produce protein complexes such as VLPs for vaccine production (Belyaev and Roy, 1993; Zhang *et al.*, 2018; Mi *et al.*, 2021).

However, one limitation of many dual expression vectors is their use of multiple copies of the *p10* or *polh* promoters. This can cause instability in the recombinant virus, resulting in the loss or rearrangement of the foreign gene sequences due to homologous recombination between the duplicated promoter sequences (R. Possee, personal communication). Consequently, discovery of other strong promoters for the expression of foreign genes is desirable. As discussed above, the production of EGFP from the *chiA/lacO* promoters over time was assessed. Western blots (Figure 4.12) indicated the cumulative production of EGFP up to 72 h.p.i., showing LacO83 to yield greater EGFP levels than that from the very late *polh* promoter. This enhancement of the *chiA/cath* promoter's activity could therefore make it an attractive alternative promoter option for BEVS dual expression. Its use could reduce instability caused by using multiple copies of the same promoter, whose use can increase the possibility of gene loss via homologous recombination between identical promoter sequences

Furthermore, the production of large quantities of protein during the very late phase of baculovirus infection, when post-translational modification and protein quality control mechanisms decline, can result in suboptimal protein yields and the production of dysfunctional or aggregated proteins (Ishiyama and Ikeda, 2010; reviewed by Grose *et al.*, 2021, Bruder and Aucoin, 2022). Proteins susceptible to aggregation or requiring advanced

post-translational modifications, such as VLPs, have been shown to benefit from continuous stable expression using earlier promoters (Ishiyama and Ikeda, 2010, Bruder and Aucoin, 2022). Consequently, with its earlier and expanded temporal phase, which results in a greater overall production of protein in comparison to the *polh* promoter, initial results suggest that the LacO83 promoter could be an ideal candidate for expression of these “difficult” proteins.

4.4 Conclusion

Overall, the work of this chapter confirmed the integrity of the single and dual expression *chiA/lacO* promoters. Basal gene expression from the *chiA/cath*^(syn) promoter was affected by *lacO* positioning relative to the TAAG motif. Insertion of *lacO* after the TAAG motif significantly increased promoter activity whilst insertion before significantly decreased activity. Insertion of a second *lacO* sequence after the TAAG motif further increased promoter activity, although this was not found to be significant. Results showed that insertion of one *lacO* after the TAAG motif delayed promoter activity and that the increase in promoter activity was due to an increase in promoter strength. Addition of a second *lacO* after TAAG however, did not increase promoter activity by further increasing promoter strength, but instead by extending the length of time the promoter was active. The basal activity of the dual expression promoter was also increased following insertion of *lacO* after each TAAG motif.

The next chapter focused on the production of a stable cell line that constitutively produces the LacI repressor protein. Subsequent infection of this cell line with the recombinant viruses produced in this chapter containing the *chiA/lacO* promoters should allow for the formation of *lacO/LacI* complexes resulting in the inhibition of downstream gene transcription. Hence, further experiments were carried out that explored which of the *chiA/lacO* promoter constructs would inhibit linked gene expression the best in the presence of LacI, and whether this correlated with the promoter that had the best levels of basal gene expression in the absence of LacI. Following this, the most appropriate *chiA/lacO* promoter construct was chosen for use in the LacI/O Repressor System for inhibition of the key genes required for BV production identified previously in Chapter 3.

Chapter 5:

**Development of a cell line that
continuously expresses *lacI***

5.1 Introduction

Previous research by Fuerst *et al.* (1989) showed potential for elements of the *lac* operon to be used to regulate gene expression in vaccinia virus. However, this was achieved in mammalian CV-1 cells where viral replication took place in the cell cytoplasm. Therefore, the work of this chapter aimed to assess whether a similar LacI/O repressor system could be established within Sf9 insect cells to repress gene expression from AcMNPV where replication occurs in the nuclei of cells.

For the development of the BEVS LacI/O Repressor System, an Sf9 cell line continuously expressing *lacI* was developed. Rather than incorporating *lacI* into the recombinant AcMNPV genome, which would only allow for constant repression of key gene expression, infection of a cell line constitutively expressing *lacI* would allow for selective repression of key genes. Infection of these cells with a recombinant virus containing key genes under a *chiA/lacO* promoter construct (Chapter 4), would allow LacI to bind the *lacO* and block the downstream transcription of those genes. Consequently, selective abrogation of BV production during recombinant protein/VLP production may be possible. However, infection of Sf9 cells (lacking LacI) would allow for selective expression of the key genes when BV production was desired (e.g. for virus stock amplification).

5.2 Results

5.2.1 Expressing *lacI* in Sf9 cells

First, it was established that the LacI protein could be produced in Sf9 cells. To this aim, Sf9 cells were co-transfected with pOET2-LacI (Figure 5.1A) and *flashBAC* ULTRA™ DNA to produce a recombinant virus with *lacI* under the *polh* promoter (AcLacI). AcLacI was used to infect Sf9 cells (MOI 5) in duplicate and *lacI* expression assessed by western blot 72 h.p.i., which confirmed production of LacI within Sf9 cells (Figure 5.1B). AcMNPV and mock-infections were carried out as negative controls for LacI production.

5.2.2 Nuclear targeting of LacI in Sf9 cells

After establishing that the LacI protein could be produced within Sf9 cells, the next step was to determine that it could be targeted to the nuclei of the cells as this is the site of

AcMNPV DNA replication where LacI is required to bind the *lacO* of the modified promoter. As shown in Figure 5.1A, the pOET2-LacI plasmid was designed to contain a nuclear localisation signal (NLS) to target LacI to the nucleus. Sf9 cells were infected with AcLacI (MOI 5) and harvested at 72 h.p.i. AcMNPV and mock-infections were also carried out as negative controls for LacI production. Cell pellets were fractionated by treatment with 1%

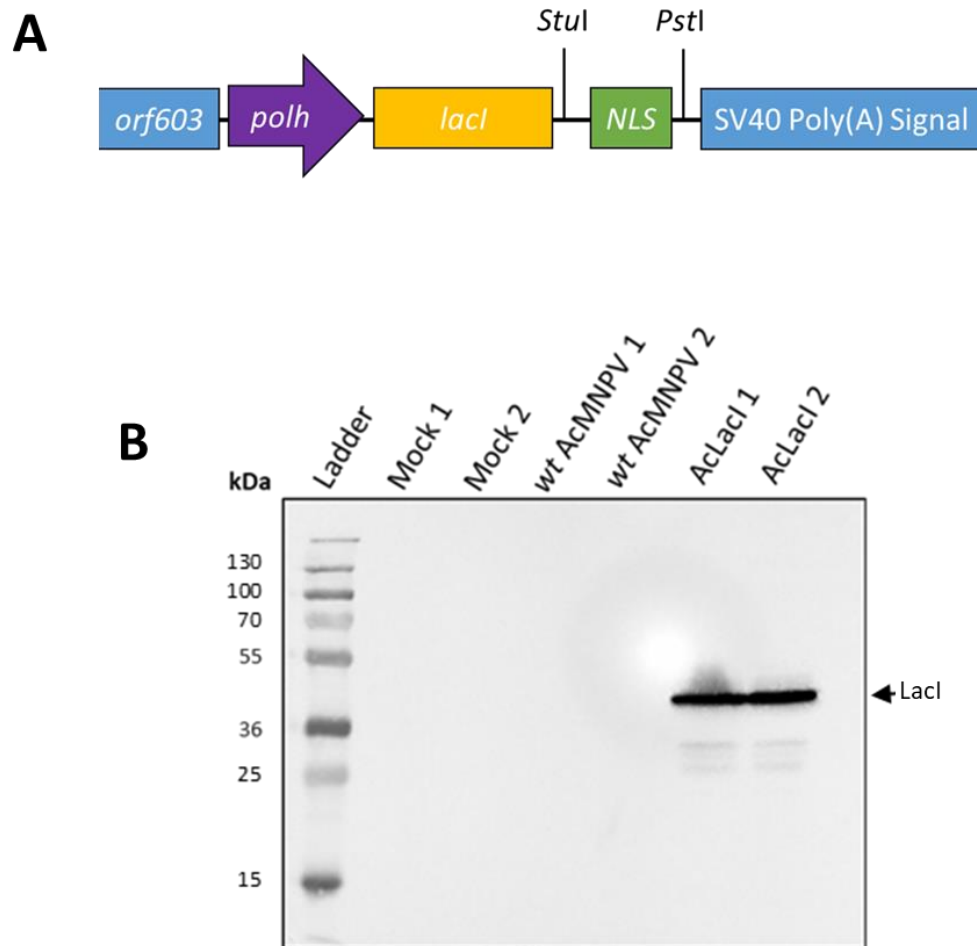


Figure 5.1- Production of LacI in Sf9 cells.

(A) Schematic representation of pOET2-LacI. The *lacI* gene is under the *polh* promoter. A nuclear localisation signal (NLS) in tandem with the *lacI* gene promotes LacI protein localisation to the nucleus of cells. **(B)** Western blot assessing the production of LacI (38.6kDa, arrowed) in Sf9 cells at 72 h.p.i. Duplicate dishes of Sf9 cells were infected with either AcLacI, AcMNPV (MOI 5) or were mock-infected. Mouse monoclonal antibody to LacI (1:1000) and anti-mouse HRP (horse radish peroxidase conjugated antibody) were used as primary and secondary antibodies, respectively. Molecular mass ladder, shown left.

NP40 lysis buffer to produce total, nuclear and cytoplasmic fractions. LacI presence in each of the fractions was then assessed by western blots (Figure 5.2) using either anti-GP64

(panel A) or anti-LacI (panel B) as primary antibody. The GP64 blot was a control for the fractionation process and GP64 was only observed in the total and cytoplasmic fractions as expected (panel A). Whilst some LacI was observed within the cytoplasmic fraction of the AcLacI-infected cells, the majority of the protein was targeted to the nuclear fraction (panel B).

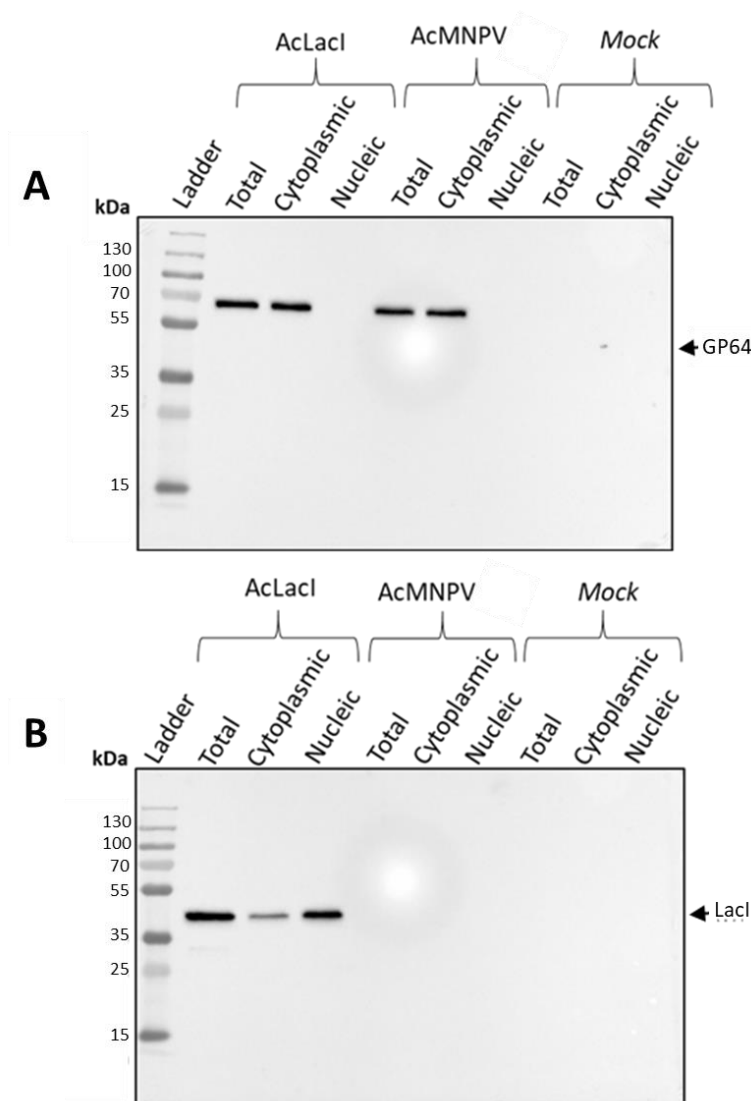


Figure 5.2- Distribution of LacI throughout Sf9 cells.

Western blots targeting **(A)** GP64 (64 kDa, arrowed) and **(B)** LacI (38.6kDa, arrowed) in Sf9 cells, lysed and separated into total, nuclear and cytoplasmic fractions. Sf9 cells were infected with AcLacI, AcMNPV (MOI 5) or mock-infected and harvested 72 h.p.i. before fractionation with 1% NP40 lysis buffer. Mouse monoclonal to GP64 (1:1000) **(A)** or mouse monoclonal to LacI (1:1000) **(B)** were used as primary antibodies. anti-mouse HRP was used as secondary antibody for both blots. Molecular mass ladder, shown left.

5.2.3 The nuclear localisation signal is required to target LacI to the nuclei of Sf9 cells

To confirm that the NLS was required to target LacI to the nucleus of Sf9 cells, pOET2-LacI was digested to remove the NLS, which was replaced by an oligonucleotide containing an essential stop codon to derive pOET2-LacI-ΔNLS (Figure 5.3A-C). Sf9 cells were co-transfected with pOET2-LacI-ΔNLS and *flashBAC* ULTRA™ DNA to produce a recombinant virus with *lacI* lacking a NLS under the *polh* promoter (AcLacI-ΔNLS).

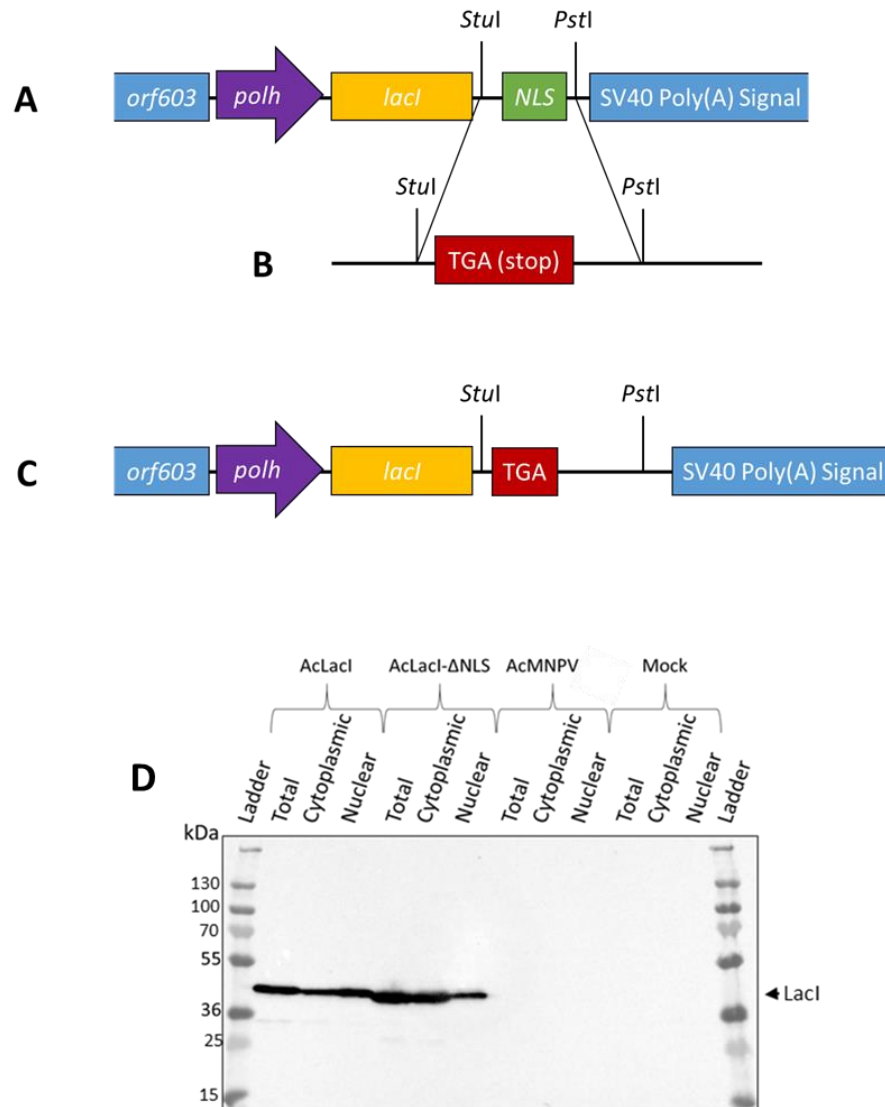


Figure 5.3- A nuclear localisation signal is required to target LacI to the nucleus of Sf9 cells. pOET2-LacI (**A**) was digested with *PstI* and *StuI* to remove the NLS. Two oligos (RDP1332 and RDP1333), were annealed to form a small fragment with *PstI* and *StuI* complementary ends and a stop codon (**B**), which was inserted into pOET2-LacI to replace the NLS sequence. The plasmid DNA, pOET2-LacI-ΔNLS (**C**), was purified and sequenced. (**D**) Western blot targeting LacI (38.6kDa, arrowed) in Sf9 cells fractionated into total, nuclear and cytoplasmic fractions. Sf9 cells were infected with either AcLacI, AcLacI-ΔNLS, AcMNPV (MOI 5) or mock-infected and harvested at 72 h.p.i. before fractionation with 1% NP40 lysis buffer. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder, shown left and right.

Sf9 cells were infected with AcLacI- Δ NLS and control viruses as described in section 5.2.2. Cell pellets were fractionated by treatment with 1% NP40 lysis buffer to produce total, nuclear and cytoplasmic fractions before LacI localisation was assessed by western blot (Figure 5.3D). Results showed that, following removal of the NLS, there was some localisation of LacI to the nuclei of cells. However, the majority of LacI was now clearly confined to the cytoplasmic fraction of the cells. It can therefore be concluded that the NLS is required to enhance LacI transport to the nucleus.

5.2.4 Transient expression of *lacI* from pIE1K-LacI+

The main objective of this chapter was to explore the synthesis of LacI in Sf9 cells, with the intention of producing a stable cell line continuously expressing *lacI*, which would constitute a vital component of a repressible system to inhibit key gene expression/BV production. Consequently, Sf9 cells were transfected with a plasmid containing *lacI* under the immediate early 1 (*Ac-ie1*) promoter (Figure 5.4A). The integration of *lacI* into the host cell chromosome under the *Ac-ie1* promoter would allow for continuous expression of *lacI* as the immediate early genes are transcribed by the host cell RNA polymerase II (Hoopes and Rohrmann, 1991; Blissard *et al.*, 1992).

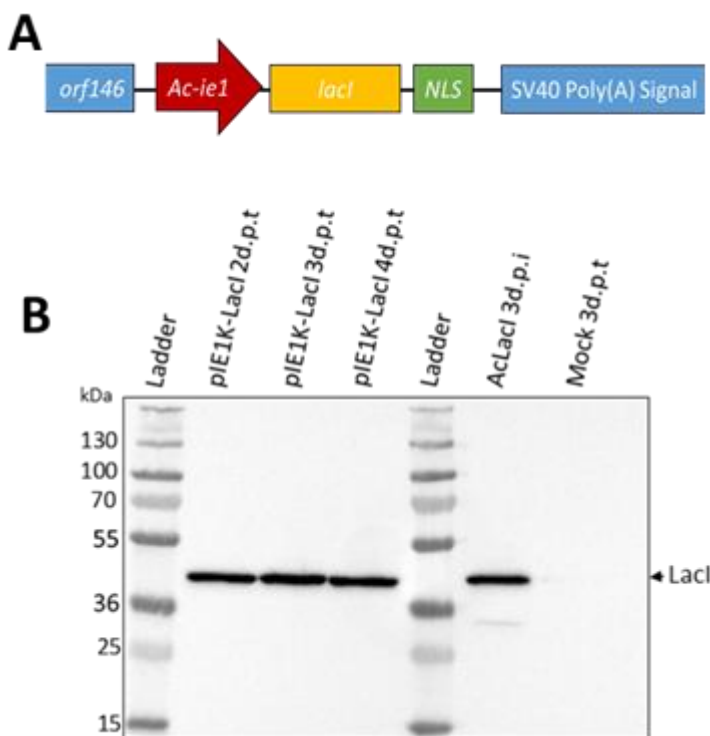


Figure 5.4- Transient production of LacI in Sf9 cells using the AcMNPV immediate early 1 gene promoter. (A) Schematic representation of pIE1K-LacI+, which contains *lacI* and a nuclear localisation signal under the control of the immediate early 1 (*Ac-ie1*) promoter. **(B)** Transient production of LacI in Sf9 cells transfected with pIE1K-LacI+. Western blot targeting LacI (38.6kDa, arrowed) in Sf9 cells transfected with pIE1K-LacI+ and harvested 48, 72, or 96 h.p.t. Control cells were infected with AcLacI (MOI 3) or mock-transfected. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder, shown left.

To confirm that LacI could be transiently produced within Sf9 cells when *lacI* was under the *Ac-ie1* promoter, Sf9 cells were transfected with pIE1K-LacI+ (Figure 5.4A), or infected with AcLacI (MOI 3) or mock-transfected as positive and negative controls for LacI production, respectively. pIE1K-LacI+ transfected cells were harvested 48, 72 and 96 h.p.t. and LacI presence assessed by western blot (Figure 5.4B). Control cells were harvested 72 h.p.i/t. Results confirmed that LacI could be transiently produced within Sf9 cells when under the control of the *Ac-ie1* promoter, with levels of production appearing stable over a short period of time.

5.2.5 Production of a polyclonal cell line expressing *lacI*

Having established that *lacI* could be transiently expressed from pIE1 LacI+, Sf9 cells were co-transfected with pIE1K-LacI+ and pIEK1-1neo to produce a stable cell line transiently producing LacI. pIEK1-1neo (Figure 5.5) contained *KanR*, a resistance gene that confers resistance to both the antibiotics kanamycin (for bacterial cell selection) and G418 (for eukaryotic cell selection). Co-transfected cells growing under G418 selection pressure would have successfully integrated the *KanR* gene and should therefore have also integrated the *lacI* gene too, as has been previously published (Hollister *et al.*, 1998).



Figure 5.5- Schematic representation of pIEK1-1neo.

The pIEK1-1neo contains *KanR*, a kanamycin resistance gene that confers resistance to the antibiotic G418. *KanR* is under the control of the immediate early 1 (*Ac-ie1*) promoter.

A titration of G418 was carried out in Sf9 cells to confirm their susceptibility to G418 and determine the minimum concentration of the antibiotic required to kill all non-resistant cells. Cells were seeded in a 12-well plate in duplicate and incubated at 28°C overnight. Cell media was replaced with 1mL of medium containing G418, with concentrations ranging from 2 to 0mg/mL (at 0.4mg/mL intervals). After approximately 72h, all cells growing in

G418 concentrations of 0.4mg/mL and above had been killed. Consequently, it was decided that co-transfections of Sf9 cells with pIE1K-LacI⁺ and pIEK1-1neo would be carried out at 0.4, 0.8 and 1.2mg/mL G418.

Sf9 cells were co-transfected with 500ng of pIE1K-LacI⁺ and pIEK1-1neo for each of the previously determined G418 concentrations. Control cells were transfected with just one of the plasmids or mock-transfected. However, after approximately 2 weeks post-transfection, the growth of resistant cell colonies in the pIE1K-LacI⁺ and pIEK1-1neo transfected wells had halted. Therefore, the co-transfections were repeated as before but this time with G418 at 0.4, 0.2 and 0.1mg/mL to reduce the levels of stress on the cells and encourage G418 resistant cell growth. An extra control was included in which cells were co-transfected with pIEK1-1neo and an alternative second plasmid (pOET6-GFP; *gfp* under control of the *polh* promoter) to rule out the possibility that transfecting with two plasmids was restricting colony growth. After three days, all non-resistant cells in the mock- and pIE1K-LacI⁺ transfected control wells had been killed and G418-antibiotic resistant colonies in the pIE1K-LacI⁺ and pIEK1-1neo transfected wells were growing steadily.

After two weeks, resistant cells in the pIE1K-LacI⁺ and pIEK1-1neo transfected wells reached confluency and two wells of cells (0.4mg/mL G418) were transferred into a 6-well plate. Following further growth, the cells continued to be scaled up until they were maintained in 25mL suspension flasks (data not shown).

During scale-up, cells were screened by PCR, which confirmed the integration of the *KanR* and *lacI* genes (Figure 5.6A). The production of LacI from the cells was also confirmed by western blot (Figure 5.6B).

5.2.6 Production of a clonal cell line expressing *lacI*

Now that a polyclonal cell line had been established, work was undertaken to produce a clonal cell line to ensure all cells expressed both the *lacI* and *KanR* genes. The polyclonal cells were diluted across a 96-well plate (2-fold dilution down each column and across each row) until wells contained single cells. Cells were grown in ESF-921 +10% FCS media comprised of 50% fresh and 50% conditioned media to support single cell growth. Three

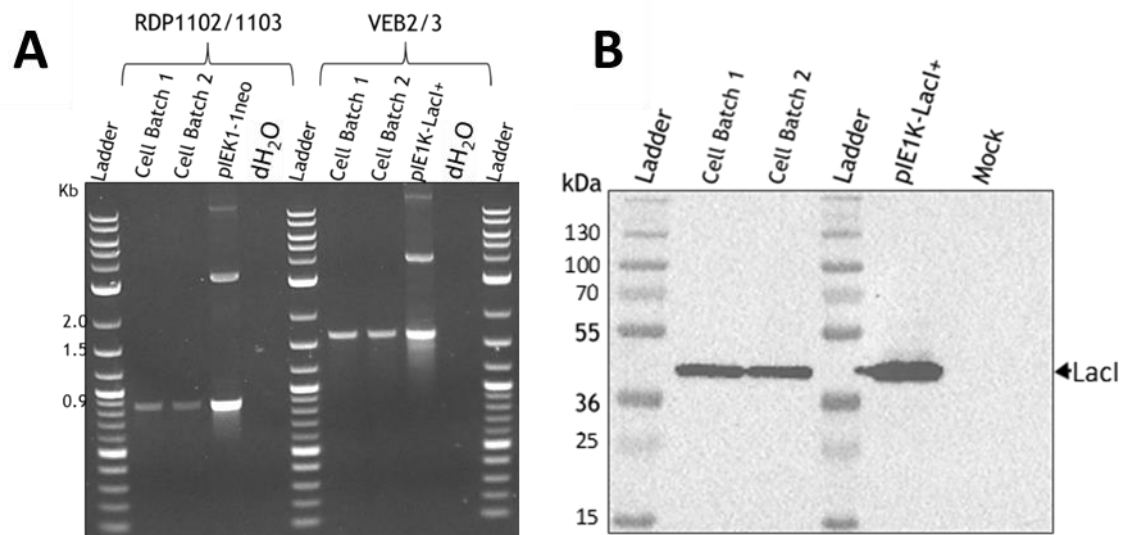


Figure 5.6- Analysis of two cultures of polyclonal Sf9 cells producing LacI following co-transfection with pIE1K-LacI+ and pIEK1-1neo. Sf9 cells were co-transfected with 500ng pIE1K-LacI+ and pIEK1-1neo and grown under G418 selection pressure (0.4mg/mL). Cells were scaled from 12-well plates into 25mL suspension flasks. During scale up, two cultures of cells were screened by PCR **(A)** and western blot **(B)** to confirm *lacI* and *KanR* incorporation into the cell genome and LacI production (38.6kDa, arrowed), respectively. **(A)** PCR used primers RDP1102 and RDP1103 or VEB2 and VEB3 to confirm the presence of the *KanR* and *lacI* genes, respectively. pIE1K-LacI+ and pIEK1-1neo were also screened with the appropriate primers as positive controls whilst dH₂O samples were included as negative controls. **(B)** Western blot targeting LacI (38.6kDa, arrowed). As experimental controls, some cells were transfected with pIE1K-LacI+ or mock-transfected (no DNA) and harvested at 72 h.p.i/t. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder shown left.

wells with a single cell were permitted to grow into colonies and then to confluency and screened by PCR to confirm the presence of the *KanR* and *lacI* genes (Figure 5.7). Results found cell colonies 1 and 3 to contain both the *KanR* and *lacI* genes, whilst colony 2 lacked the *lacI* gene and was therefore not maintained.

Single cell-derived colonies 1 and 3 were then scaled-up to establish clonal cell lines expressing *lacI*. However, this proved to be difficult as cell colonies were unable to withstand transfer from the 96- to a 48-well plate. Multiple attempts resulted in cell death. Consequently, to remove the need to cultivate a clonal cell line from a single cell, a new cell line was produced by transfecting cells with a dual expression plasmid containing both the *KanR* and *lacI* genes (section 5.2.7).

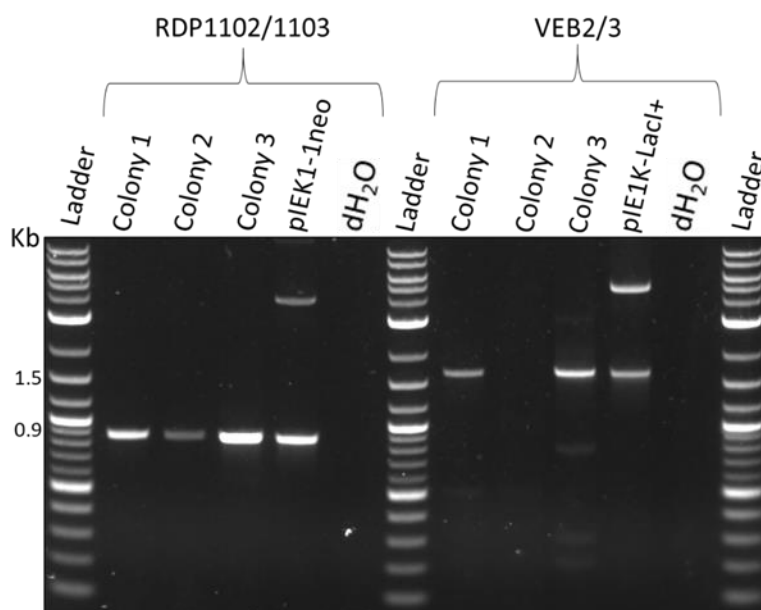


Figure 5.7- PCR screen of clonal Sf9 cell colonies expressing *lacI*.

Single cells were derived from the polyclonal Sf9 cell line expressing *lacI* following co-transfection with pIEK1-LacI+ and pIEK1-1neo. Three single cell colonies were grown to confluency and screened by PCR with RDP1102 and RDP1103 or VEB2 and VEB3 to confirm the presence of *KanR* and *lacI*, respectively. pIEK1-LacI+ and pIEK1-1neo were also screened with the appropriate primers as positive controls, whilst dH₂O samples were included as negative controls.

5.2.7 Transient expression of *lacI* from pOpIE1-neo-LacI

To eliminate the need to expand a clonal cell line from a single cell, a dual expression plasmid was made containing both the *KanR* and *lacI* genes under the control of the *Ac-ie1* and *Op-ie2* promoters, respectively (pOpIE1-neo-LacI, Figure 5.8). This would ensure the simultaneous uptake of both genes together, so any cells resistant to G418 would also express *lacI*.

Initially, it was confirmed that the LacI protein could be transiently produced within Sf9 cells from pOpIE1-neo-LacI. Sf9 cells were transfected with pOpIE1-neo-LacI, or infected with AcLacI (MOI 3) or mock-transfected as positive and negative controls for LacI production. pOpIE1-neo-LacI transfected cells were harvested at 48, 72 and 96 h.p.t. and LacI production assessed by western blot (Figure 5.9). Results confirmed that LacI could be transiently produced within Sf9 cells when under the control of the *Op-ie2* promoter, with levels of synthesis appearing stable over a short period of time.

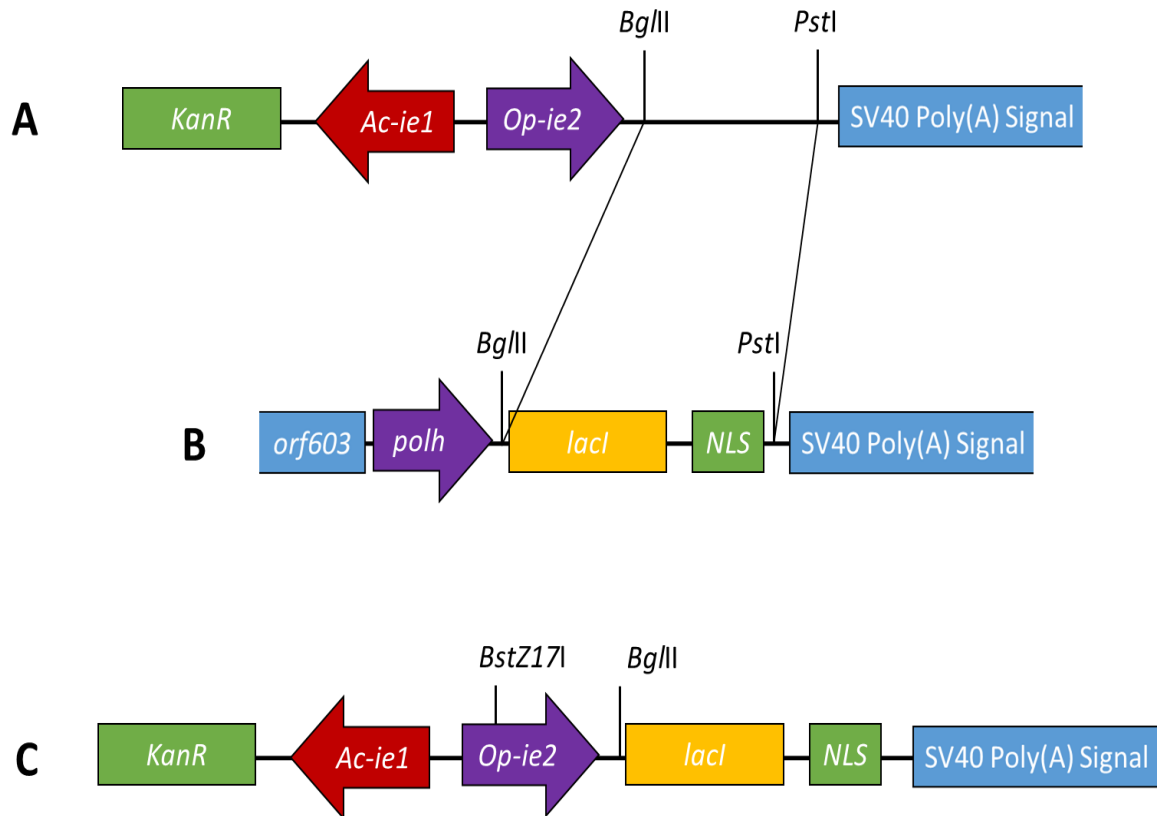


Figure 5.8- Generation of OpIE1-neo-LacI.

lacI was excised from pOET2-LacI using *Bgl*II and *Pst*I (**B**) and cloned into pOpIE1-neo digested with *Bgl*II and *Pst*I and desphosphorylated (**A**). Following ligation, the product was transformed into DH5α *E. coli* competent cells to derive pOpIE1-neo-LacI (**C**), which was confirmed by digestion with *Bst*Z17I and *Nhe*I followed by DNA sequencing.

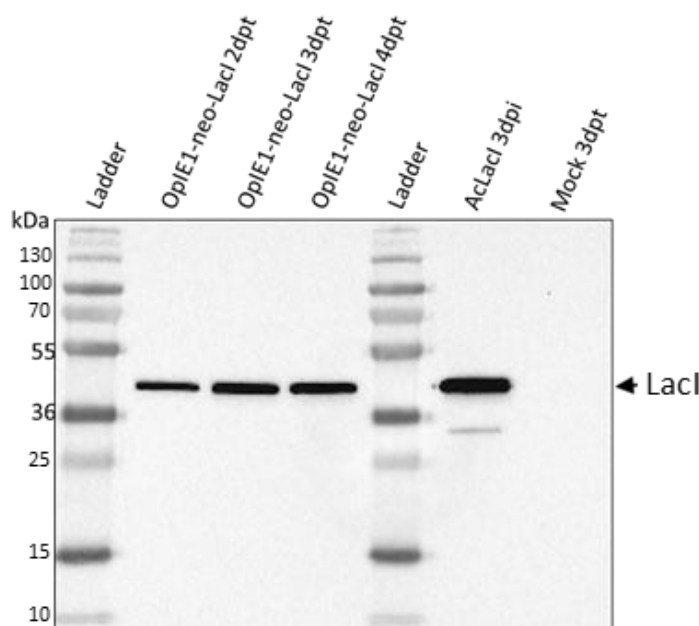


Figure 5.9- Transient expression of *lacI* in Sf9 cells from pOpIE1-neo-LacI. Western blot targeting LacI (38.6 kDa, arrowed) in Sf9 cells transfected with 2 μg pOpIE1-neo-LacI and harvested at 48, 72, or 96 h.p.t. Control cells were infected with AcLacI (MOI 3) or mock-transfected and harvested 72 h.p.i/t. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder, shown left.

5.2.8 Production of the SfLacI stable cell line

Having established that *lacI* could be transiently expressed in Sf9 cells from pOpIE1-neo-LacI, it was then used in the production of a stable cell line constitutively producing LacI (SfLacI cells). Sf9 cells were transfected with 500ng pOpIE1-neo-LacI. Control cells were transfected with either pIE1K-LacI+ (Figure 5.4A) or pIEK1-1neo (Figure 5.5) for individual *lacI* or *KanR* expression, respectively, or mock transfected. Having previously established the optimum G418 concentration for use on Sf9 cells as 0.4mg/mL (5.2.5), the transfections were carried out at 0.6, 0.4 or 0.2mg/mL. After five days, all non-resistant cells in the mock- and pIE1K-LacI+ transfected control wells grown at 0.6mg/mL G418 had been killed, and resistant colonies of cells in the pOpIE1-neo-LacI transfected wells under the same selection pressure grew steadily.

After two weeks, cells in the pOpIE1-neo-LacI transfected wells reached confluency and two wells of cells (0.6mg/mL G418) were transferred into a 6-well plate. Following further growth, the cells continued to be scaled up to 25mL suspension flasks. During scale-up, cells were screened by PCR to confirm the integration of the *KanR* and *lacI* genes (Figure 5.10A). The production of LacI from the cells was also confirmed by western blot (Figure 5.10B).

5.2.9 Stability of *lacI* expression

Two SfLacI cell cultures were monitored over time to assess the stability of the cell line and the continued production of LacI. These were analysed by western blot at passage 20 (Figure 5.11A) and again at passage 40 (Figure 5.11B). Results showed clear continued production of LacI in both cell cultures at passage 20. However, by passage 40 only cell culture 2 was still producing LacI. The cell cultures were frozen for long term storage in liquid nitrogen and were not maintained past passage 40. To maintain *lacI* expression, future SfLacI cell cultures were grown under G418 selection pressure until passage 12 and then reapplied every 10 passages.

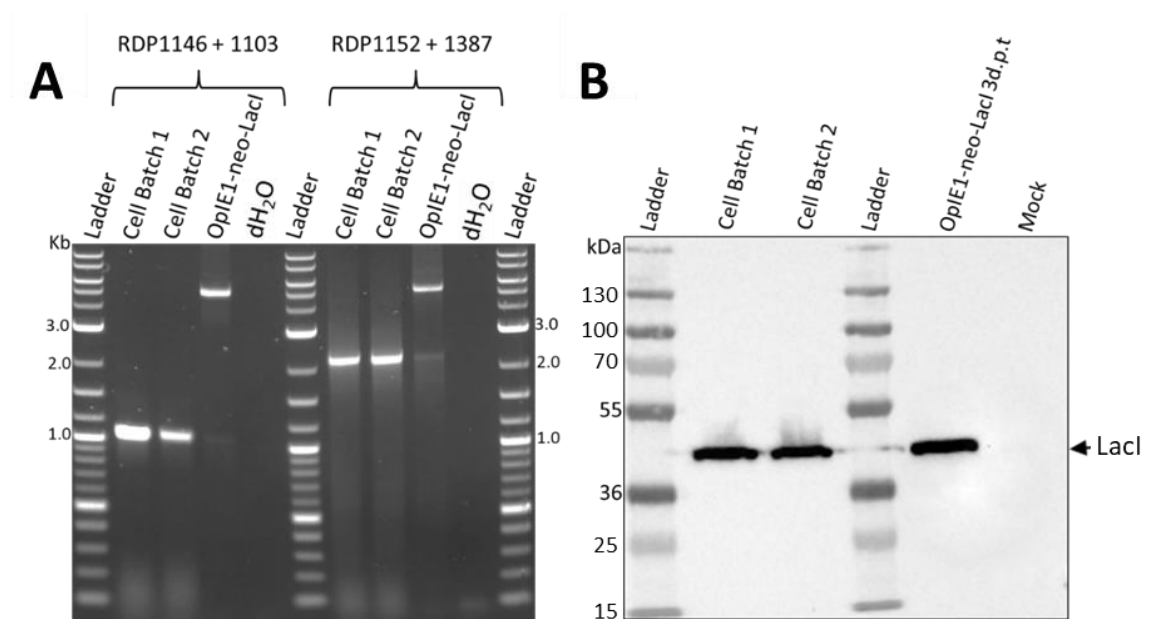


Figure 5.10- Analysis of two cultures of pOpIE1-neo-LacI transfected Sf9 cells.

Sf9 cells were transfected with 500ng pOpIE1-neo-LacI and grown under G418 selection pressure (0.6mg/mL). Cells were scaled up from 12-well plates into 25mL suspension flasks. During scale up, two cultures of cells were screened by PCR (**A**) and western blot (**B**) to confirm *lacI* and *KanR* incorporation into the cell genome and LacI production (38.6kDa, arrowed), respectively. (**A**) PCR was performed with RDP1146 and RDP1103 or RDP1152 and RDP1387 to confirm the presence of the *KanR* and *lacI* genes, respectively. pOpIE1-neo-LacI was also screened with the appropriate primers as a positive control whilst dH₂O samples were included as negative controls. (**B**) Western blot targeting LacI (38.6kDa, arrowed). Control cells were transfected with pOpIE1-neo-LacI or mock-transfected and harvested 3 d.p.t. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder shown left.

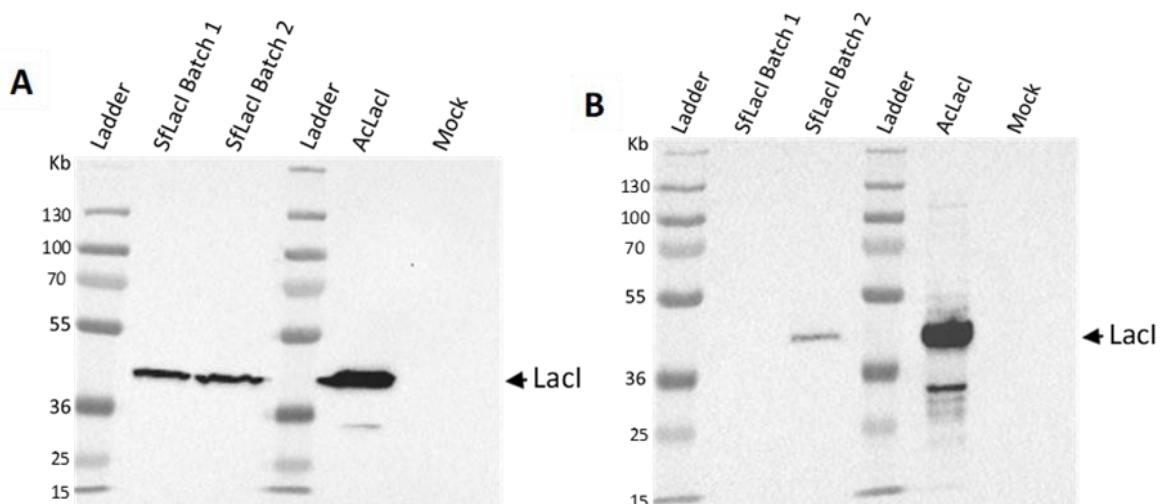


Figure 5.11- Stability of the SfLacI cell line over time.

The production of LacI (38.6kDa, arrowed) in two cultures of SfLacI cells was assessed by western blot at passage 20 (**A**) and passage 40 (**B**). Control Sf9 cells were infected with AclacI or mock-infected and harvested 2 d.p.i. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder shown left.

5.3 Discussion

Fuerst *et al.* (1989) demonstrated the potential for elements of the *lac* operon to be used to regulate gene expression in vaccinia virus. However, this was achieved in mammalian CV-1 cells where viral replication took place in the cytoplasm. Therefore, the work of this chapter aimed to assess whether a similar LacI/O repressor system could be established and implemented within Sf9 insect cells to repress gene expression from AcMNPV when replication occurs in the nuclei of cells. Results established that LacI can be produced within Sf9 cells when under the strong *polh* promoter. Versions with and without a nuclear localisation signal appeared to localise to the nuclei of cells although this signal did appear to significantly enhance transport to these organelles and was therefore used in subsequent plasmid constructs.

It was established that *lacI* can be transiently expressed within Sf9 cells when under the *Ac-ie1* and *Op-ie2* promoters. Synthesis of LacI was confirmed by western blot in Sf9 cells transfected with either the pIE1K-LacI+ or pOpIE1-neo-LacI plasmids. As immediate early (ie) gene promoters, the *Ac-ie1* and *Op-ie2* promoters were selected to drive *lacI* expression. Along with the *Bombyx mori* (*Bm*) MNPV (reviewed by Huybrechts *et al.*, 1997), the AcMNPV (Jarvis *et al.*, 1996) and *Orgyia pseudotsugata* (*Op*) MNPV (Theilmann and Stewart, 1992) immediate early promoters are the most frequently selected promoters to drive heterologous gene expression as they provide high transcriptional rates in most lepidopteran and dipteran cell lines (Pfeifer *et al.*, 1997). Moreover, the immediate early promoters bind and transcribe their associated genes from the host cell RNA polymerase II allowing for immediate expression in uninfected Sf9 cells. Further to this, *ie1* and *ie2* are transcribed throughout infection unlike other AcMNPV genes which are temporarily expressed and then shut off in the later stages of infection (Kovacs *et al.*, 1991; reviewed by Rohrman, 2019). The *Ac-ie1* and *Op-ie2* promoters would therefore allow for continued *lacI* expression following infection of SfLacI cells in the later stages of this project.

A common strategy to produce a stably transformed insect cell line is the co-transfection of an expression plasmid containing the gene of interest with a second plasmid encoding an antibiotic resistance gene (Huybrechts *et al.*, 1997; Makridou *et al.*, 2003). A clonal cell line can then be established from a single cell confirmed to have integrated both genes. Therefore, for the purpose of producing a polyclonal cell line expressing *lacI*, Sf9 cells were

initially co-transfected with two plasmids; pIE1K-LacI⁺ containing *lacI* under the *Ac-*ie1** promoter and pIEK1-1neo containing the *KanR* gene which confers resistance to the antibiotic G418. However, as described above, production of a clonal cell line via scale up from a single cell was unsuccessful as cells were unable to withstand transfer from a 96- to a 48-well plate. Consequently, to remove the need to cultivate a clonal cell line from a single cell, an alternative common method to produce a stably transformed insect cell line from a single vector was used (Sequeira *et al.*, 2018; Puente-Massaguer *et al.*, 2021).

A new cell line was produced by transfecting cells with the pOpIE1-neo-LacI dual expression plasmid containing both the *KanR* and *lacI* genes. This method ensured that all resistant cells had incorporated the desired *lacI* gene. Whilst sufficient for the purposes of this research, the quality of the new stable SfLacI cell line could have further been improved by increasing its level of LacI production, as greater amounts of LacI may be more efficient at binding *lacO* and inhibiting downstream transcription of genes. As a polyclonal cell line there was variation in the amount of LacI produced from individual cells of the SfLacI cell line, with some producing high levels and others lower levels. This could have been achieved by selecting for and continuing growth from only the cells producing high amounts of LacI. For example, Wu *et al.* (2019) produced a plasmid containing their desired gene along with a BSD antibiotic resistance gene fused with an *egfp* reporter gene. Cells with more integrated copies of the *egfp* gene (and therefore more integrated copies of their desired gene) would yield greater EGFP fluorescence. Wu *et al.* (2019) then carried out fluorescence-activated cell sorting (FACS) to isolate cells having green fluorescence intensity that fell within the top 10% of all fluorescence-positive cells. This allowed them to isolate the cells that had the highest copy number of the desired gene and to therefore establish a high-yield stable cell line. Sorting the SfLacI cells described in this work in a similar manner could have allowed for production of a high-yield cell line with less variation within the cell population. Indeed, cells with more integrated copies of the *lacI* gene and greater production of LacI may be more efficient at binding *lacO* and inhibiting downstream transcription of genes upon infection.

Finally, previous research suggested that the continued stability of transformed cell lines can decline after many passages. Hegedus *et al.* (1998) showed p97 and β -galactosidase production to be stable from their transfected Sf9 cell lines until passage 12. However, a

significant decline in β -galactosidase production was seen after 20 passages. Therefore, the stability of the SfLacI cell line was monitored over approximately 5 months until passage 40 to assess its stability and the continued production of LacI. Two cultures of SfLacI cells were grown with culture 2 maintained under constant G418 selection pressure until passage 18. By passage 20 both SfLacI cultures were still producing LacI and in similar quantities (Figure 11A). However, by passage 40, only culture 2 was still producing LacI (Figure 11B). Consequently, to ensure maintained LacI production, future SfLacI cell cultures were all grown under G418 selection pressure until passage 12. The G418 selection pressure was then reapplied after every 10 passages for 2 consecutive passages.

5.4 Conclusion

Overall, the results of this chapter have established that the LacI protein can be produced within Sf9 cells from the strong *polh* promoter. Versions with and without a Nuclear Localisation Signal appeared to localise to the nuclei of cells although this localisation signal did appear to significantly enhance transport to these organelles. It was further established that *lacI* could be transiently expressed within Sf9 cells from the *Ac-ie1* and *Op-ie2* promoters. Finally, a stable cell line constitutively producing LacI (SfLacI) was established with LacI production stability observed until at least passage 40. Next, the work of Chapter 6 presents the level of gene inhibition when infecting the SfLacI cells with recombinant viruses containing the *chiA/lacO* promoter constructs established previously in Chapter 4.

Chapter 6:

Inhibition of gene expression using the LacI/O Repressor System

6.1 Introduction

The main aim of this thesis was to produce a repressible expression system that can block the transcription of key genes essential for BV synthesis (identified in section 3.2.4), thereby inhibiting or significantly reducing BV production. In Chapters 4 and 5, the SfLacI cell line constitutively expressing *lacI* was produced, and promoter constructs containing *lacO* at different positions were designed and their integrity in the absence of LacI confirmed. Subsequently, the work of this chapter explored the inhibition of gene expression using these elements.

Firstly, repression of gene expression from the single and dual *chiA/lacO* promoters in the presence of LacI was assessed. The level of gene repression with regards to *lacO* position within the promoter was explored and compared to the corresponding level of promoter activity observed in the absence of LacI (Chapter 4). The aim was to identify the promoter construct with the greatest potential for gene inhibition in the presence of LacI, whilst retaining ample levels of expression in its absence. Along with the SfLacI cell line, these two elements formed the basis of a functional LacI/O repression system in insect cells (termed the LacI/O Repressor System).

Next, abrogation of BV production by the LacI/O Repressor System when repressing transcription of one of the key genes identified in section 3.2.4 was assessed. Of the five key genes identified (*gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*), *gp64* was selected as the first to undergo inhibition as substantial research has emphasised the essential role GP64 plays in BV infectivity and release, with previous reports by others showing its inhibition to block BV production (Blissard and Rohrmann, 1989; Monsma *et al.*, 1996; Oomens and Blissard, 1999; Valdes *et al.*, 2003; Washburn *et al.*, 2003; Lee *et al.*, 2015). Consequently, inhibition of *gp64* expression was likely to result in a substantial reduction of BV production. Finally, the production of normal levels of BV from the LacI/O Repressor System in the absence of LacI was assessed to test the feasibility of amplification and maintenance of viral stocks.

6.2 Results

6.2.1 Inhibition of gene expression by the *chiA/lacO* promoters

To determine if the *chiA/lacO* single expression promoters described in section 4.2.3 (LacO79, LacO81, LacO77 and LacO83) could inhibit downstream gene expression, Sf9 and SfLacI cells were infected with AcChiA-EGFP, AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP or AcLacO83-EGFP (all at MOI 5). AcPolh-EGFP, AcMNPV and mock-infections were also carried out as positive and negative controls for EGFP production. At 48 h.p.i., flow cytometry analysis (Figure 6.1) showed a reduction in EGFP fluorescence intensity from all the promoter constructs when infecting SfLacI cells compared to Sf9 (42.8%, 53.5%, 59.9% and 44.2% reduction from the LacO77, LacO79, LacO81 and LacO83 promoters, respectively). Statistical analysis found the reduction from the LacO77 promoter was not significant. Due to large sample variance, the reduction from the LacO81 promoter was also not significant. However, reduction in EGFP fluorescence intensity was significant at $p < 0.05$ and $p < 0.01$ from the LacO79 and LacO83 promoters, respectively. Further analysis found no significant difference between the reductions in EGFP fluorescence intensity from the LacO79 and LacO83 promoters or the LacO79 and LacO81 promoters, suggesting the addition of a second *lacO* sequence before or after the TAAG motif did not significantly affect levels of gene inhibition.

EGFP production at 48 h.p.i. was further assessed by western blotting (Figure 6.2). These results supported the flow cytometry data, showing a reduction in EGFP production from each of the *chiA/lacO* promoters in the presence of LacI. Western blot analysis at 48 h.p.i. also confirmed continued production of LacI in the SfLacI cells following infection (Figure 6.3).

6.2.2 Inhibition of gene expression by the dual *chiA/lacO* promoter

Previous data (section 3.2.4.2) showed inhibition of two key genes rather than just one to significantly increase reductions in BV production. To determine if the *chiA/lacO* dual expression promoter (LacO85) described in section 4.2.7 was capable of inhibiting downstream expression of two different genes, Sf9 and SfLacI cells were infected with AcLacO85-EGFP/mCherry or AcChiA_TAAG-EGFP/mCherry as a comparative control (MOI 5). AcpOET5-EGFP/mCherry, AcMNPV and mock-infections were also carried out as positive

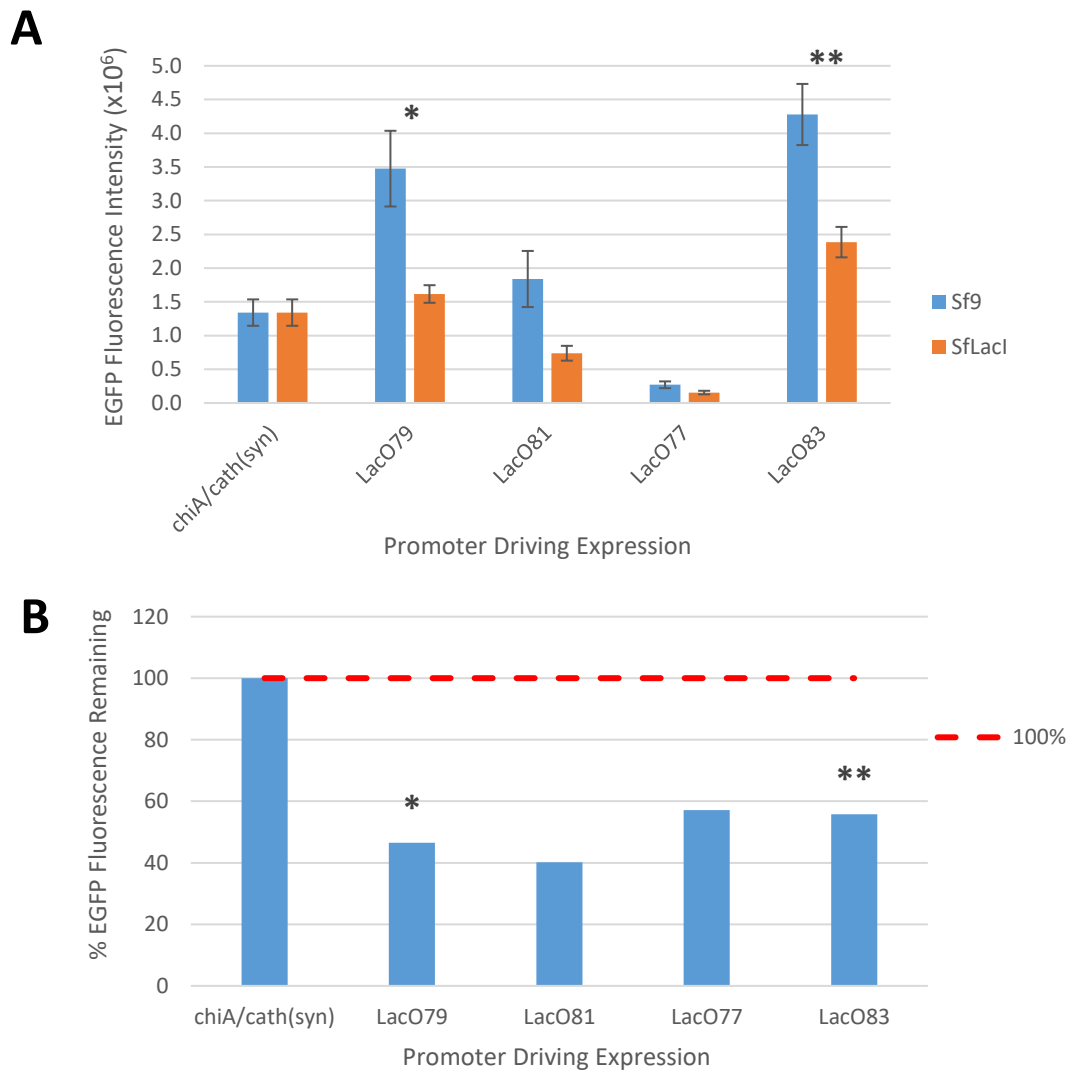


Figure 6.1- EGFP production from the *chiA/lacO* promoters in the presence of LacI.

Sf9 and SfLacI cells were infected with AcChiA-EGFP, AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP, AcLacO83-EGF, AcPolh-EGFP, AcMNPV (all at MOI 5), or were mock-infected. At 48 h.p.i., EGFP fluorescence intensity was analysed by flow cytometry. Data shown for each promoter represents the average of five experimental replicates. AcMNPV-infected cells defined the negative/positive threshold for EGFP fluorescence, from which the fluorescence intensity of the other infected cell samples was calculated. Significant reduction in EGFP fluorescence intensity is indicated (*= $p < 0.05$, **= $p < 0.01$). (A) Average EGFP fluorescence intensity 48h.p.i. Error bars indicate the standard error of the mean (SEM). (B) Average percentage of EGFP fluorescence remaining when infecting SfLacI cells compared to that of the corresponding Sf9 cell infections.

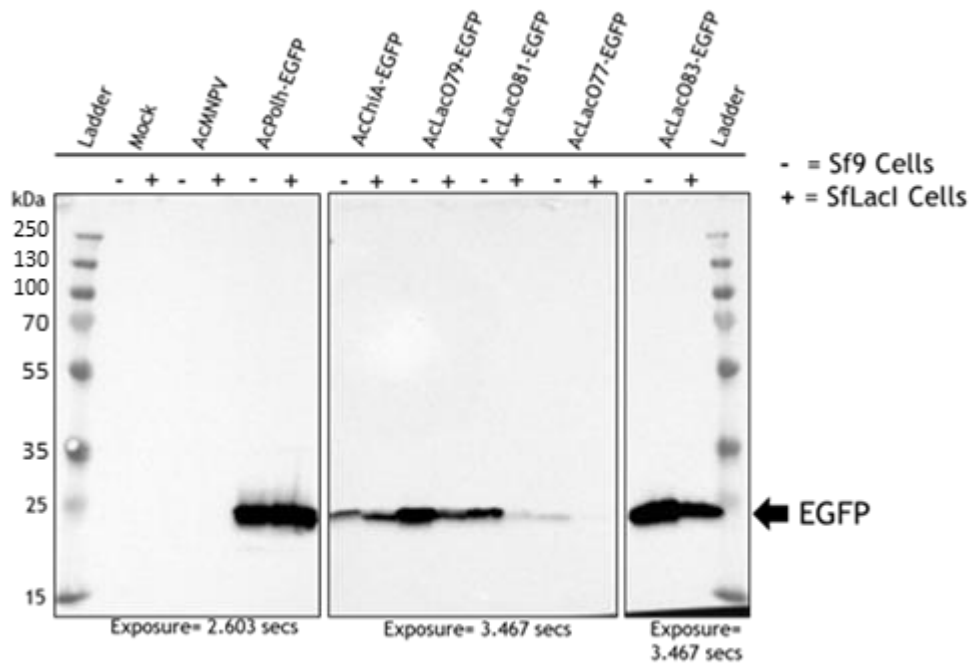


Figure 6.2- Western blot analysis of EGFP production from the *chiA/lacO* promoters in the presence of LacI. Sf9 and SfLacI cells were infected with AcChiA-EGFP, AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP, AcLacO83-EGFP, AcPolh-EGFP, AcMNPV (all at MOI 5) or were mock-infected. At 48 h.p.i., EGFP (24.6kDa, arrowed) production was assessed. Mouse monoclonal to EGFP (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder shown left.

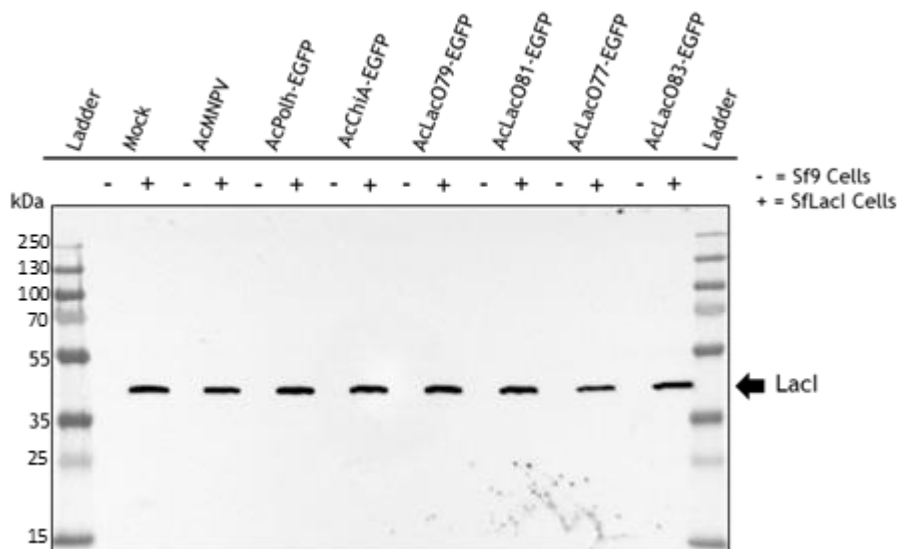


Figure 6.3- LacI production from Sf9 and SfLacI cells infected with the *chiA/lacO* promoter viruses. Sf9 and SfLacI cells were infected with AcChiA-EGFP, AcLacO77-EGFP, AcLacO79-EGFP, AcLacO81-EGFP, AcLacO83-EGFP, AcPolh-EGFP, AcMNPV (all at MOI 5) or were mock-infected. At 48 h.p.i., LacI production (38.6kDa, arrowed) was assessed to confirm absence in the Sf9 cells but continued production from the SfLacI cells during infection. Mouse monoclonal to LacI (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder, shown left.

and negative controls for EGFP and mCherry production. At 48 h.p.i., plate reader analyses (Figure 6.4) showed a reduction in both EGFP and mCherry fluorescence intensity when infecting SfLacI cells compared to Sf9. EGFP fluorescence was significantly reduced ($p < 0.005$) by 66.3% and mCherry fluorescence was significantly reduced ($p < 0.05$) by 43.9%.

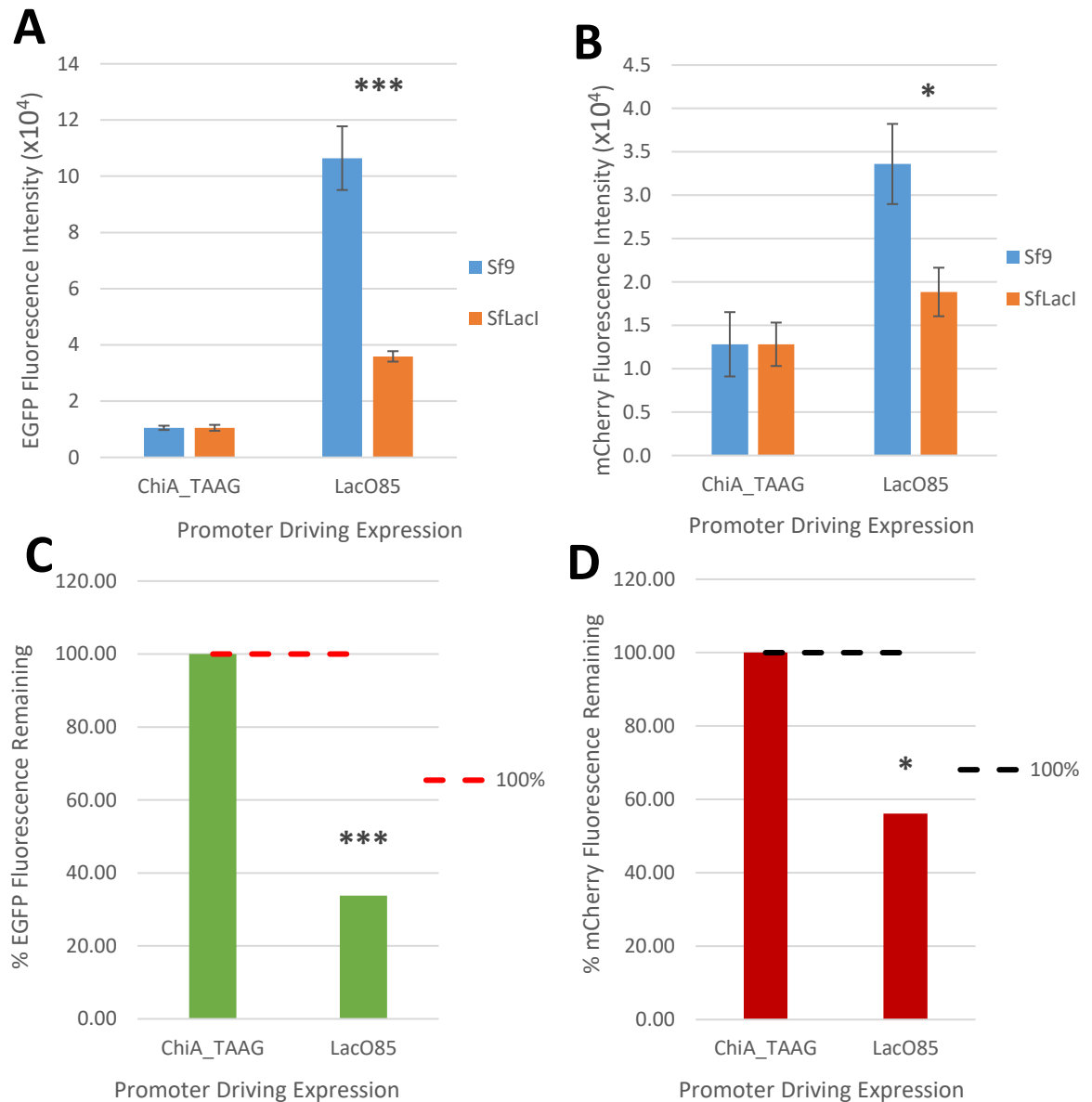


Figure 6.4- EGFP and mCherry production from the dual expression LacO85 promoter in the presence of LacI. Sf9 and SfLacI cells were infected with AcChiA_TAAG-EGFP/mCherry, AcLacO85-EGFP/mCherry, AcPOET5-EGFP/mCherry, AcMNPV (all at MOI 5) or were mock-infected. At 48 h.p.i., the EGFP and mCherry fluorescence intensity was analysed using a plate reader. Data shown represents the average of three experimental replicates. AcMNPV-infected cells defined the negative/positive threshold for EGFP or mCherry fluorescence, from which the fluorescence intensity of the other infected cell samples was calculated. Significant reductions in EGFP or mCherry fluorescence intensity are indicated (*= $p < 0.05$, ***= $p < 0.001$). **(A)** Average EGFP or **(B)** mCherry fluorescence intensity 48 h.p.i. Error bars indicate the standard error of the mean (SEM). **(C)** Average percentage of EGFP or **(D)** mCherry fluorescence remaining when infecting SfLacI cells compared to that of the corresponding Sf9 cell infections.

The EGFP and mCherry production from the cells was further assessed by western blotting (Figure 6.5). These results supported the absorbance measurements showing a reduction in both EGFP and mCherry production from the LacO85 promoter when infecting SfLacI cells compared to Sf9.

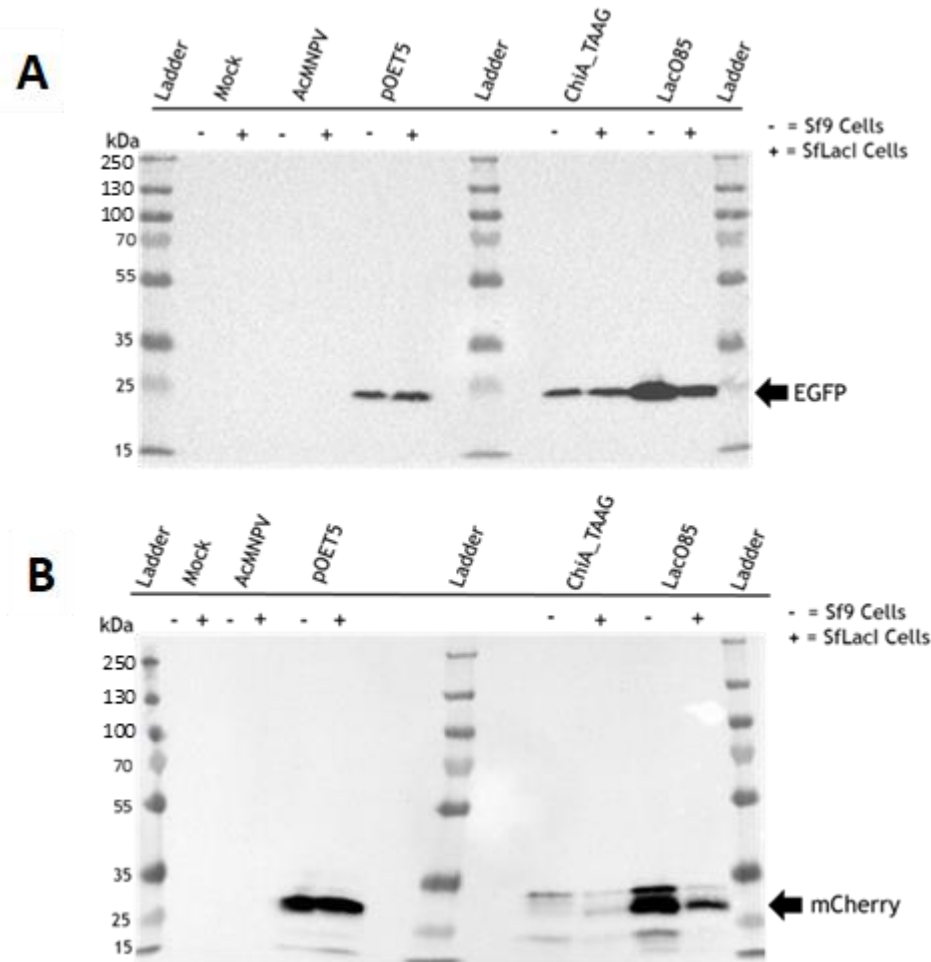


Figure 6.5- Western blot analysis of EGFP and mCherry production from the dual expression LacO85 promoter when infecting SfLacI or Sf9 cells. Sf9 and SfLacI cells were infected with AcChiA_TAAG-EGFP/mCherry, AcLacO85-EGFP/mCherry, AcPOET5-EGFP/mCherry, AcMNPV (all at MOI 5) or were mock-infected. At 48 h.p.i., **(A)** EGFP (24.6kDa, arrowed) and **(B)** mCherry (26kDa, arrowed) production was analysed. **(A)** Mouse monoclonal to EGFP (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. **(B)** Mouse monoclonal to mCherry (1:1000) and anti-mouse HRP were used as primary and secondary antibodies, respectively. **(A + B)** Molecular mass ladder shown left.

6.2.3 Budded virus production from the LacI/O Repressor System

The results of section 6.2.1 identified the LacO79 promoter construct as the single expression promoter with the greatest potential for downstream gene inhibition when infecting SfLacI cells. These two elements would therefore form the basis of the LacI/O

Repressor System. This section assessed the abrogation of BV by the LacI/O Repressor System when blocking transcription of key genes previously identified in section 3.2.4. Of the five key genes identified (*gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*), *gp64* was selected as the first to undergo inhibition by the LacI/O Repressor System as described above (6.1).

6.2.3.1 Inhibition of *gp64* using the LacI/O Repressor System

Firstly, it was confirmed that the LacI/O Repressor System could inhibit expression of *gp64*. Sf9 and SfLacI cells were infected with AcLacO79-*gp64* (described in section 4.2.5.2) at MOI 5. AcChiA-*gp64*, AcMNPV and mock-infections were also carried out as positive and negative controls for *gp64* expression. GP64 production was assessed at 72 h.p.i. by western blotting. Results (Figure 6.6) showed clear reduction in GP64 production when SfLacI cells were infected with AcLacO79-*gp64*, compared to Sf9, confirming the ability of the LacI/O Repressor System to downregulate *gp64* expression. However, even though reduced in comparison to the Sf9 infected cells, GP64 produced from the AcLacO79-*gp64* infected SfLacI cells seemed comparable to that from the AcChiA-*gp64* infected SfLacI cells. This suggested that the reduction in *gp64* expression may not be enough to reduce BV production. Western blot analysis for LacI was also carried out for each infected cell sample at 72 h.p.i. and confirmed continued production in the SfLacI cells throughout infection (data not shown).

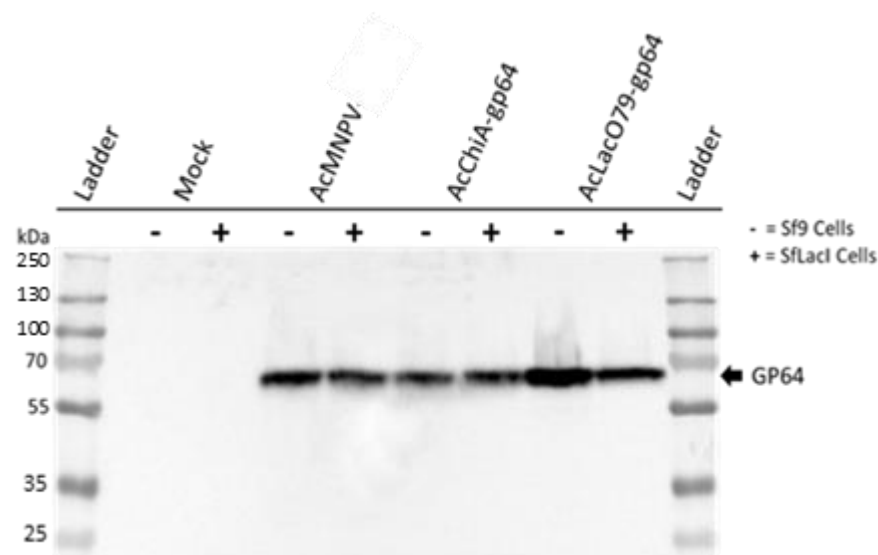


Figure 6.6- Western blot analysis of GP64 production from the LacI/O Repressor System.

Sf9 and SfLacI cells were infected with AcLacO79-*gp64*, AcChiA-*gp64*, AcMNPV (all at MOI 5) or were mock-infected. GP64 production (64kDa, arrowed) was analysed 72 h.p.i. to assess the reduction seen from the LacI/O Repressor System in the presence of LacI. Mouse monoclonal to GP64 (1:2800) and anti-mouse HRP were used as primary and secondary antibodies, respectively. Molecular mass ladder shown left.

6.2.3.2 Budded virus production when inhibiting the “key gene” *gp64* using the LacI/O Repressor System

Following confirmation that the LacI/O Repressor System could reduce expression of *gp64*, it was assessed whether this level of gene inhibition was sufficient to reduce BV production and by how much. SfLacI cells were infected with AcLacO79-*gp64* (MOI 5). AcChiA-*gp64*, AcMNPV and mock-infections were carried out as comparative, positive and negative controls for BV production, respectively. At 72 h.p.i., BV titre was determined by plaque assay. Results (Figure 6.7) showed a significant ($p < 0.05$) reduction in BV production, with titres reduced by 50% when *gp64* expression was downregulated using the LacI/O Repressor System.

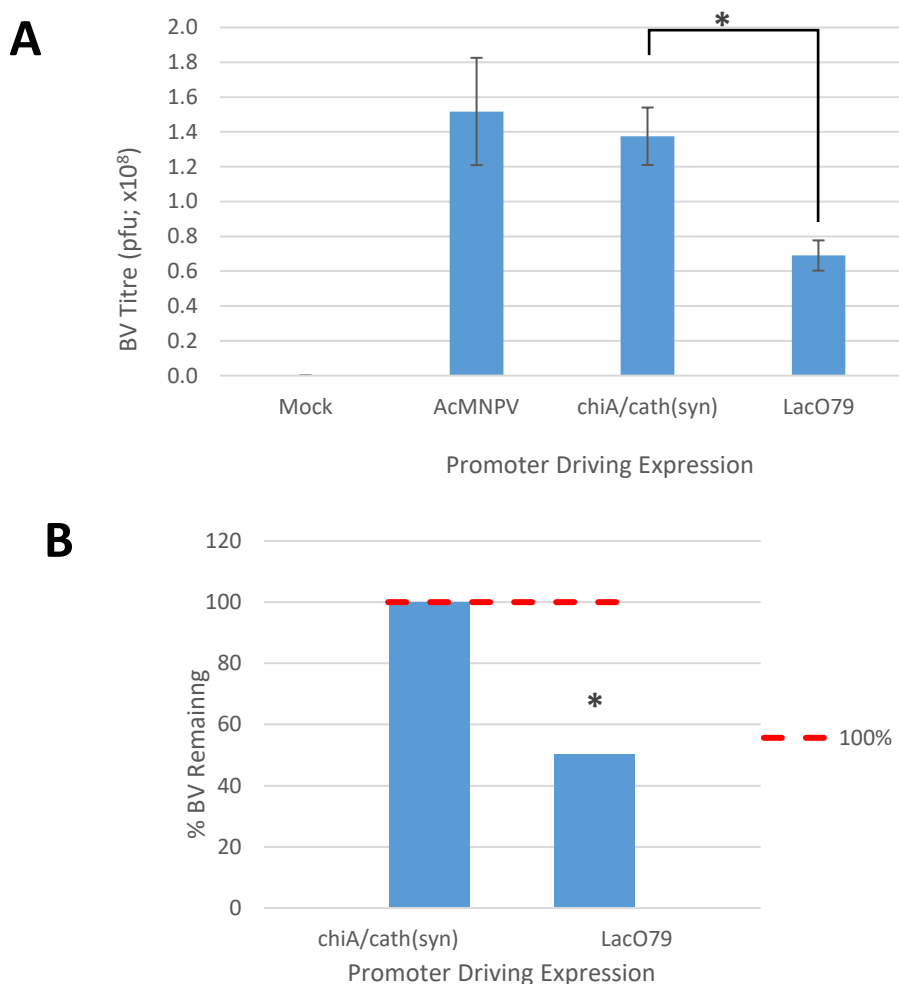


Figure 6.7- BV production when using the LacI/O Repressor System to inhibit *gp64* expression. SfLacI cells were infected with AcLacO79-*gp64*, AcChiA-*gp64*, AcMNPV (all at MOI 5), or were mock-infected. At 72 h.p.i., BV titre was determined by plaque assay. Data shown represents the average of four experimental replicates. Significant reductions in BV titre are indicated ($*=p < 0.05$). **(A)** Average BV titre 72h.p.i. Error bars indicate the standard error of the mean (SEM). **(B)** The average percentage of BV remaining when *gp64* expression was repressed from LacO79 promoter compared to when *gp64* expression was driven by the *chiA/cath*^(syn) promoter.

6.2.3.3 Restoration of budded virus production when infecting Sf9 cells

Whilst the inhibition of BV production during VLP production is the main aim of the LacI/O Repressor System, another essential feature is its ability to allow for normal levels of BV production in the absence of LacI. Therefore, when infecting an alternative to the SfLacI cell line (i.e. Sf9 cells), normal levels of BV production could be resumed for amplification of viral stocks. To confirm this was possible, Sf9 cells were infected with AcLacO79-gp64 (MOI 5). AcChiA-gp64, AcMNPV and mock-infections were carried out as comparative, positive and negative controls for BV production, respectively. At 72 h.p.i., BV titre was determined by plaque assay (Figure 6.8) which showed no significant ($p>0.05$) change in BV titre when infecting Sf9 cells with AcLacO79-gp64 or AcChiA-gp64. Results actually showed a slight increase in BV production when infecting with AcLacO79-gp64.

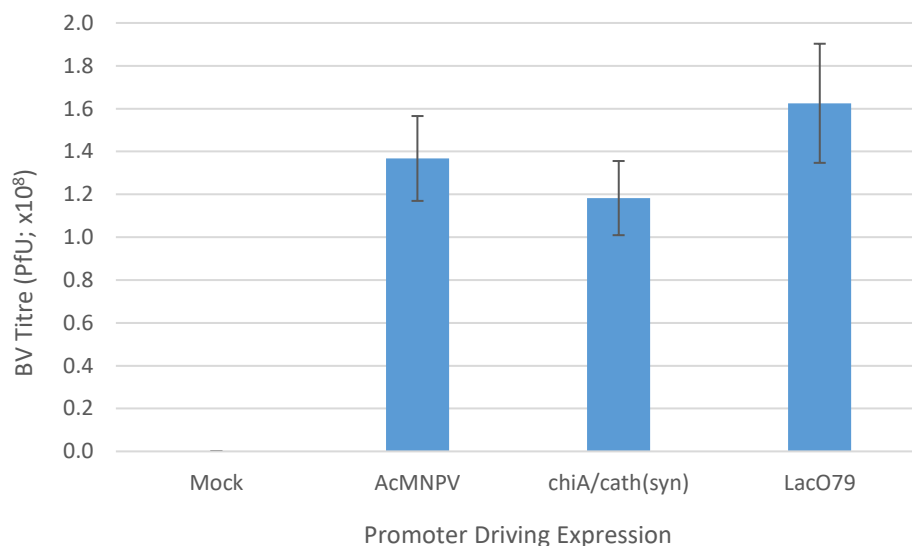


Figure 6.8- BV production when using the LacI/O Repressor System to drive *gp64* expression in Sf9 cells. Sf9 cells were infected with AcLacO79-gp64, AcChiA-gp64, AcMNPV (all at MOI 5) or were mock-infected. At 72 h.p.i., BV titre was determined by plaque assay. Data shown represents the average of four experimental replicates and error bars indicate the standard error of the mean (SEM). Statistical analysis found no significant difference between BV titres of each sample.

6.3 Discussion

The work of this chapter focused on the inhibition of gene expression using an SfLacI cell line (Chapter 5) and *chiA/lacO* promoter constructs (Chapter 4). Initially, the single expression promoter construct with the greatest potential for gene inhibition was

identified, so that along with the SfLacI cell line these elements would form the basis of the LacI/O Repressor System. Firstly, flow cytometry analysis found all the single expression *chiA/lacO* promoter constructs (LacO79, LacO81, LacO77 and LacO83) were capable of downregulating *egfp* expression upon infection of SfLacI cells. It was therefore concluded that insertion of the *lacO* into the *chiA/cath^(syn)* promoter allowed for inhibition of downstream gene expression regardless of its insertion site. However, it was only following insertion of *lacO* after the conserved TAAG motif that significant levels ($p < 0.05$) of gene inhibition were observed (e.g., EGFP fluorescence intensity was significantly reduced from the LacO79 ($p < 0.05$) and LacO83 ($p < 0.01$) promoters). Whilst there was still a reduction in gene expression when the *lacO* sequence was inserted prior to the TAAG motif (42.8% and 59.9% reduction from the LacO77 and LacO81 promoters, respectively), these levels were not found to be significant.

These results seem to support and follow the data and model of inhibition proposed by Lanzer and Bujard (1988), who also observed greater repression of gene expression when *lacO* was inserted downstream of the conserved site of transcription initiation compared to when inserted upstream. They found that RNA polymerase (RNAP) and LacI compete for promoter binding, and upon binding, RNAP covers 70bp (-50 to +20 of the conserved transcription initiation site). Through substantial analysis, Lanzer and Bujard (1988) concluded that following initiation of transcription, an upstream *lacO* is uncovered and LacI can bind before a second RNAP can bind to the promoter (as the transcribing RNAP is still covering large portions of the promoter further downstream). After promoter clearance, RNAP and LacI compete for their binding sites in promoter constructs containing a downstream *lacO*, and distribute according to their rate of complex formation/binding rate and the concentration of free proteins. Furthermore, their results suggest that RNAP can still bind to the promoter when LacI is bound to *lacO* at either upstream or downstream positions. However, LacI bound at a downstream position efficiently prevents RNAP initiating transcription whereas when bound at an upstream position RNAP was still capable of initiating transcription, though at low efficiency. This would explain the results of section 6.2.1 in which significant levels of repression were observed from the LacO79 and LacO83 constructs (*lacO* inserted after the TAAG motif), whilst lesser and insignificant levels of repression were observed from the LacO77 construct (*lacO* inserted before the TAAG motif).

When *lacO* was inserted downstream of the conserved TAAG motif, gene expression was reduced by 53.5%. Previous research employing different repressor systems including the *tet* (O'Connel *et al.*, 2020) and *lac* (Fuerst *et al.*, 1989; Zhang and Moss, 1991) systems also reported significant levels of gene repression when the relevant operator sequence was inserted downstream of the conserved site of transcription initiation. However, all these studies and specifically those employing the *lac* repressor system, achieved greater levels of reduction than those achieved in this chapter. For example, Fuerst *et al.* (1989) saw a 97% reduction in β -galactosidase activity with *lacO* inserted after the conserved TAAAT motif of the Vaccinia virus F17R (*P11*) late promoter. Zhang and Moss (1991) also reported a 95% reduction in gene transcription when *lacO* was inserted after the TAAAT motif of the same promoter. This difference in levels of reduction could, in part, be explained by the exact positioning of the *lacO* sequence after the start site of transcription. Both Fuerst *et al.* and Zhang and Moss inserted *lacO* immediately after the transcriptional start site (+1 and +3 bp, respectively). However, in the LacO79 construct, *lacO* was inserted 11bp downstream of the TAAG motif reflecting the natural positioning of *lacO* in *E. coli* (+11bp from the RNAP binding site) (Reviewed by Lewis, 2005). However, it is possible that the closer positioning of the *lacO*:LacI complex to the RNAP binding site results in a more stringent inhibition of RNAP activity.

Furthermore, both repressor systems evaluated gene repression from the strong Vaccinia virus F17R (*P11*) late promoter. It has been shown that there is a correlation between the strength of a promoter and the level of repression achieved when employing the *lac* repressor system, with stronger promoters yielding a stronger level of repression (Lanzer and Bujard, 1988). This is because strong promoters' high rate of promoter clearance lowers the occupancy of the promoter by RNAP, thus increasing opportunity for LacI binding and subsequently increasing repression. Higher levels of repression would therefore be expected from the stronger F17R (*P11*) late promoter compared to the baculovirus *chiA/cath* promoter.

It should be noted that when designing the *chiA/lacO* promoter constructs, a promoter with *lacO* overlapping the conserved TAAG motif was not designed as previous studies by others had consistently shown this to completely abolish promoter activity (Lanzer and Bujard, 1988; Fuerst *et al.*, 1989). Whilst this would indeed eliminate downstream gene

expression, it would also prevent the reinstatement of basal levels of expression in the absence of LacI, thus rendering the LacI/O Repressor System pointless for the purposes required in this study.

Interestingly, the results of the single gene inhibition experiments seem to correlate with the results obtained in Chapter 4 (section 4.2.5). The promoters that provided significant increases in activity (LacO79 and LacO83) also demonstrated significant reductions in gene expression, whereas the promoter that saw a significant decrease in activity (LacO77) also had the worst and insignificant levels of reduction in gene expression. This again seems to support the data of Lanzer and Bujard (1988) who found that when employing the *lac* repressor system, stronger promoters yielded a stronger level of gene repression. Their data showed it was the high rate of promoter clearance which, by lowering the occupancy of the promoter by RNAP and increasing opportunity for LacI binding, was responsible for the increased levels of repression rather than being due to a low rate of complex formation between RNAP and the promoter. Thus, it can be hypothesised that the insertion of *lacO* into the *chiA/cath* promoter after the TAAG motif increased promoter activity by speeding up the rate of RNAP clearance, rather than by decreasing the rate of RNAP binding. This, therefore, results in a stronger repression from the promoter due to the increased opportunity for LacI binding. More research is required to establish the exact mechanism by which insertion of the *lacO* sequence after the TAAG motif increases RNAP clearance. By analogy, it could further be concluded that the insertion of *lacO* before the TAAG motif decreased promoter activity and gene repression from the promoter by slowing down the rate of RNAP clearance.

There is much evidence that the presence of multiple operators within a regulatory system enhances levels of gene repression. For example, research into numerous bacterial regulatory systems including the *deo* (Dandanell *et al.*, 1987), the *araBAD* (Dunn *et al.*, 1984) and the *gal* operons (Fritz *et al.*, 1983; Haber and Adhya, 1988) has shown multiple operators to contribute 10 to 20-fold to full repression. Vidal-Ingigliari and Raibaud (1985) also showed that promoters containing only the first *lacO* were just 5-fold repressed by LacI compared to the >1000 fold repression achieved by the wild-type *lac* repressor system. Interestingly however, the results presented in this thesis showed that the addition of a second *lacO* sequence after the conserved TAAG motif did not significantly affect levels of

gene inhibition. Indeed, there was no significant difference between the reduction of EGFP fluorescence intensity from the LacO79 and LacO83 promoters. This again seems to correlate well with the results presented and discussed in section 4.2.5, in which the addition of a second *lacO* sequence after the TAAG motif did not significantly increase the activity of the *chiA/cath^(syn)* promoter. However, it is worth noting that whilst the addition of a second *lacO* sequence did not significantly affect the levels of gene inhibition, it did increase the level of significance of the reduction in EGFP fluorescence due to reduced levels of variation between experimental replicates. The addition of a second *lacO* sequence therefore, made gene repression more reliable, possibly by increasing the rate of repressor/operator binding, or by increasing the stability of that complex (i.e reducing repressor clearance).

It is likely that the lack of increase in repression observed following the addition of a second *lacO* after the TAAG motif in the LacO83 construct can be explained by the distance it was placed from the first *lacO*. For each of the different bacterial repressor systems described above, the additional operator sites were placed much further apart from each other (minimum distance of 114bp from centres of symmetry) than those in the LacO83 construct (0bp apart). Indeed, the quality of the O2 and O3 *lac* operators of the wild-type *lac* repressor system has been shown to correspond inversely to their position from O1. O2 binds LacI 10-fold less well than O1 and is positioned 401 bp away. O3 however, is positioned only 92 bp from O1, but its affinity for LacI is approximately another 30-fold lower than that of O2 (Oehler *et al.*, 1994). This suggests that the LacO83 construct would have yielded greater LacI affinity and thereby gene repression if the second *lacO* had been placed further away from the first.

Furthermore, in previous studies it has been shown that repression from the *lac* operon is facilitated by the formation of DNA loops, possible by the presence of multiple *lacO* sites (Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018). Kania and Muller-Hill (1977) proposed that dimeric LacI was capable of binding *lacO* and therefore, wild-type tetrameric LacI must be able to bind two *lac* operator sequences simultaneously. Moreover, *in vitro* experiments demonstrated that LacI is indeed able to bind simultaneously to two operator sequences located on one DNA fragment and binding of O1 and O2 or O1 and O3 forces the intervening DNA into a loop structure (Krämer *et al.*,

1987; Flashner and Gralla, 1988; Oehler *et al.* 1990). However, the positioning of the operators from each other is key to successful loop formation. A length of 400bp was found optimal for DNA looping (Shore *et al.*, 1981), although formation of loops has been shown possible at a wide range of *lacO* distances, from as little as 41bp to hundreds of bps apart (Krämer *et al.*, 1987; Haber and Adhya, 1988, Becker *et al.*, 2013). DNA looping is dependent on DNA torsion. DNA has natural torsional rigidity and develops a resistive torque when it is twisted. Therefore, bending smaller lengths of DNA (*lacO* sites placed close together) requires greater torsional energy than that for larger lengths of DNA (*lacO* sites placed further apart) (Reviewed by Cournac and Plumbridge, 2013; Figure 6.9). Furthermore, changing the spacing between the two *lacO* sites rotates the binding sites around the DNA helix relative to each other. Torsional energy is then also required to twist the DNA so that the operators are available to be simultaneously bound by LacI. Therefore, some spacings are better for looping induced by LacI binding and yield better repression than others (Krämer *et al.*, 1987; Becker *et al.*, 2013; Tungter *et al.*, 2018). A periodicity of ~10bp ensures the *lacO* sites are in the same phase on the DNA double helix (Shore and Baldwin, 1983) hence why, along with the need to be placed further apart to allow for easier formation of loops, their current position in the LacO83 construct also meant the two operators were not in phase with each other, further inhibiting the formation of a loop.

The other promoter construct containing multiple copies of *lacO* was the LacO81 promoter. In contrast to the LacO83 construct, which had both *lacO* sequences after the TAAG motif, the LacO81 construct had one *lacO* both before and after the TAAG motif. Of all the single expression promoters, LacO81 yielded the greatest reduction in *egfp* expression (59.9%) when infecting SfLacI cells. However, this reduction was found not to be significant due to high variation between experimental replicates. Previous results (section 4.2.5.1) found the activity of the LacO81 construct was increased in comparison to the unmodified *chiA/cath^(syn)* promoter. However, this increase was not significant or as much as that of the LacO79 and LacO83 constructs. Therefore, an increased rate in RNAP clearance is not enough to explain the level of reduction seen from this promoter. Consequently, this data hints at the formation of a DNA loop. Indeed, as discussed above multitudes of research has shown DNA looping to contribute significantly to gene repression from the *lac* repressor system (Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018) by increasing operator occupancy (Mossing and

Record, 1986). Presence of just one *lacO* represses gene expression only 20-fold, whereas presence of a second *lacO* increases repression >47-fold (Oehler *et al.*, 1990). Unlike the LacO83 promoter, which had both *lacO*'s directly next to each other thus preventing loop formation, the *lacO*'s in the LacO81 promoter were spaced 42bp apart from their centres of symmetry meaning loop formation may have been possible although unstable (Figure 6.9). Krämer *et al.* (1987) showed loop formation to be possible when operators were spaced 52bp apart and Becker *et al.* (2013) found it possible at distances no less than 41bp. However, these structures were rarer than larger loops due to the effects of DNA torsion. A 42bp loop in the LacO81 construct may therefore have been possible, which would explain the larger reduction in *egfp* expression seen. The rarity and instability of its formation due to DNA torsion would further explain the large variation of results between experimental replicates. The reduction in *egfp* expression seen from the LacO81 promoter construct therefore, suggests that the presence of multiple *lacO*'s would increase gene repression from the *chiA/cath* promoter via the formation of DNA loops. However, for significant and stable gene repression operators should be positioned further apart.

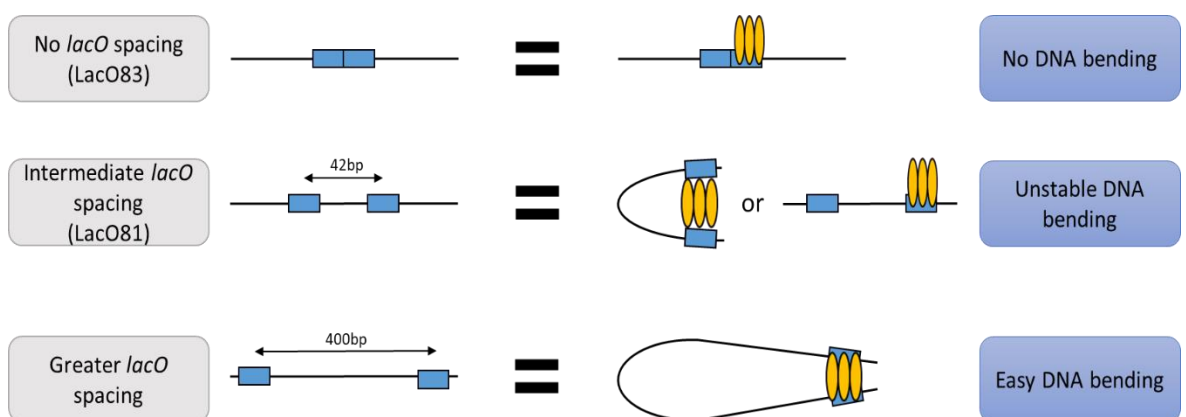


Figure 6.9- Schematic representation of how *lacO* spacing impacts DNA bending and loop formation. LacI (represented as three yellow ovals) is capable of simultaneously binding to two operator sequences (represented as a blue box) located on one DNA fragment and forcing the intervening DNA into a loop structure (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Oehler *et al.* 1990). However, the positioning of the operators from each other is key to successful loop formation, as looping is dependent on DNA torsion. DNA has natural torsional rigidity and develops a resistive torque when twisted. Consequently, bending shorter lengths of DNA (*lacO* sites placed close together e.g. the LacO81 promoter) requires greater torsional energy than that for larger lengths of DNA (*lacO* sites placed further apart). Research has suggested a spacing of 400bp to be optimal for DNA looping from two *lacO* sequences (Shore *et al.*, 1981). When *lacO* sites are placed directly next to each other, no DNA looping is possible.

As discussed previously, results in section 3.2.4.2 suggested that reduction in BV production from the LacI/O Repressor System would be significantly greater ($p < 0.05$) if two key genes were silenced rather than just one. The *chiA/cath* promoter was therefore selected for modification due to its ability to act as a bi-directional transcription unit with late promoter TAAG motifs on either strand to regulate expression of two genes. This would allow for subsequent inhibition of two of the previously identified key genes rather than just one, which should enable a greater inhibition of BV production. Results of the dual promoter inhibition experiments confirmed dual gene inhibition was possible from the modified *chiA/cath* promoter, LacO85. Both *egfp* and *mCherry* expression was significantly reduced ($p < 0.005$ and $p < 0.05$, respectively) when infecting SfLacI cells. A slightly stronger level of repression was observed for the *egfp* gene (66.3%) than the *mCherry* gene (43.9%). As with the single gene inhibition studies, these levels of gene inhibition seem to correlate well with the results presented in Chapter 4. In section 4.2.8, data showed a near 10-fold increase in EGFP production from the lacO85 promoter, whilst a lesser but still significant 3-fold increase in mCherry production was seen. As before, this data suggested that greater levels of gene expression would result in greater levels of gene inhibition when employing the *lac* repressor system, again concurring with the work of Lanzer and Bujard (1988).

Interestingly, the level of gene inhibition from the sense TAAG motif controlling *egfp* transcription was not reduced following the reinstatement of the antisense TAAG motif as may have been expected due to the decrease in the ratio of RNAP binding *versus* LacI available. Repression and its significance level was instead increased compared to that from the LacO79 or LacO81 promoters (which both had one *lacO* inserted after the sense TAAG motif). It is likely that, as with the LacO81 promoter compared to the LacO79 promoter, the level of repression was increased due to the presence of a second *lacO* sequence within the promoter allowing for the formation of a DNA loop which enhances repression from the *lac* repressor system (Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018). However, unlike the LacO81 construct, the two *lac* operators in the LacO85 promoter were spaced further apart (69bp from their centres of symmetry). This increased distance compared to the LacO81 promoter would have increased the probability and stability of a DNA loop forming due to decreased DNA tension, thus evoking more likely and consistent repression.

Although significant, the reduction in gene expression from the best single expression promoter (LacO79) and the dual expression promoter (LacO85) was not 100%. Indeed, assessment of each of the four single expression promoters has already shown the importance *lacO* positioning has on the levels of gene repression achieved. Furthermore, as discussed above, evaluation of the LacO81 and LacO85 promoter constructs has suggested that multiple copies of *lacO* within a promoter will increase levels of repression by allowing for DNA loop formation. Numerous studies have also shown that the presence of multiple *lac* operators enables DNA looping, which significantly increases gene repression from the *lac* repressor system (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018) and that the positioning of these operators affects the frequency and stability of these loops forming (Shore *et al.*, 1981; Shore and Baldwin, 1983; Krämer *et al.*, 1987; Oehler *et al.*, 1994; Becker *et al.*, 2013). Therefore, repression from the LacO79 and LacO85 promoters could be increased by inserting additional copies of the *lacO* into the promoters and by ensuring their positions are optimal for loop formation.

Previous research has suggested that increased levels of LacI increases levels of repression by increasing operator occupancy. Fuerst *et al.* (1989) showed during coinfection that a 1:1 LacI to *lacO* ratio resulted in 97% repression of B-galactosidase, whilst a 2:1 ratio resulted in >99% reduction. Oehler *et al.* (1990) also found high amounts of LacI increased *LacZ* repression from just the O1 *lac* operator by 19-fold. The same conditions led to a 20-fold increase in repression from the O3 operator alone. Therefore, increasing LacI production from the SfLacI cell line could increase the levels of repression observed from the LacO79 and LacO85 promoters. This could be achieved by carrying out fluorescence-activated cell sorting (FACS). Indeed, as previously discussed (Chapter 5), when producing a high-yield cell line, Wu *et al.* (2019) produced a plasmid containing their desired gene fused with an *egfp* reporter gene. Cells with more integrated copies of the *egfp* gene (and therefore more integrated copies of their desired gene) yielded greater EGFP fluorescence. They then used FACS to isolate cells with green fluorescence intensity that fell within the top 10% of all fluorescence-positive cells to establish a high-yield stable cell line. Modifying and sorting the SfLacI cells in a similar manner could allow for production of a high-yield cell line with less variation within the cell population enabling greater levels of repression. However, some research has shown that too much LacI favours single occupancy of multiple operator

sites *in vitro*, resulting in inhibition of DNA loop formation (Krämer *et al.*, 1987). Therefore, whilst increasing LacI might be good for a promoter construct containing just one *lacO* such as the LacO79 construct, it may decrease repression from promoters containing two or more *lacO* sequences, such as the LacO85 construct, by inhibiting the considerable levels of repression facilitated by DNA loop formation.

Much research has provided evidence that the presence of the two “pseudo operators” (O2 and O3) of the *lac* operon increase gene repression via DNA looping (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018). However, there is little evidence as to whether it is the precise combination of O2 or O3 operators with the O1 operator which enables efficient DNA looping and repression, or if multiple copies of the same operator could yield the same or increased levels of looping and gene repression. It has been suggested that the repressor binding affinities of these operators plays a key role in their ability to form loops with each other and thereby repress gene expression (Oehler *et al.*, 1990). The *in vitro* affinity of O2 for LacI has been determined to be 10-fold less than that of O1 (Pfahl *et al.*, 1979; Winter and von Hippel, 1981), and the *in vitro* affinity of O3 between 16- and 1000-fold lower than that of O1 (Pfahl *et al.*, 1979; Fried and Crothers, 1981; Winter and von Hippel, 1981). Oehler *et al.*, (1990) has shown that looping from the weakest O2 and O3 operators alone exhibits only minor repression whereas looping of O2 or O3 with the strong O1 operator increased repression by 47-48-fold. It has also been suggested that cooperative repression by weaker operators can be achieved by increasing their proximity to each other. Oehler *et al.* (1994) found a short distance of 92bp allowed for effective repression by two weak O3 operators. Altogether, this raises the question as to whether, *in vitro*, the presence of multiple copies of only the strongest O1 operator would increase the rate and stability of DNA loop formation and by extension increase repression. Furthermore, the work of Sadler *et al.* (1983) concluded that the natural operator is ‘flawed’ with respect to DNA binding. They demonstrated that a completely symmetric DNA fragment containing a true inverted repeat and a sequence corresponding to the left half of the O1 operator binds more tightly to LacI than the natural O1 operator sequence. The *in vitro* affinity of this “ideal” operator was found to be 8-fold greater than that of O1 (Sadler *et al.*, 1983; Simons *et al.*, 1984). Consequently, a promoter construct containing multiple copies of this “ideal” operator may

perhaps form DNA loops at even greater rates and with even greater stability, thus further reducing gene expression.

Of the four single expression promoter constructs, the LacO79 and LacO83 promoters both provided significant ($p < 0.05$) repression in the presence of LacI without inhibiting basal gene expression in its absence. As there was no significant difference between their levels of repression, the LacO79 promoter construct was chosen to be used in the LacI/O Repressor system as it demonstrated the larger average percentage reduction in gene expression. The next stage of research explored whether the levels of gene inhibition currently achieved by the LacO79 promoter were significant enough to effectively reduce BV production when blocking transcription of key genes previously identified in section 3.2.4. Of the five key genes identified (*gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*), *gp64* was selected as the first to undergo inhibition by the LacI/O Repressor System due to an abundance of research also showing *gp64* silencing to inhibit BV production (Monsma *et al.*, 1996; Oomens and Blissard, 1999; Lee *et al.*, 2015) as well as having access to a *gp64* knockout plasmid (supplied by OET Ltd) that allowed for immediate production of *gp64*ΔFBG.

The results of section 6.2.3.1 confirmed that along with EGFP, production of the key structural protein GP64 could also be reduced when expression was controlled by the LacO79 promoter in the presence of LacI. Future work employing a BCA assay or analysis by flow cytometry would be particularly interesting to establish the exact percentage reduction in GP64 production and compare this to the reduction levels seen for EGFP. The western blot data also showed that, as with EGFP, reduction was not 100%. Furthermore, the blot seemed to show that whilst the production of GP64 was reduced when infecting SfLacI cells with AcLacO79-*gp64*, GP64 production still seemed to be comparable to that seen from the AcChiA-*gp64*-infected SfLacI cells. This suggested that the current level of gene expression inhibition from the LacO79 promoter may not be enough to reduce BV production.

The work of section 6.2.3.2, therefore, focused on whether the current levels of gene inhibition achieved by the LacO79 promoter were substantial enough to significantly reduce BV production when blocking transcription of the key gene, *gp64*. Following cell infections, BV titres were assessed at 72 h.p.i., as this would correlate with the time point

when recombinant proteins or VLPs produced from the LacI/O Repressor System would be harvested. Results found there to be a significant ($p < 0.05$) reduction in BV production, with titres reduced by 50% when *gp64* expression was downregulated using the LacI/O Repressor System. This is a significant starting point for the development of a BV free expression system for the production of VLP based vaccines.

This current level of reduction in BV production is most likely due to two main elements. First is the level of gene repression from the LacO79 promoter not yet being 100%, as discussed above. There are multiple reasons why this may be and how it could be improved including the addition of a second *lacO* at a suitable distance from the first to allow for DNA loop formation, increasing the production of LacI from the SfLacI cell line to achieve optimal levels of LacI:*lacO* ratios, and exploring the use of *lac* operators with stronger LacI binding affinity. Furthermore, as the results of this chapter and findings reported by others suggests, using stronger promoters may yield a stronger level of repression (Lanzer and Bujard, 1988), so additional modifications to the LacO79 promoter to insert sequences that further increase promoter activity should be explored. Indeed, it has been shown that association of enhancer sequences, such as the *p10* and *polh* promoter A/T rich “burst sequence” or *hrs*, with baculovirus late promoters can significantly enhance activity (Habib *et al.*, 2009; Lee *et al.*, 2018; reviewed by Grose *et al.*, 2021). Similar modification of the LacO79 promoter could further increase its activity, subsequently increasing its level of gene and BV repression.

The second element restraining the level of reduction in BV production is the current inhibition of only a single key gene. As discussed previously, the data presented in this thesis has shown that silencing of two key genes compared to one significantly reduced BV production. Of the five key genes identified in Chapter 3, those that provided statistically significant combinations for reducing BV production when knocked down in combination with *gp64* were *Ac83(a)* or *Ac142*. Why exactly these two genes provide greater inhibition of BV production when silenced alongside *gp64* than the other key genes is currently unclear and would require further research. However, as discussed in Chapter 3 (section 3.3) it is possible that limitations of the RNAi procedure with regards to RNA uptake efficiency and competition potency could have impacted the results. It may, therefore, be possible that all of the key genes are capable of significantly reducing BV production when

inhibited in combination with *gp64* by the LacI/O Repressor System and the limitations of the RNAi procedure are removed. Due to overlapping of *Ac142* with the essential *IE1* gene of the AcMNPV genome, the design of an *Ac142* knockout bacmid would be difficult. Therefore, the next step in the development of the LacI/O Repressor System would be to use the LacO85 dual expression promoter to inhibit *gp64* along with *Ac83(a)*.

Whilst the inhibition of BV production during VLP/protein production is the main aim of the LacI/O Repressor System, another essential feature is its ability to allow for normal levels of BV production in the absence of LacI. This would enable selective production of BV which would be required for continued amplification/ maintenance of viral stocks to ensure they do not run out/degrade. Results showed no significant change in BV titre when infecting Sf9 cells with AcLacO79-gp64 compared to AcChiA-gp64 confirming the ability of the LacI/O Repressor System to resume normal levels of BV production in the absence of LacI. Results actually showed a slight increase in BV production when infecting with AcLacO79-gp64. This was expected as previous results had shown gene expression from the LacO79 promoter to be increased in comparison to the unmodified *chiA/cath^(syn)* promoter (Chapter 4). Therefore, assuming it was the limiting factor, an increased production of the key structural protein GP64 would be expected to result in an increase in BV production. This unintended benefit would actually aid in future production of AcLacO79-gp64 stocks.

6.4 Conclusion

The aim of this chapter was to determine the levels of gene repression from the *chiA/lacO* promoter constructs in the presence of LacI and to subsequently assess the abrogation of BV production from the LacI/O Repressor System. All the single expression *chiA/lacO* promoter constructs were capable of downregulating gene expression in the presence of LacI. However, only insertion of *lacO* after the TAAG motif yielded significant levels ($p < 0.05$) of gene inhibition. Increased promoter activity seemed to correlate with greater levels of gene downregulation, which was likely due to the increased rates of RNAP clearance increasing opportunity for LacI binding to *lacO* in the promoter. Addition of a second *lacO* sequence after the TAAG motif made the level of gene repression more constant between experimental replicates, possibly by increasing the rate of repressor/operator binding, or by increasing the stability of that complex (i.e reducing repressor clearance). The presence

of two *lacOs* spaced further apart appeared to enhance gene repression, possibly by enabling the formation of DNA loops. Results confirmed that significant downregulation of two genes was also possible from the dual LacO85 promoter construct in the presence of LacI.

Whilst gene repression was not 100% from any of the *chiA/lacO* promoters, it was enough to significantly reduce BV production when employing the LacI/O Repressor System to downregulate key gene expression. BV titres were reduced by 50% when *gp64* expression was controlled by the LacO79 promoter in the presence of LacI. This is a significant starting point for the development of a BV free expression system. Next steps to achieve greater levels of BV inhibition should be to improve the current levels of gene repression from the LacI/O Repressor System, by modifying the structure/design of the *chiA/lacO* promoter constructs (to allow for DNA loop formation or increased LacI/*lacO* binding) as well as exploring dual gene repression of the *gp64* and *Ac83(a)* genes.

Chapter 7:

Final discussion/ future work

7.1 Introduction

The BEVS is a valuable system for the production of VLPs and other recombinant proteins. A significant limitation of the BEVS, however, is the co-production and therefore contamination of VLPs with the baculovirus BV. BV contamination of biopharmaceuticals is a particular concern to regulatory bodies as they are enriched with the glycoprotein GP64, which is immunogenic in mammals (Luo *et al.*, 2013). Consequently, before VLP or other protein preparations can be used for scientific or medical purposes, they must be biophysically or biochemically separated from BVs. However, similarities in size, architecture, and physical properties (Wagner *et al.*, 2014) makes the consistent, scalable and economically feasible isolation of VLPs from BVs particularly difficult (reviewed by Pijlman, 2015). Furthermore, harsh purification can reduce VLP potency (particularly that of enveloped VLPs) and yield by harming their physical structure (Huhti *et al.*, 2010; Pijlman, 2015).

Consequently, this thesis aimed to develop an upstream solution that inhibited or significantly reduced BV production during VLP/protein production, thus eliminating the need for stringent downstream VLP processing, which would save significant time and money. A novel, repressible BEVS was developed that employed components of the *E. coli lac* operon to down-regulate the expression of essential genes required for BV production (identified using RNAi) during VLP/ protein production (the LacI/O Repressor System). This system comprised the SfLacI cell line (constitutively expressing *lacI*) and a promoter construct containing *lacO* (LacO79). Upon infection of the SfLacI cells with recombinant virus containing an essential gene under the modified promoter, LacI binds *lacO* and reduces the downstream transcription of the key gene, significantly reducing BV production. However, in the absence of LacI the key gene is not repressed, allowing for normal levels of BV production to resume, which is required for the continued amplification/ maintenance of viral stocks. This LacI/O “switch” allows for the selective repression of genes, so those required for BV production can be temporarily downregulated during VLP/protein production to significantly reduce BV synthesis.

7.2 Final discussion/ future work

Future work should now focus on further improving the potential of the LacI/O Repressor System. There are many things that could still be done to achieve this. Firstly, much more research could be done into the structure and design of the *chiA/lacO* promoter constructs. Modifications that increase their level of gene repression would most likely further reduce BV production. Initially, a broad screening of *lacO* insertion at each bp position of the *chiA/cath* promoter would identify the ideal insertion point, as well as provide further insights into the structure and function of late promoters. The current method for analysing gene expression from each promoter construct could be scaled down to a 48- or 96-well plate system to allow for high-throughput screening of multiple promoter constructs.

The results of Chapter 6 imply the formation of DNA loops during gene expression from the LacO81 promoter. DNA looping between two *lacOs* in the *E. coli lac* operon has been well documented and shown to increase the levels of repression (Krämer *et al.*, 1987; Flashner and Gralla, 1988; Haber and Adhya, 1988; Oehler *et al.*, 1990; Becker *et al.*, 2013; Tungter *et al.*, 2018). Consequently, the greater but inconsistent levels of gene repression from the LacO81 promoter hint at unstable loop formation due to suboptimal *lacO* spacing restricting DNA bending. Future work should explore this theory. Firstly, formation of DNA loops from the LacO81 promoter could be confirmed by electron microscopy. Next, more promoter constructs should be designed with the spacing/phasing of two *lacOs* adjusted to facilitate DNA looping. Spacing of 400bp was found optimal for DNA looping (Shore *et al.*, 1981), although formation of loops has been shown possible at a wide range of *lacO* distances (Krämer *et al.*, 1987; Haber and Adhya, 1988, Becker *et al.*, 2013). It could also be interesting to assess *lacO* spacings that mimic their natural position within the *E. coli lac* operon. Furthermore, DNA looping relative to the transcription initiation site of the promoter and the effect this has on repression levels should also be explored. Constructs should be designed with appropriately spaced *lacOs* inserted either both after the TAAG motif, or one before and one after.

Sadler *et al.* (1983) found the natural *lacO* to be ‘flawed’ with respect to DNA binding. They demonstrated that a completely symmetric DNA fragment containing a true inverted repeat and a sequence corresponding to the left half of the O1 operator binds more tightly to LacI than the natural O1 operator sequence. The *in vitro* affinity of this “ideal” operator

was found to be 8-fold greater than that of O1 (Sadler *et al.*, 1983; Simons *et al.*, 1984). Consequently, future research should explore the levels of repression that could be achieved if a promoter construct containing this “ideal” operator was produced as increased LacI affinity is likely to enhance gene repression.

It would also be interesting to explore the effects of *lacO* insertion on alternative late baculovirus promoters, and whether the same influence on promoter activity and repression is observed. Indeed, the data of Lanzer and Bujard (1988) suggests that when employing the *lac* repressor system, stronger promoters yield a stronger level of gene repression. Modification of a stronger promoter may, therefore, allow for stronger levels of gene repression.

Future research into the mechanisms behind the change in promoter activity induced by insertion of *lacO* is also essential. It may help to explain why insertion before TAAG reduces activity whilst after TAAG increases activity. Better understanding of the mechanisms of action could also provide key insights into the optimal position for *lacO* insertion within the *chiA/cath* promoter, allowing for the best possible levels of gene repression. Firstly, DNA shape analysis using bioinformatics tools such as that proposed by Zhou *et al.* (2013) could provide information on how insertion of *lacO* into the promoter affects DNA shape. Indeed, research has shown DNA shape to impact both binding affinity and binding specificity of TFs (reviewed by Rohs *et al.*, 2010; reviewed by Slattery *et al.*, 2014). The *lacO* sequence may also be facilitating DNA unwinding, thus increasing access to the DNA by RNA polymerase and further enhancing transcription. Additionally, analysis of DNA-protein binding and protein-protein interactions could provide insights into if and how the *lacO* affects TF binding at the promoter.

The addition of the *lacO* sequence after the TAAG motif of the *chiA/cath* promoter increases the length of the 5'UTR. It is possible that it is this increase in length that is facilitating the increase in promoter activity and, therefore, any arbitrary sequence added after the TAAG motif may enhance *chiA/cath* activity. The results of the dual expression promoter analysis (section 4.2.8), however, suggest otherwise. Whilst the length of both the sense and antisense TAAG 5'UTRs was equal (53bp), the increase in promoter activity was not. Activity from the sense TAAG motif was increased 10-fold whilst activity from the antisense TAAG motif was increased 3-fold. This implies that there is a more complex

mechanism of influence induced by the presence of the *lacO* sequence, than simply acting to increase 5'UTR length. Indeed, the *lacO* sequence was 11bp away from the sense TAAG motif but 24bp away from the antisense motif. It is possible that the *lacO* sequence enhances DNA unwinding, and therefore unwinding at positions closer to the TAAG motif may facilitate RNA polymerase binding. More research, however, into the mechanisms behind the influence of the *lacO* sequence is needed to better understand the effects insertion of *lacO* has on individual late promoters including dual promoters. Experiments should explore the effects of *lacO* insertion into the *chiA/cath* and alternative baculovirus promoters, and compare to effects observed following insertion of an arbitrary sequence.

Further development of the SfLacI cell line could also increase the repression potential of the LacI/O Repressor System. Research has shown higher levels of LacI to increase levels of repression by increasing operator occupancy (Fuerst *et al.*, 1989; Oehler *et al.*, 1990). Therefore, increasing LacI production from the SfLacI cell line could increase the levels of repression seen from the *chiA/lacO* promoters. Much like Wu *et al.* (2019), this could be achieved via fluorescence-activated cell sorting (FACS). Following immunostaining of the SfLacI cells for LacI, FACS could be used to isolate cells having the top 10% fluorescence intensity of all fluorescence-positive cells and therefore, the top 10% of cells producing LacI to establish a high-yield cell line.

In Chapter 3, RNAi for numerous late BV structural genes led to the identification of five “key genes” capable of reducing BV by over 80% if their expression was inhibited. These were *gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*. However, whilst the list of BV structural genes to be targeted for knockdown by RNAi was extensive (Table 3.2), it was not exhaustive and therefore other structural genes not explored in this thesis may also induce significant reduction in BV production if downregulated by the LacI/O Repressor System. For example, research has shown the inhibition of *Ac51* to result in a 1000-fold reduction in BV production (Qiu *et al.*, 2019). *Ac141* knockout viruses also saw a 99% reduction in BV production (Dai *et al.*, 2004; Fang *et al.*, 2007). Inhibition of the *Ac76*, *Ac77*, *Ac82*, and *Ac93* genes also resulted in significant reductions in BV production (Vanarsdall *et al.*, 2006; Hu *et al.*, 2010; Yaun *et al.*, 2011; Gauthier *et al.*, 2012). Therefore, future research could explore the knockdown potential of these and other promising BV structural genes not yet assessed in this thesis.

Of the five key genes identified in Chapter 3 (*gp64*, *vp39*, *Ac83(a)*, *Ac92*, and *Ac142*), *gp64* was selected as the first to undergo regulation by the LacI/O Repressor System as substantial research has emphasised the essential role it plays in BV infectivity and release with data showing its inhibition to block BV production (Blissard and Rohrmann, 1989; Monsma *et al.*, 1996; Oomens and Blissard, 1999; Valdes *et al.*, 2003; Washburn *et al.*, 2003; Lee *et al.*, 2015). Ultimately, downregulation of *gp64* from the LacI/O Repressor System significantly reduced BV production by 50%. The next logical step in exploring the potential of the LacI/O Repressor System would be to inhibit the four other key genes (*vp39*, *Ac83(a)*, *Ac92*, and *Ac142*). Indeed, whilst *gp64* was chosen for initial analysis of the LacI/O Repressor System, data showed the other key genes to induce a greater reduction in BV production with *Ac83(a)* knockdown yielding the greatest reduction of 92%. Consequently, the downregulation of these genes may result in a greater reduction of BV production than that currently achieved by *gp64* downregulation.

Following on from downregulation of all the identified individual key genes, experiments should also explore the knockdown of two of the key genes simultaneously from the LacO85 dual promoter construct. Currently, data showed a 50% reduction in BV production when using the LacO79 promoter to downregulate *gp64* expression. However, the data of Chapter 3 showed that dual inhibition of the following key gene pairs yields a significantly greater reduction in BV production than individual inhibition: *gp64* and *Ac83(a)*, *gp64* and *Ac142*, *vp39* and *Ac83(a)*, *vp39* and *Ac142*, and *Ac83(a)* and *Ac142*. Consequently, dual gene knockdown from the LacO85 promoter should greatly improve the current potential for BV reduction by the LacI/O Repressor System.

With the results of Chapter 3 demonstrating that inhibition of a second key gene would significantly enhance the reduction in BV production, it would be reasonable to assume that the inhibition of three or more key genes would further increase this BV inhibition. Currently, the work of this thesis has only presented a system capable of inhibiting up to two key genes. However, if future research into establishing other AcMNPV promoters containing a *lacO* is successful, then it may be possible to produce recombinant virus with three or more key genes under the control of multiple “*lacO*” promoters. However, whether the production of other “*lacO*” promoters is possible is unknown. Furthermore, the extent to which inhibition of more than two key genes would aid the reduction of BV

production would also need further research. Indeed, it has not yet been established whether the effects of inhibiting multiple key genes are synergistic or additional in nature. If enhanced BV reduction was a result of synergistic relations between the two inhibited genes, then the knockdown of more than two genes may not yield as greater reduction in BV production than if the relationship was additional in nature. Furthermore, other limiting factors such as the current level of repression of each gene from the promoters may reduce the effect that inhibiting more genes has.

Finally, the use of the LacI/O Repressor System should be demonstrated during VLP or protein production to illustrate its potential.

The LacI/O Repressor System also has potential for use in other applications. If *lacO* integration into the *polyhedrin* or *p10* promoters is successful, the repression system could be used to regulate the expression of foreign genes. This would be particularly useful for cytotoxic gene products, which can render making the original recombinant virus difficult. Infection of the SfLacI cell line would allow for downregulation of the cytotoxic gene during amplification. Subsequent infection of Sf9 cells would then allow for expression of the cytotoxic gene when required.

7.3 Concluding Remarks

Overall, this thesis has provided key insights into baculovirus genomics and specifically late promoter structure and function. Multiple genes were identified as essential and required for BV synthesis. Insertion of the short 24bp *lacO* sequence into the late *chiA/cath* promoter had significant impact on promoter activity. Insertion upstream of the transcriptional start site (TAAG) reduced promoter activity by 80.2% whereas insertion downstream delayed activity but increased it by 146.6%. Insertion of two copies of *lacO* after the TAAG motif, however, prolonged promoter activity allowing for earlier and later gene expression, which resulted in an increase in promoter activity by 218.7%. Insertion of *lacO* downstream of each TAAG motif of the dual *chiA/cath* promoter induced a 10-fold increase in activity from the sense TAAG motif and a 3-fold increase from the antisense TAAG motif. It is not yet clear as to the mechanisms by which *lacO* insertion acts to influence promoter activity but its presence after the TAAG motif is likely to increase promoter clearance by RNA polymerase.

Gene repression from all the *chiA/lacO* promoter constructs was possible regardless of *lacO* position relative to the transcription start site when in the presence of LacI. However, repression was only significant when *lacO* was inserted downstream of the TAAG motif with gene repression of 53.3% achieved. Significant gene repression was possible from the dual *chiA/cath* promoter with *lacO* inserted after each TAAG motif: gene expression was reduced by 66.3% from the sense TAAG motif and 43.9% from the antisense TAAG motif. There were strong indications from the data that the presence of two *lacO* sequences spaced at an appropriate distance (whether both after the TAAG motif or one before and one after), would further increase gene repression by allowing the formation of DNA loops.

Finally, a novel, repressible BEVS (the LacI/O Repressor System) has been established. This system allows for the selective repression of genes, so that those required for BV production can be temporarily downregulated during VLP/protein production, thus significantly reducing BV synthesis. Currently, through downregulation of *gp64*, the LacI/O Repressor System is capable of reducing BV production by 50%. In summary, this is a significant starting point for the development of a BV free expression system. There is still much more that could be done to further improve the potential of the LacI/O Repressor System and increase the inhibition of BV production. Potential for a dual LacI/O Repressor System has also been demonstrated.

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