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Final Report for the Australian Government Department of the Environment and Heritage

Development of Recombinant Proteins as a Candidate Vaccine for Psittacine Beak and Feather Disease

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Non-technical Summary

The main aims of the project that Murdoch University undertook included:

1. Develop a Beak and Feather virus using bacteria.
2. See if antibodies recognise the engineered virus from naturally infected bird species.
3. Determine if the engineered virus will induce an antibody response in sheep?
4. And will these antibodies recognise native virus?
5. Will psittacine species antibodies respond to the engineered virus?
6. Identify and prioritise the gaps in existing knowledge relating to this field of research and provide recommendations as to areas of future research activity that will address those gaps identified.

This research project successfully engineered a Beak and Feather Disease virus that was recognised by anti-BFDV-antibodies raised in chickens and cockatoos. Additionally, two sheep and several psittacine birds were infected with the engineered virus and their antibody response was monitored. It was found that both sheep and birds inoculated with the synthesized virus induced the production of antibodies that recognized native BFDV. These results show that the engineered virus is able to induce an antibody response and those characteristics of the native BFDV have been conserved. The engineered virus has valuable potential future applications in immunisation of parrots, lorikeets and cockatoos. In addition, this experiment has resulted in a large stock of sheep-anti-Beak and Feather Disease Vaccine sera that can be used for diagnostic testing of psittacine birds.

Technical Summary

Full length and C-terminal truncated recombinant beak and feather disease virus (BFDV) capsid proteins were expressed using the bacterial expression system PinPoint™. The full length recombinant protein reacted with sera from naturally immune cockatoo and chicken experimentally inoculated with BFDV. Reactivity proved antigenic epitopes of the BFDV had been conserved in the recombinant protein. The full length recombinant BFDV capsid protein induced an antibody response in two inoculated sheep. Antibodies raised against the recombinant capsid in the sheep recognized native viral inclusions in skin sections from a chronically infected cockatoo by immunohistochemistry. These results confirmed that antigenic epitopes of the BFDV had been conserved in the recombinant protein and are involved in the generation of an antibody response. Haemagglutination inhibition assay demonstrated that some psittacine birds vaccinated with the recombinant protein in conjunction with Freund's incomplete adjuvant produced antibodies that inhibited the haemagglutinating activity of BDFV. This is the first reported evidence of the potential value of a recombinant protein in vaccination and protection of psittacine species against the detrimental effects of BFDV and its future application for preservation of Australian Psittaciforme biodiversity.

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Abbreviations

a.a.	amino acid
ATP	adenosinetriphosphate
BFDV	beak and feather disease virus
bp	base pairs
BSA	bovine serum albumine
CAV	chicken anaemia virus
C-terminus	carboxy terminus
DNA	dioxy ribonucleic acid
ddNTP	dideoxynucleotidetriphosphate
dNTP	deoxynucleotidetriphosphate
DTT	dithiothreitol
ds	double stranded
EDTA	ethylenediaminetetra acetic acid
E. coli	*Escherichia coli*
GIT	gastrointestinal tract
GTP	guanosinetriphosphate
HCl	hydrogen chloride
HI	haemagglutination inhibition
HIU	HI units
HRP	horse radish peroxidase
IHC	immunohistochemistry
IPTG	isopropyl β-D-thiogalactopyranoside
Jca	Jembrana Disease Virus capsid protein
KCl	potassium chloride
KOA	potassium acetate

LB	Luria Bertani
$MgCl_2$	magnesium chloride
NaOH	sodium hydroxyde
NaCl	sodium chloride
nt	nucleotide
N-terminus	aminoterminus
OD	optical density
ORF	open reading frame
ORF C1	open reading frame C1 that codes for BFDV capsid protein
ORF V1	open reading frame V1 that codes for BFDV replication protein
PBFD	psittacine beak and feather disease
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	porcine circovirus
PMSF	phenylmethylsylphonyl fluoride
PolyA	poly adenylation
RCR	rolling circle replication
Rep	replication associated protein
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
ss	single stranded
TAE	Tris acetate EDTA
TBS	Tris buffered saline
TBST	Tris buffered saline with tween
TE	Tris EDTA

UV	ultra violet
v/v	volume per volume
w/v	weight per volume

Units

g	relative centrifugation force [=($\omega^2 \times r$) / 980 cm/s^{-2}]
g/L	grams per litre
g/mL	grams per millilitre
kb	kilo bases
kDa	kilo Dalton
mA	milliampere
mg/mL	milligram per millilitre
mL	millilitres
min	minutes
mM	millimolar
mW	milliwatts
ng	nanograms
rpm	rounds per minute
s	seconds
ρmol	picomoles
µL	microliters
µg/mL	micrograms per millilitre
µM	micromoles
U	units

1 Review of literature on BFDV

This review will consider the current knowledge on beak and feather disease virus (BFDV), its associated disease Psittacine Beak and Feather Disease (PBFD) and other literature relevant to this honours project: Development of Recombinant Beak and Feather Disease Virus Capsid Protein as a Candidate Vaccine for Psittacine Beak and Feather Disease. The review is composed of four sections. Section one will consider the family *Circoviridae* to which BFDV belongs, section two accounts for the pathogenicity, transmission, epidemiology of BFDV and techniques currently available for diagnosing PBFD. Section three accounts for genetics and proteomics of BFDV, and finally section four discusses recombinant protein technology and the application of recombinant proteins.

The nomenclature used in this thesis is that used by Bassami *et al.* (1998) when concerning convention of numbering nucleotide (nt) positions. Nomenclature as described by Niagro *et al.* (1998) is used for labeling open reading frames (ORFs). Finally, taxonomy is by the convention of the International Committee on Taxonomy of Viruses (van Regelmortel *et al.*, 2000).

1.1 Introduction

The most common viral disease of psittacine birds in Australia is PBFD, endemically affecting both wild (Raidal *et al.*, 1993b; McOrist *et al.*, 1984) and captive species (Studdert, 1993). BFDV is listed as a key threatening process for five endangered psittacine species in Australia (Raidal 2004, per comm.) and causes significant problems with breeding of Psittaciformes (Studdert, 1993; Jacobson *et al.*, 1986; Kock *et al.*, 1993).

1.2 The *Circoviridae*

BFDV is a member of the family *Circoviridae*. Viruses of the *Circoviridae* are characterized by a single stranded (ss) circular DNA genome and non-enveloped virions with icosahedral morphology (van Regelmortel *et al.*, 2000). The members of this family appear to be host specific. Family *Circoviridae* currently holds two genuses, *circovirus* and *gyrovirus*. BFDV, porcine circovirus type 1 (PCV1) and porcine circovirus type 2 (PCV2) are gathered in the genus *circovirus*. Chicken anaemia virus (CAV) is the only member of the genus *gyrovirus* (van Regelmortel *et al.*, 2000). CAV is classed in a separate genus within the *Circoviridae* due to some features significantly different to the other circoviruses, including a single polycistronic message encoding all viral proteins, lack of a stemloop structure, and not possessing an ambisense genome. There are no common antigens reported between these animal circoviruses (van Regelmortel *et al.*, 2000). Circoviruses cause a wide range of disease in different animal hosts. CAV causes transient anemia and immunosuppression in young chicks (van Regelmortel *et al.*, 2000). PCV1 was first discovered in the continuous porcine kidney cell line PK-15, however no disease has been associated with the virus (Tischer *et al.*, 1986). PCV2 in comparison is believed to be associated with post weaning multisystemic wasting syndrome (van Regelmortel *et al.*, 2000).

1.3 Clinical presentation and pathology of PBFD

PBFD is most often seen in young captive birds under the age of three years. The condition is apparently irreversible, and lasts from several months to a year or even several years (Pass and Perry, 1984; Jacobson *et al.*, 1986). PBFD occurs as a chronic or acute condition. Symmetrical loss and dystrophy of contour, crest, tail and down feathers are typically seen in chronic PBFD (Figure 1-1) and mainly occurs in older birds. Wing feathers may also less commonly be affected. The abnormal feathers may

present with at least one of the following characteristics: retained feather sheath, feather sheath filled with blood, short clubbed feathers, curled and deformed feathers, feathers with circumferential constriction, and stress lines in the vane (Pass and Perry, 1984; McOrist *et al.*, 1984; Jergens *et al.*, 1988; Jacobson *et al.*, 1986). Feather lesions are only seen in feathers developed after infection with BFDV (Wylie, 1991). Therefore birds with readily developed feathers may not exhibit feather lesions until the bird has moulted.

Figure 1-1. Two sulphur crested cockatoos chronically infected with BFDV displaying gross clinical signs of feather loss.

The beak may exhibit changes in colour, progressive elongation, palatine necrosis, and longitudial and transverse fractures (Figure 1-2) (Pass and Perry, 1984; Jergens *et al.*, 1988; McOrist *et al.*, 1984). Beak and feather pathology are due to epidermal cell necrosis, epidermal hyperplasia and hyperkeratosis (Pass and Perry, 1984; McOrist *et al.*, 1984).

Figure 1-2. A galah chronically infected with BFDV displaying gross clinical signs of beak fracture.

Acute disease, most commonly observed in fledgling and immature birds, differs distinctly from the chronic form as it does not usually manifest with beak and feather abnormalities, however is characterised by lethargy, depression, diarrhoea and often results in death (Raidal, 1994; Ritchie *et al.*, 1989a). Severe leukopaenia, anaemia, or pancytopaenia and liver necrosis, have been reported in birds diagnosed with peracute PBFD. These birds, as with acutely infected birds, lacked feather and beak abnormalities (Schoemaker *et al.*, 2000). Route of infection, virus titre, age, condition of the bird at the time of infection and species have been postulated as factors determining whether the disease has a chronic or an acute outcome (Ritchie *et al.*, 1989a; Wylie and Pass, 1987; Raidal and Cross, 1995).

The target organ of BFDV has been shown to be the epithelium, primary site of virus replication most likely being the bursa of Fabricius, but may also occur in internal organs. BFDV antigens have also been demonstrated in liver, kidneys, thymus, bone marrow and other internal organs (Pass and Perry, 1984). Evidence by Raidal *et al.* (1993a) suggests that the liver is an important site for BFDV replication in both acute and chronic disease. Experimental infection of nestling sulphur-crested cockatoos by Raidal and Cross (1995) and studies on African grey parrots (*Psittacus erithacus*

erithacus) by Schoemaker *et al.* (2000) showed that BFDV infections may cause necrotizing hepatitis. Additionally, the latter authors report findings of severe leukopaenia in such parrots naturally infected with BFDV.

1.4 Immunology associated with PBFD

Damaged lymphoid tissue and immune suppression occurs typically with BFDV-infection. Populations of both helper ($CD4^+$) and cytotoxic ($CD8^+$) T cells are depleted, due to the virus targeting precursor T cells. This results in a predisposition to secondary bacterial and fungal infections, which may result in mortality (Schoemaker *et al.*, 2000; Latimer *et al.*, 1992). Wylie and Pass (1987) found occasional infiltration of heterophils and lymphocytes into the pulp of feathers during experimental reproduction of PBFD using budgerigars. These findings have been further supported by Jacobson *et al.* (1986), indicating that feather lesions and abnormalities are associated with the immune response.

1.5 Transmission of BFDV

Feather dust has been proposed as a major vehicle of transmission, due to the high amount of viral inclusion bodies that may be found in the follicular and feather epithelium of BFDV positive birds (Pass and Perry, 1984; McOrist *et al.*, 1984; Ritchie *et al.*, 1991). Necrotic epithelial cells in the outer layers of hyperkeratotic feather sheaths contain intracytoplasmic viral inclusion bodies (Pass and Perry, 1984), supporting the theory of feather dust as a vehicle for transmission. One likely mode of transmission may occur by ingestion during preening activities (Ritchie *et al.*, 1991). The second possible mode of transmission is the faecal-oral route. The gastrointestinal tract (GIT) has been postulated as a site of viral replication, due to findings of viral-induced inclusion bodies in several locations of the GIT, and therefore explains findings

of BFDV in the faeces of infected birds. Thus transmission is likely to occur via the faecal-oral route (Ritchie *et al.*, 1991; Raidal and Cross, 1995). Thirdly, crop washings have also been shown to contain BFDV. Even though the concentration of virus was low, it is possible that the virus can be transmitted from adults to neonates during feeding activities involving regurgitation of food and crop epithelium (Gerlach, 1994). Supporting this theory, vertical transmission of BFDV has been demonstrated with an infected hen, where the chicks consistently developed lesions associated with PBFD (Gerlach, 1994). A fourth possible mode of transmission is, according to Raidal (1994), direct deposition of contaminated material into the cloaca of nestling birds. Nestlings have weak legs and poor balance, and are forced to sit tripod like on their legs and abdomen. When they defecate, nestlings slide their cloaca over nesting material, thus possibly contracting infections.

1.6 Epidemiology of BFDV

Between 1887 and 1888 Ashby (1907) observed a decline in the population of *Psephotus* parrots in the Adelaide Hills, and described "feathering abnormalities" causing impaired flight as the reason for this decline. This first reported outbreak of feather disease in wild parrots was probably due to BFDV (Raidal *et al.*, 1993b). PBFD occurs endemically in both captive and wild psittacine species (Raidal *et al.*, 1993b; Raidal, 1994) and has been reported in both old and new world captive psittacine species (Ritchie *et al.*, 1991; Kock *et al.*, 1993; Raidal, 1995). Histological and clinical observations suggesting PBFD have been described in 42 psittacine species (Gerlach, 1994). Approximately 30 and perhaps all 50 species of Australian psittacine birds, both captive and wild, are affected by PBFD (Studdert, 1993). The disease is largely found in sulphur-crested cockatoos (*Cacatua galerita*). Based on gross pathology and histology, McOrist *et al.* (1984) found that several flocks of sulphur-crested cockatoos in Victoria

had a 10-20% incidence of PBFD. In some wild populations, seroprevalence as high as 94% has been reported, however the exact number of affected birds in a flock is hard to determine due to an unknown number of birds affected by PBFD dying in the nest (Raidal *et al.*, 1993b). PBFD has also been demonstrated in galahs (*Cacatua roseicapilla*), little corella (*Cacatua sanguinea*), Major Mitchell's cockatoo (*Cacatua leadbeateri*), lovebirds (*Agapornis spp*.), budgerigars (*Melopsittacus undulatus*), African grey parrot (*Psitticus erithacus erithacus*), short-billed corella (*Cacatua sanguinea*), eastern long-billed corella (*Cacatua tenuirostris*), blue bonnet (*Psephotus haematogaster*) rainbow lorikeet (*Trichoglossus haematodus*) and other psittacine species (Schoemaker *et al.*, 2000; Raidal *et al.*, 1993b; Ritchie *et al.*, 2003). PBFD is the most commonly encountered disease in captive and wild psittacine birds in Australia, where it currently threatens five species [(orange-bellied parrot (*Neophema chrysogaster*), swift parrot (*Lathamus discolour*), Norfolk Island green parrot (*Cyanoramphus neozelandiae cookie*), superb parrot (*Polytelis swainsonii)* and Eastern regent parrot (*Polytelis anthopeplus monarchoides*)] (Raidal 2004, pers comm).

1.7 Diagnosis of PBFD

The primary procedure for diagnosis of PBFD is clinical examination on the basis of gross pathology. Diagnosis based on observation of clinical presentation is not in itsself sufficient for an accurate diagnosis, as many practitioners fail to detect pathognomonic feather lesions in early clinical cases (Raidal *et al.*, 1993c) and clinical signs may be confused with polyomavirus infections and other conditions (reviewed in Riddoch (1996)). Observing epidermal hyperplasia and hyperkeratosis of feather follicles using histopathological examination of feather follicles (Pass and Perry, 1984) may be used as a routine measurement, but requires biopsy of feather follicles, causing injury to the bird. Histopathology is of also limited use when testing flocks (Raidal *et al.*, 1993c).

Polymerase chain reaction (PCR) (Ypelaar *et al.*, 1999) and haemagglutination (Raidal and Cross, 1994b) will detect BFDV nucleic acid and antigen, respectively, and a haemaglutination inhibition assay (HI) (Raidal *et al.*, 1993c) has been developed for the detection of BFDV antibody. Diagnostic techniques such as immunohistochemistry (IHC), transmission electronmicroscopy, DNA dot-blot hybridization (Raidal *et al.*, 1993c) *in situ* hybridisation (Ramis *et al.*, 1994) and feather enzyme immunoassay (Raidal, 1994) have been described.

1.8 Vaccination and control of PBFD

Raidal and Cross (1994a) proved vaccination to be an effective means of controlling PBFD. This experiment used an aviary flock of 77 *Agapornis spp* and vaccine produced from the feathers of PBFD-affected sulphur-crested cockatoos. Subsequent generations after vaccination did not develop disease either (Raidal and Cross, 1994a). A double-oil emulsion killed vaccine, produced by Raidal *et al.* (1993a) was successful at protecting nestling galahs. These experimental findings indicate that PBFD can be controlled in individual, as well as flocks of psittacine species by vaccination.

Attempts at culturing BFDV in numerous culture systems have been unsuccessful (Pass and Perry, 1985). Thus development of a recombinant protein vaccine would be a useful tool in the control of this endemic disease in wild and aviary flocks as well as pet psittacine birds.

1.9 Genetic variation in BFDV

Initially Ritchie *et al.* (1989b) determined that the genome of BFDV was a single ssDNA between 1.7 to 1.9 kb in size. Bassami *et al.* (1998) determined a more accurate size of 1993 nt. Further research by Bassami *et al.* (2001) compared eight new isolates

of Australian BFDV, to the isolate (BFDV-AUS) described by Bassami *et al.* (1998) and the BFDV-USA isolate derived from pooled BFDV reported in the USA by Niagro *et al.* (1998). The eight new isolates ranged between 1992 and 2018nt in genomic size and had an overall nt identity between 84 and 97% compared to the BFDV-AUS isolate. The nt sequence identity of ORF C1 in the eight Australian isolates studied by Bassami *et al.* (2001) varied from 80 to 99% when compared to the BFDV-AUS isolate. Further, of the seven ORFs detected in the BFDV-AUS isolate, only three (ORF V1, ORF C1 and ORF5) were consistently detected in all 10 isolates compared. Point mutations and several deletions and insertions from 1 to 17 nt in size in both coding and non-coding regions accounted for the variation in these isolates.

When considering location of ORFs, hairpin structure, nonanucleotide motif, the three motifs in the rep protein required for rolling circle replication (RCR), the P-loop motif and poly adenylation (polyA) signal downstream of ORF V1 and ORF C1, and the octanucleotide motif downstream of the hairpin structure, all these isolates were homologous, except for in one isolate from a blue bonnet (*Psephotus haematogaster*) in Western Australia, where there was a base substitution in the second octanucleotide motif.

1.9.1 BFDV genome

Further discussion of BFDV genome related topics will be based on the BFDV-AUS isolate. BFDV has an ambisense genome, with a size of 1993 nt (Bassami *et al.*, 1998; Niagro *et al.*, 1998). Seven ORFs, three on the viral strand and four on the complementary strand (Figure 1-3) tentatively encoding proteins of >8.7kDa have been reported (Bassami *et al.*, 1998). Flanked by ORF C1 and V1 in the replicative form, the genome of BFDV has a nt motif with the capacity to form a stem loop structure at nt position 1976-1993 and 1-12 (Figure 1-4). The apex of the BFDV stemloop is the

nonanucleotide motif 5'-TAGTATTAC-3'. This motif is highly conserved within the *Circoviridae* and other circular ssDNA viruses (Table 1-1). Immediately downstream of the stemloop structure is an octanuclotide repeat sequence 5'-GGGCACCG-3' (Figure 1-4) (Bassami *et al.*, 1998).

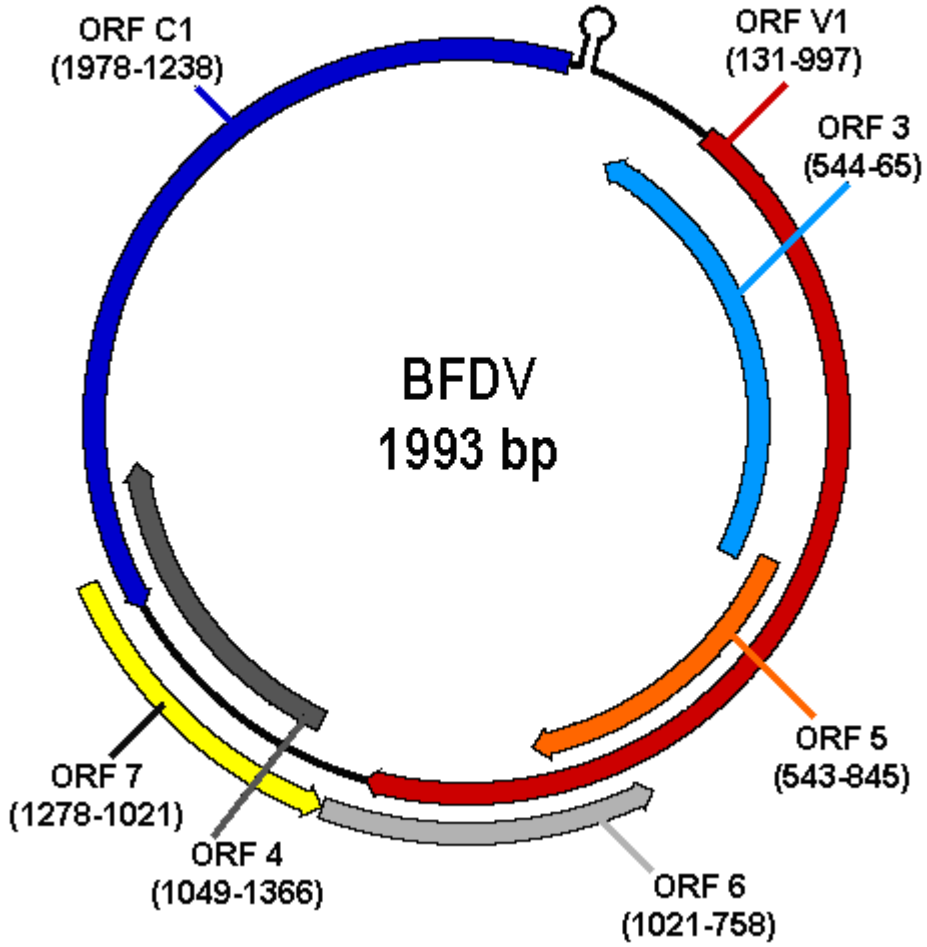

Figure 1-3. Schematic diagram of BFDV double stranded replicative form displaying location of ORFs as outlined Bassami et al. (1998).

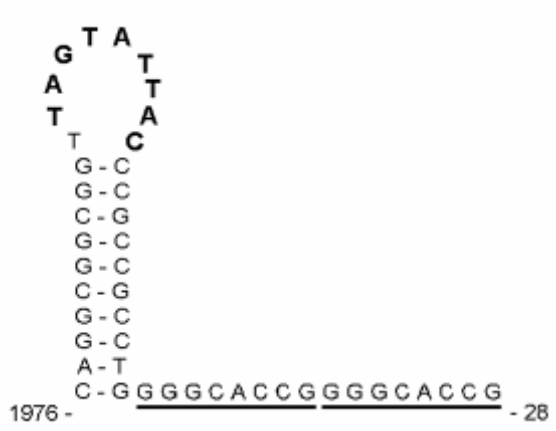

Figure 1-4. The potential stemloop structure of BFDV. In bold is the nonanucleotide motif and underlined is the octanucleotide repeat sequence. Adopted from Bassami (1998).

Table 1-1. Sequence alignment of nonanuclotide motif of BFDV (accession No. AF080560) (Bassami *et al.*, 1998), PCV (accession No. U49186) (Meehan *et al.*, 1997), CAV (accession No. D10068) and the geminiviruses: Tomato golden mosaic virus (TGMV), squash leaf curl virus (SLCV) and dicot-infecting geminiviruses (DIG) (Arguello-Astorga *et al.*, 1994). *, indicates conserved nt within the motifs.

Virus species	**Nt sequence (5' - 3')**
BFDV	TAGTATTAC
CAV	TACTATTCC
PCV	TAGTATTAC
TGMV	TAATATTAC
SLCV	TAATATTAC
DIG	TAATATTAC
	* * * ***** *

The viral strand has two potential TATA boxes, TATA at nt 86-89 and TATAAAA at nt 680-686 upstream of the start codon for ORF V1, and two polyA signals downstream of the stopcodon for ORF V1 at nt 1019-1024 and 1196-1201. The complementary strand of the replicative form features a polyA signal, AATAAA at nt 758-763, only one nt downstream of ORF C1 (Bassami *et al.*, 1998). The function of these TATA boxes was not determined or hypothesized.

1.9.2 ORFs and protein products

1.9.2.1 ORF C1

The 741 nt ORF C1 is located on the complementary strand of the double stranded (ds) replicative form (nt position 1978-1238). This ORF encodes a potential 28.9 kDa protein product hypothesized to be the capsid protein (Bassami *et al.*, 1998). The predicted protein product of the equivalent ORF in PCV was identified on the complementary strand, and shared 29.1% amino acid (a.a.) sequence identity with BFDV. The ORF starts within or close to the stemloop structure of the respective viruses, indicating similarity in function (Bassami *et al.*, 1998). BFDV and PCV ORF C1 predicted protein products feature a highly conserved 14 a.a. sequence close to the C-terminus with an adjacent myristylation site (Niagro *et al.*, 1998). The highly conserved nature of these features pleads their vast necessity for protein function. PCV2 ORF C1 cloned into baculovirus expression vector by Nawagitgul *et al.* (2000) resulted in expression of a 30 kDa product. Similar results were achieved with purified virus particles. Electron microscopic viewing of the recombinant protein detected a self-assembled protein forming capsid-like particles. Similar results have been reported by Liu *et al.* (2001)**.** These reports are experimental proof for PCV2 ORF C1 encoding a capsid protein, and strengthens the assumption that BFDV ORF C1 also encodes a

capsid protein when correlating these results with the 29.1% sequence identity found between ORF C1 of BFDV and PCV reported by Bassami *et al.* (1998).

1.9.2.2 ORF V1

ORF V1 is 867nt long, located on the viral strand and was predicted to encode a 33.3 kDa protein product by Bassami *et al.* (1998). This putative protein features all sequence motifs associated with proteins involved in RCR. A P-loop motif has been identified in the reading frame (Niagro *et al.*, 1998). P-loop motifs and ATP/GTP binding motifs are found in proteins with helicase activity (Hodgeman, 1988). A potential pyrophosphatase domain is found in the predicted a.a. sequence of this protein (Niagro *et al.*, 1998). Bassami *et al.* (1998) demonstrated a high a.a. similarity (45.6%) between the product of BFDV ORF V1 and the replication-associated protein of PCV, subterranean clover stunt virus and faba bean necrotic yellows virus. Thus, it is likely that ORF V1 of BFDV also encodes a replication-associated protein.

1.10 BFDV replication

BFDV has a very small genome and a limited protein expression capacity; therefore DNA replication must be heavily dependant on the host cell machinery (Todd, 2000). No reports have been published on the exact mechanism of replication in BFDV. Circular ssDNA viruses replicate using a mechanism known as RCR (Gutierrez *et al.*, 2004; Gronenborn, 2004). The predicted replication-associated protein of BFDV, possesses all motifs involved in RCR (Niagro *et al.*, 1998), thus it is most likely that BFDV also replicates via this mechanism to produce progeny virus. In addition, the stem loop structure and nonanuclotide motif previously described are common features of ssDNA viruses that replicate via RCR. Geminiviruses are ssDNA viruses proven to replicate via RCR. They feature the nonanucleotide motif with high sequence homology

to BFDV and PCV (Table 1-1) and, as with BFDV, have a short inverted repeat sequence flanking the nonanucleotide motif (Arguello-Astorga *et al.*, 1994; Bassami *et al.*, 1998). CAV, even though not possessing the stemloop structure, has a very similar nonanucloutide motif to BFDV. ORF V1 of PCV, which is closely related to BFDV, encodes a putative replication-associated protein. This protein carries all the sequence motifs commonly found and required for RCR, and additionally a P-loop motif (Niagro *et al.*, 1998). Although experimental proof is not available, these findings in BFDV and its similarities to other circular ssDNA viruses, suggest that BFDV replicates via RCR.

1.11 Recombinant protein technology

1.11.1 Recombinant protein expression systems

Many systems have been developed for production of recombinant proteins, including bacterial, yeast, mammalian cell, insect cell and cell free systems. Today's knowledge of *E. coli* genetics, molecular biology and biochemistry far exceeds that of any other organism. Bacteria can be grown and genetically manipulated in an easy and inexpensive manner, and many foreign proteins are expressed at a high level (Sambrook and Russel, 2001). Due to these characteristics, bacterial systems and their phages are the preferred choice of laboratories engaging in development of recombinant proteins. Conversely, use of bacterial systems for expression of biologically active proteins may incur problems, due to the absence of posttranslational modification in these systems. Additionally, the foreign proteins may become tied up in inclusion bodies, making purification of the protein intricate (Coligan *et al.*, 2002; Sambrook and Russel, 2001).

Problems associated with bacterial recombinant protein expression can be overcome with the use of eukaryotic systems including mammalian and insect cell expression systems. For example baculovirus expression systems utilize insect cells, which are

eukaryotic cells. Therefore protein modifications, processing and transport systems are similar to those found in higher eukaryotes. Large amounts of recombinant protein can be relatively easy produced in baculovirus systems by using a helper independent virus that can be propagated to high titres in insect cells adapted for growth in suspension culture (Coligan *et al.*, 2002). In contrast to bacterial systems, most of this over expressed protein will stay soluble in the cells. Thus baculovirus is increasingly becoming a popular choice in recombinant protein technology (Coligan *et al.*, 2002). The majority of protein expressed in this system occurs during acute lytic infection of the insect cells, resulting in addition to more effort and expertise, a requirement for constant generation of new virus stocks and cells (Sambrook and Russel, 2001).

Yeast systems, also eukaryotic, have for a long time been utilised for expressing recombinant proteins, however they have been found to be vastly inefficient for protein expression, producing low quantities of protein and are therefore not ideal for experiments where the aim is to produce significant recombinant protein for use in, for example, vaccination (Sambrook and Russel, 2001).

For this honours project, an *E. coli* based system was chosen. The reason for this is the ease with which recombinant bacteria can be made, use of inducible promoters and fusion tags allowing for controlled expression and purification of the protein, and finally the low cost of using an *E. coli* based system.

1.11.2 Application of recombinant proteins

Recombinant proteins have several uses. Haemaglutination inhibition (HI) is a specific and sensitive technique used for detection of circulating antibodies in animals, indicating whether the animal has previously been challenged with an infection of a haemagglutinating virus. The technique is a standard serological test for many

haemagglutinating viruses. BFDV is a haemagglutinating virus (Raidal and Cross, 1994b) and a HI technique has been developed (Raidal *et al.*, 1993c). However, this technique requires access to native virus, and thus limits the use of this technique for wide spread diagnosis of BFDV infection. A recombinant BFDV capsid protein would allow for more extensive use of this technique.

Enzyme-linked immunosorbent assay (ELISA) is another technique used for detection of circulating antibodies and is a valuable tool in disease diagnosis (Roitt *et al.*, 2001). Recently, Johne *et al.* (2004) developed an ELISA and an immunoblotting technique successful in detecting antibodies to BFDV in psittacine sera, using a truncated recombinant BFDV capsid protein expressed in *E. coli*. ELISA based on Baculovirus-expressed PCV-2 capsid protein has also been described (Liu *et al.*, 2004).

Recombinant proteins may also be applied as vaccines for control of viral, bacterial, parasitic and other diseases. The truncated recombinant BFDV capsid protein developed by Johne *et al.* (2004) was shown to be successful in raising an antibody response in a chicken. Immune response to recombinant hemagglutinin-neuraminidase glycoprotein of *Perste des petits ruminants* virus in goats has been reported (Sinnathamby *et al.*, 2001). The cathepsin B-like protein of juvenile *Fasciola hepatica* has been cloned and expressed in yeast and shown to be antigenic in vaccinated rats (Law *et al.*, 2003). Vaccination against *Fasciola hepatica* resulting in significant protection using a recombinant antigen, has been reported (Almeida *et al.*, 2003).

1.12 Hypothesis

A recombinant BFDV capsid protein expressed using a bacterial system is antigenic, causing the host to respond by producing antibodies that would recognize both the recombinant protein and native virus particles.

1.13 Aims

The aims of this honours project were to:

1) Produce a recombinant BFDV capsid protein, using a bacterial expression system.

2) Determine whether the recombinant protein is recognised by sera from naturally infected and challenged bird species.

3) Determine whether the protein induces an antibody response in sheep and whether these antibodies will recognize native virus.

4) Determine whether the protein induces an antibody response in psittacine birds.

2 Materials and Methods

2.1 Viral DNA

Feathers or blood samples known to contain BFDV were provided by the Diagnostic Pathology Service of Murdoch University Veterinary Hospital. The QIAamp DNA Blood Mini kit (QIAGEN, Australia) was used to extract BFDV DNA according to the directions provided by the manufacturer as follows. Twenty microliters of proteinase K was pipetted into the bottom of a 1.5 mL microcentrifuge tube and 200 µL whole blood added. Two hundred microliters of buffer AL was further added and mixed by pulse-vortexing for 15 s. The sample was then incubated for 10 min at 56°C and then microcentrifuged briefly to remove drops from the inside of the lid. Two hundred microliters of 100% ethanol was added, mixed by 15 s pulse-vortexing and briefly centrifuged again. The mixture was then transferred to a QIAamp spin column inserted in a 2 mL collection tube and centrifuged at 6,000 *g* for 1 min. The spin column was transferred to a new collection tube and centrifuged for 1 min at 6,000 *g*. Five hundred microliters of buffer AW1 was added to the tube and centrifuged as before. The spin column was then transferred to a clean collection tube, 500 µL buffer AW2 added and centrifuged at 20,800 *g* for 3 min. The spin column was transferred to a new 1.5 mL microcentrifuge tube, 200 µL buffer AE added, incubated at room temperature for 1 min and finally centrifuged for 1 min at 6,000 *g*.

2.2 Cells used for expression of recombinant proteins

High efficiency JM109 competent *Escherichia coli* cells (Stratagene, Australia) were used for transformation and expression of recombinant BFDV capsid protein. This *E.*

coli strain is of the genotype *e14-(McrA-) recA1 endA1 gyrA96 thi-1 hsdR17*(rK- mK+) *supE44 relA1 Δ(lac-proAB)* [F' *traD36 proAB lacI*^{q}*ZΔM15]*.

2.3 Amplification of BFDV ORF C1

PCR was used to amplify BFDV ORF C1 from extracted viral DNA. All reactions were performed using thermal cyclers: Applied Biosystems GeneAmp PCR system 2400, BioRad MyCycler or the Eppendorf Mastercycler Gradient thermal cycler in 0.2 mL microcentifuge tubes (Sarstedt, Germany). Specific primers were designed using computer programs Amplify for Analysing PCR Experiments (Engels, 1992) and Primer Express (version 1.0, ABI Prism, Applied Biosystems) based on the BFDV-AUS isolate (accession number: AF080560) and analysed using blastn (Altschul *et al.*, 1997) in BioManager by ANGIS (http://www.angis.org.au). The region of ORF C1 for producing full length 5' truncated and 3' truncated recombinants was chosen on the basis of published sequence data (accession number: AF080560) and predicted antigenicity and hydrophilicity charts using the algorithms Antigenic index and Kyle/Doolittle hydrophilicity in MacVector (version 6.5.1, Oxford Molecular Group, Madison, WI, USA). These predictions can be viewed in Figure 2-1. The latter would result in expression of a carboxy-terminus (C-terminus) truncated recombinant capsid protein. The design of 5' truncated recombinants was based on predicted hydrophilic regions within the protein and would express an aminoterminus (N-terminus) truncated recombinant capsid protein. The three regions of ORF C1 were amplified using: primers Forward 1 and Reverse 1 (primer set A) for full length; Forward 1 and Reverse 2 (primer set B) for 3' truncated and primers Forward 2 and Reverse 1 (primer set C) for 5' truncated amplification of the ORF C1. Position and orientation of primers can be seen in Figure 2-2 and primer details can be seen in Table 2-1.

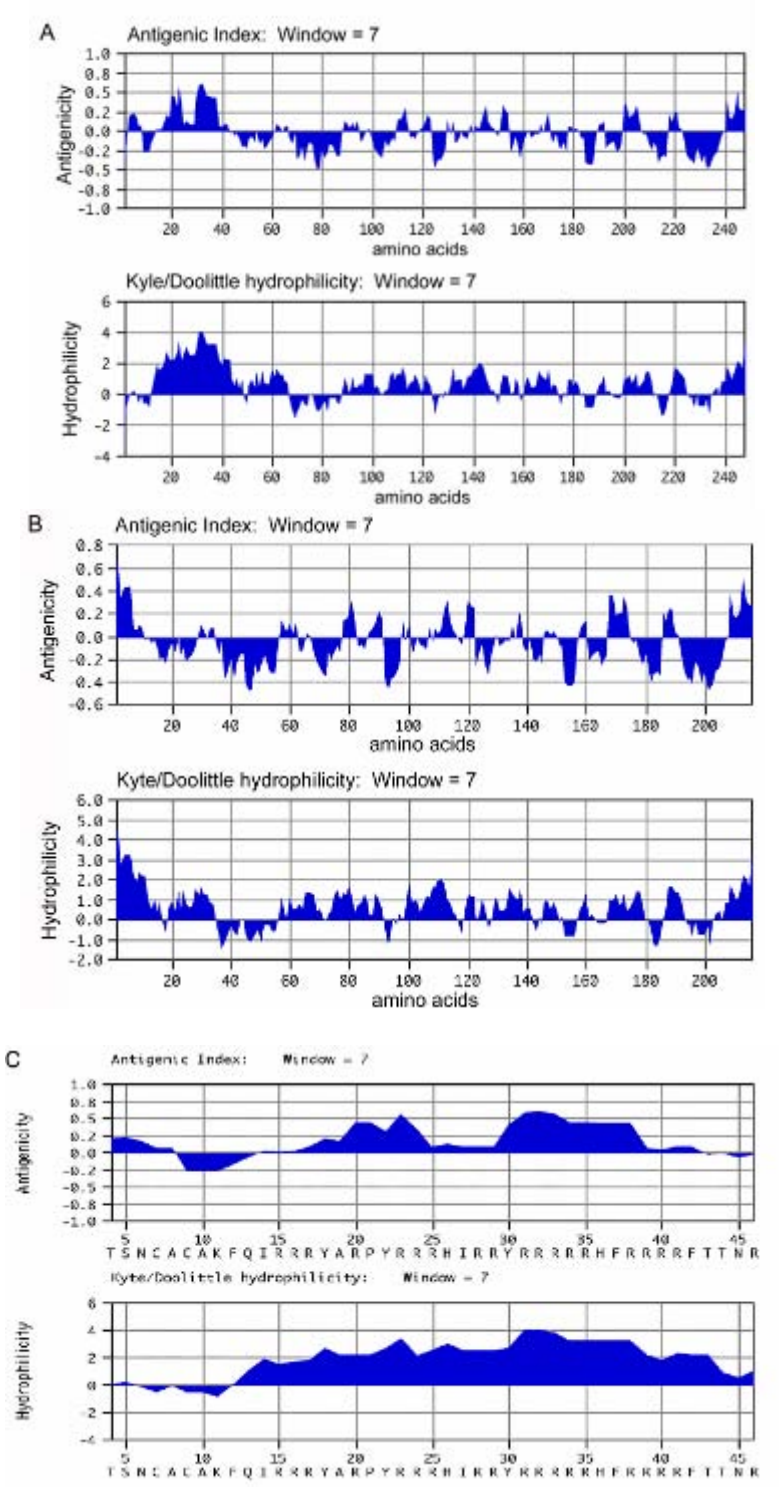

Figure 2-1. Antigenicity and hydrophilicity charts of A) full length BFDV capsid protein, B) region chosen for 5' truncated BFDV capsid protein and C) region chosen for 3' truncated BFDV capsid protein.

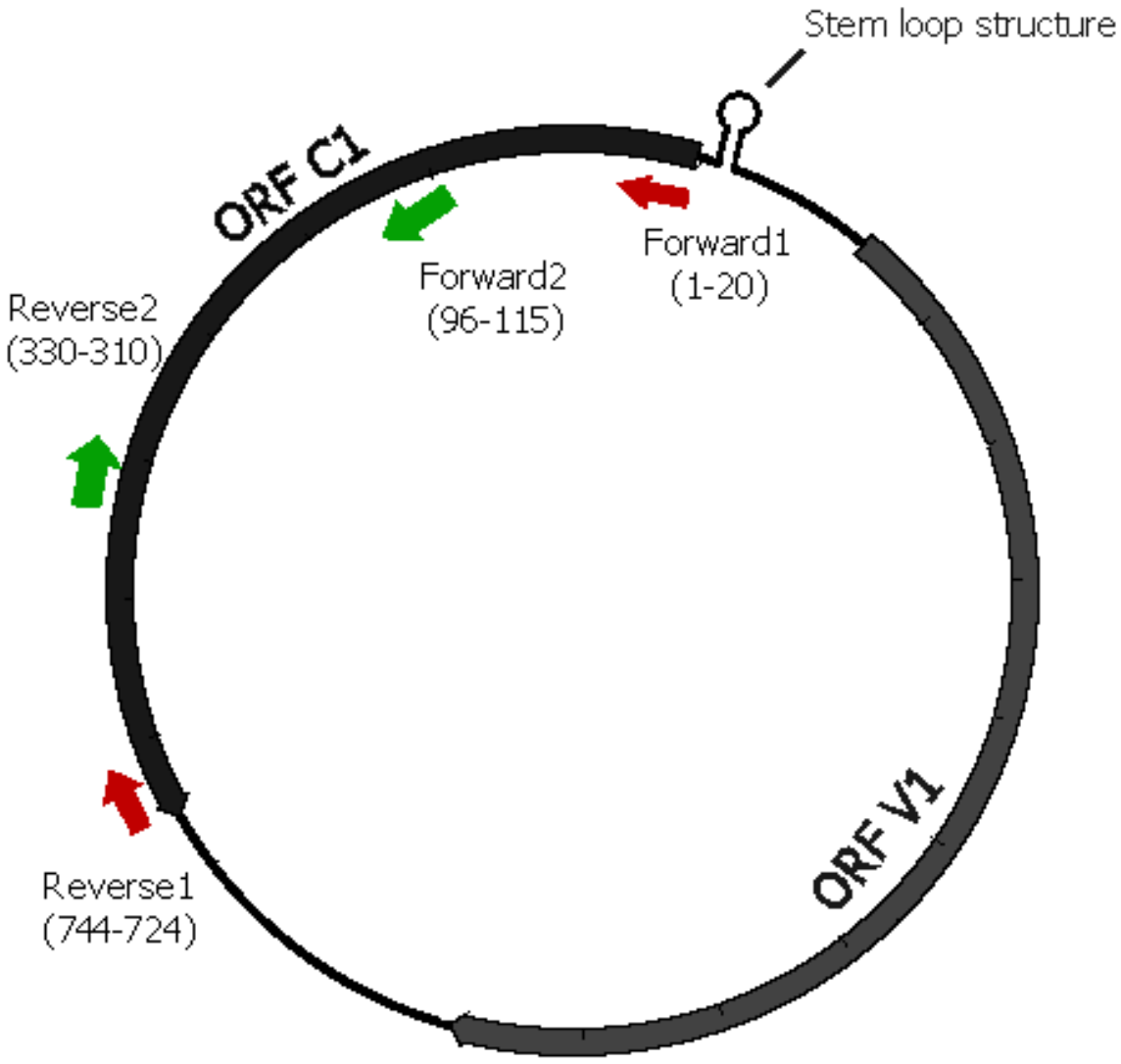

Figure 2-2. Position and orientation of primers in the BFDV genome (ds replicative form) used for full length and truncated ORF C1 amplification.

Table 2-1. Primer sequences used to amplify ORF C1 full length and truncated fragments of the BFDV genome. The binding position of the primer within ORF C1 is given (nt).

Primer	Primer sequence	nt position in ORF C1 (primers: Forward 1 and 2, and Reverse 1 and 2) and nt position in vector (primers: SP6 and pinseq)
Forward 1	5'cat gtg ggg cac ctc taa ct3'	1-20
Reverse 1	5'tta agt gct ggg att gtt agg ggc3'	744-724
Forward 2	5'ccg acg tag gca ctt ccg ca3'	96-115
Reverse 2	5'ggg cct cat ttc cat ttt3'	330-310
SP6	5' tat tta ggt gac act ata g 3'	451-468
Pinseq	5' cgt gac gcg gtg cag ggc g 3'	325-343

Thermal cycle conditions were optimised for optimal primer annealing temperature using an annealing temperature gradient as described below in section 2.3.1. The PCR conditions: magnesium chloride concentration, primer concentration and dNTP concentration, were optimised according to the Taguchi method applied to PCR described by (Cobb and Clarkson, 1994) and outlined in section 2.3.2.

2.3.1 Optimization of annealing temperature

A basic PCR mixture with excess primers and dNTPs was utilised to determine the annealing temperature range that could be used for amplification. PCR reactions were carried out in a total volume of 30 μL and consisted of 0.7 U Expand High Fidelity DNA polymerase (Roche), 1× polymerisation buffer (composition not supplied, Roche), 2.083 mM $MgCl_2$ (Roche), 300-400 ng DNA template, 0.083 mM dNTPs, 25-35 ρmol

of each primer and made up to 30 μL with Ultra pure water (Fisher Biotec, Australia). Thermal cycle protocol for temperature optimisation is outlined in Table 2-2.

Theoretical optimal annealing temperature for each primer was calculated by the formula ((g+C)×4)+((A+T)×2). The annealing temperature calculated for primers Forward 1, Reverse 1 and Forward 2 appeared to be excessively high, thus a closer to normal temperature of 56°C was chosen as the mean temp. Additionally, the calculated annealing temperatures for the two primers in each pair differed significantly, thus two annealing temperatures were used in the thermal cycle to accommodate these differences. Further, the optimal annealing temperatures were found by using a temperature gradient, deviating ±5°C from the theoretical temperature determined, on an Eppendorf Mastercycler Gradient thermal cycler. Finally, the annealing temperature giving the highest yield of amplification was determined to be 56.4°C and 44.7°C for primer sets A and C, and 53.7°C and 42°C for primer set B by examining the fluorescence strength of fragments visualised by electrophoresis as described under section 2.4.

Table 2-2. Thermal cycling protocol used in optimisation of annealing temperature of the PCR.

Stage	Temperature (°C)	Time (min)	Cycles
1	95	5	1
2	95	0.5	30
	56 ± 5	0.5	
	44 ± 5	0.5	
	72	1	
3	72	10	1
4	15	HOLD	

2.3.2 Optimization of magnesium chloride, primer and dNTP concentration for PCR amplification of BFDV ORF C1

Reactions were set up at a final volume of 30 μL, containing 0.7 U of Taq DNA polymerase (Roche), 1×polymerisation buffer (composition not supplied, Roche) and 300-400 ng DNA template. Three different concentrations of $MgCl_2$, primer and dNTP (Table 2-3) were chosen and arranged in an orthogonal array (Table 2-4) to meet the requirements of the Taguchi method for PCR optimisation. In an orthogonal array, each concentration (A, B or C) occurs an equal number of times between each row, thus less reactions are required to determine the effects of each variable ($MgCl_2$, primer and dNTP) (Cobb and Clarkson, 1994). The thermal cycle condition was carried out using the protocol outlined in Table 2-2 using optimal annealing temperatures as determined.

Table 2-3. Concentrations used for optimisation of PCR magnesium chloride, primer and dNTP concentration. Concentrations of each reagent were used in combinations as seen in Table 2-4.

	Concentrations		
Reagent	***A***	***B***	***C***
dNTPs	0.033 mM	0.067 mM	0.1 mM
$MgCl_2$	0.417 mM	1.25 mM	2.08 mM
Primer	25-35 ρmole	31-44 ρmole	38-52 ρmole

Table 2-4. Orthogonal array used for optimisation of PCR magnesium chloride, primer and dNTP concentration.

	Reaction								
Reagent	***1***	***2***	***3***	***4***	***5***	***6***	***7***	***8***	***9***
dNTPs	A	A	A	B	B	B	C	C	C
$MgCl_2$	A	B	C	A	B	C	A	B	C
Primers	A	C	B	C	A	B	C	A	B

Optimal dNTP, $MgCl_2$, and primer concentrations were determined to be 0.1 mM dNTPs, 1.25 mM $MgCl_2$ and 31-44 ρmole primers for primer set A; 0.067 mM dNTPs, 2.08 mM $MgCl_2$ and 25-35 ρmole primer for primer set B; and 0.033 mM dNTPs, 1.25 mM $MgCl_2$ and 38-52 ρmole primers for primer set C by examining the fluorescence strength of fragments visualised by electrophoresis as described under section 2.4.

2.4 Agarose gel electrophoresis

Agarose gel electrophoresis was carried out using horizontal gels of 0.8 or 1.0% (w/v) agarose (Progen, Australia), TAE buffer (40 mM Tris-HCl; 20 mM glacial acetic acid; 2 mM EDTA; pH 7.0) and 10 μg/mL ethidium bromide (Sigma, Australia). PCR products and DNA were loaded into the agarose gel and electrophoresed for 30 min at 90 Volts in TAE buffer using a Minisub DNA cell (BioRad, Australia). Fragments were visualized

by UV illumination at 260 nm with a UVP transilluminator (BioRad, Australia) or the Gel Doc-1000 system and Molecular Analyst Software version 1.4 (BioRad, Australia). The size of the separated DNA fragments was determined by comparing them with a 1 kb DNA ladder (Promega, USA), 100 bp DNA ladder (Invitrogen, Australia) or a 1 kb plus DNA ladder (Invitrogen, Australia).

2.5 Ligation of PCR products into expression vector

The linear Promega pPinPoint™ Xa-1 T-vector was used to construct the recombinant vector for expressing recombinant BFDV capsid proteins. This vector has a coding region for Beta-lactamase (AmpR), multiple cloning region, biotin purification tag coding region, taq promoter, SP6 RNA polymerase promoter and a T7 RNA polymerase promoter (Figure 2-3). This vector is linear and deoxythymidine tailed, and therefore does not require restriction digest before ligation, only Taq polymerase amplification of insert resulting in the appropriate deoxyadenine overhangs.

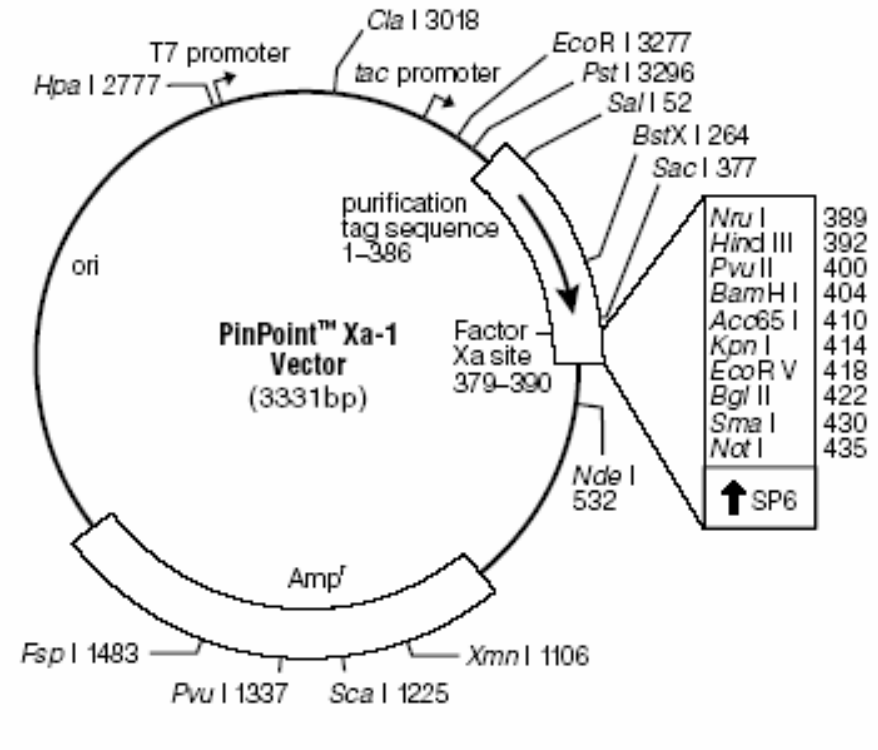

Figure 2-3. The Promega pPinPoint™ Xa-1 vector circle map and sequence reference points. The T-vector is produced by digesting the Promega pPinPoint™ Xa-1 vector with *Eco*R V and adding a 3' terminal deoxythimidine to both ends (Promega, 2000).

One ligation reaction was set up for each amplicon and the PCR product was not purified prior to ligation. Ligation reactions were set up in 0.65 mL microcentrifuge tubes with 12.5 ng vector, 1.0 U T4 DNA ligase, T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 25 μg/mL BSA; pH 7.8; New England BioLabs, Australia), 0.25 μL PCR reaction product and made to a final volume of 10 μL with distilled water. Reactions were incubated in a 15°C water bath overnight.

2.6 Transformation of JM109 competent cells with recombinant pPinPoint™ Xa-1 T-vector

Two microlitres of ligation reaction was transferred to a 1.5 mL microcentrifuge tube (Sarstedt, Germany) and centrifuged 20,800 *g* for 4 to 5 s to collect ligation mix at the

bottom. Chemically competent *E.coli* JM109 were removed from -80°C storage and thawed on ice. Fifty microlitres of cell suspension was added to the tube containing the ligation reaction and the contents of each tube mixed by gentle flicking. Tubes were then incubated on ice for 30 min prior to heat shock transformation of the JM109 *E.coli* for 50 s in a 42°C water bath and then incubated on ice for 2 min. Nine hundred and fifty microliters of SOC medium (0.02 g/mL Bacto-tryptone, 0.005 g/mL Bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM Mg^{2+} and 20 mM glucose) was added to each tube, tubes inverted to mix and incubated for 1 hour at 37 °C with shaking at 225 rpm. One hundred microliters of each transformation mix was spread onto each of two Luria Bertani (LB) agar (10 g/L Bacto-tryptone, 5g/L Bacto-yeast extract, 5g/L NaCl and 15 g/L agar) plates supplemented with 100 μg/mL ampicillin. The remaining transformation mix was pelleted at 16,000 *g* for 2 min, supernatant discarded, cells resuspended in 200 μL SOC medium and 100 μL plated onto each of two LB agar plates supplemented with 100 μg/mL ampicillin. All plates were incubated at 37°C for 48 hours.

2.7 Screening of *E.coli* transformants

Transformants to be used for expression of recombinant BFDV capsid protein were selected by screening for occurrence and orientation of insert in the plasmid vector. Screening was achieved by PCR, restriction digest and Dye-terminator sequencing of the plasmids.

2.7.1 PCR screening of transformants

Isolated colonies were subcultured onto fresh LB agar plates supplemented with 100 μg/mL ampicillin and incubated overnight at 37°C. Between 2 and 10 isolated colonies from each of the 12 plates were picked using a flame sterilized loop, pricked onto a

grided LB agar plate supplemented with 100 μg/mL ampicillin (to be used as a storage library), and then the loop was swabbed in a 0.5 mL microcentrifuge tube containing 50 μL PCR colony buffer [TE pH 8.0, 0.1 % w/v Triton X-100 (Promega, Australia)]. Colonies from the same plate were added to the same tube. Cells, resuspended in PCR colony buffer, were boiled for 5 min and centrifuged for 5 min at 20,800 *g* to pellet cell debris.

PCR reactions for screening were made to a total volume of 30 μL consisting of 0.7 U Expand High Fidelity DNA polymerase (Roche), 1×polymerisation buffer (composition not supplied, Roche), 2.083 mM $MgCl_2$ (Roche), 300 to 400ng DNA template, 0.083 mM dNTPs, 25-35 ρmol of each primer and made up to 30 μL with Ultra pure water (Fisher Biotec, Australia). Thermal cycle are outlined in Table 2-5. Colonies from the reactions found positive by the pooled PCR screening reactions were further screened individually.

Table 2-5. Thermal cycle protocol used for screening transformants for insert.

Stage	Temperature (°C)	Time (min)	Cycles
1	95	5	1
2	95	0.5	30
	50	0.5	
	46.1	0.5	
	72	1	
3	72	10	1
4	15	HOLD	

2.7.2 Alkaline miniprep technique for purification of recombinant constructs for use in restriction digest screening

Colonies found positive by PCR were grown up to stationary phase in 10 mL 2×YT broth supplemented with 100 µg/mL ampicillin overnight at 37 °C with shaking at 225 rpm. Three millilitres of each cell culture was centrifuged at 10,000 *g* for 30 s to pellet the bacteria. Supernatant was discarded and pellet resuspended in 200 µL resuspension solution [25 mM Tris, 10 mM EDTA pH 8.0, 20 µg/mL Ribonuclease A (Sigma, Australia)]. Two hundred microliters of a solution of 0.2 M NaOH, 1% w/v SDS and distilled water was added and tubes gently inverted 8 times before slowly adding 200 µL of a solution of 1.8M KOA and 11.5% (v/v) glacial acetic acid. Tubes were then gently inverted until white precipitate appeared and then centrifuged for 10 min at 10,000 *g*. Supernatant was transferred to a clean tube and 600 µL of ice cold 100% isopropanol added. Reactions were left to precipitate at room temperature for 15 min. Tubes were then centrifuged for 30 min at 12,000 *g*, supernatant discarded, pellet washed in 200 µL 70% ethanol, DNA pelleted at 12,000 *g* for 5 min, supernatant taken off by aspiration and pellet dried using a Savant speed vac concentrator. Finally, the pellet was resuspended in 50 µL TE buffer (pH 8.0).

2.7.3 Restriction digest screening of pPinPoint™ recombinant construct

Orientation of insert in full length and 3’ truncated recombinants was determined by restriction digest. The pPinPoint™ vector has a single *Bam*H I restriction site at nt 406 and the full length insert at nt 584, thus full length recombinants with correctly oriented insert should produce fragments of 598 and 3477 bp when digested with *Bam*H I. Restriction digest of full length recombinants was carried out as follows. Restriction digest reactions were made up to a final volume of 30 µL in 0.65 mL microcentrifuge tubes, consisting of 25 µL plasmid prep, 20 U *Bam*H I (New England Biolabs, Inc),

1×*Bam*H1 buffer (New England Biolabs, Inc), 1×BSA (New England Biolabs, Inc) and made up to 30 µL with distilled water. Reactions were incubated in a 37°C water bath for 1.5 hours.

The 3' truncated recombinants were digested with *Sal* I which cuts the pPinPoint™ vector at nt 52, and with *Sph* I which cuts the insert at nt position 22, thus 3' truncated recombinants with correctly oriented insert should produce fragments of 388 and 3273 bp when digested with *Sal* I and *Sph* I. Restriction digest of 3' truncated recombinants was carried out as follows. Restriction digest reactions were made up to a final volume of 30 µL in 0.65 mL microcentrifuge tubes, consisting of 20 µL plasmid prep, 10 U *Sal* I (New England Biolabs, Inc), 1×*Sal* I buffer (New England Biolabs, Inc), 1×BSA (New England Biolabs, Inc) and made up to 30 µL with distilled water. Reactions were incubated at 37°C for 21 hours. The enzyme was then heat inactivated in a 65°C water bath for 20 min, 5 U *Sph* I added and incubated at 37°C for 1 hour. Results of restriction digest were visualized as outlined under section 2.4.

2.8 Extraction and purification of plasmid for dye-terminator sequencing

The recombinant pPinPoint™ Xa-1 T-vector that contained the correctly sized and oriented insert were purified prior to sequencing using the BioRad Aurum Plasmid Mini Kit, performed exactly as instructed by the manufacturer, using 3 mL of overnight culture grown in 10 mL LB broth supplemented with 100 µg/mL ampicillin. Three millilitres (2×1.5 mL) of this culture was centrifuged for 1 min at 10,000 *g*, supernatant removed by aspiration and pellet resuspended in 250 µL resuspension solution by vortexing. Two hundred and fifty microliters of lysis solution was added and contents of tube mixed by inverting rapidly 8 times. Then, 350 µL of neutralization buffer was added and tubes inverted briskly 8 times and the neutralized lysate centrifuged at 10,000 *g* for 5 min. The supernatant was transferred by pipetting to a plasmid mini column

inserted into a 2 mL cap-less tube, and centrifuged at 10,000 *g* for 1 min. Filtrate was discarded, 750 µL wash solution added to the mini column and centrifuged for 1 min at 10,000 *g*. The wash solution was then discarded, column replaced into the tube and centrifuged again at 10,000 *g* for 1 min to remove residual wash solution. Mini column was then transferred to a 1.5 mL capped microcentrifuge tube, 50 µL elution solution added onto the membrane and allowed to saturate the membrane for 1 min. Finally, the tube was centrifuged at 10,000 *g* for 1 min to elute the plasmid, and mini column discarded.

2.9 Dye-terminator sequencing

Recombinant plasmid vectors were sequenced by the Dye-terminator sequencing method. This method of sequencing is similar to PCR with the amplification of the DNA; however, in contrast to PCR, dye-terminator sequencing utilizes only one primer. Thus elongation is only terminated when there is incorporation of a fluorescently labelled dideoxynucleotidetriphosphate (ddNTP). Such terminations will be at random, resulting in fragments of varying size labelled at the end. Amplification reactions are electrophoresed, separating the fragments on the basis of molecular weight and allowing the sequence to be determined on the basis of location of each fluorescent ddNTP.

All recombinant plasmids proven to have the insert in correct orientation were sequenced twice, once with the SP6 primer (Table 2-1) and once with the pinseq primer (Table 2-1). Reactions were made to a total volume of 10 µL in 0.2 mL PCR tubes and consisted of 2 µL BigDye terminator mix version 3.1 (Applied Biosystems, Australia, composition not given), 5×sequencing buffer (Applied Biosystems, Australia, composition not given) 3.2 ρmoles of one sequencing primer, 300-400 ng of plasmid

DNA and made up to a final volume of 10 µL with ultra pure water (Fisher Biotec, Australia). Reactions were cycled according to the protocol outlined Table 2-6.

Table 2-6. Thermalcycle protocol used for dye-terminator sequencing of recombinant plasmids.

Stage	Temperature (°C)	Time	Cycles
1	96	5 min	1
2	96	10 s	25
	55	5 s	
	60	4 min	
3	60	10 min	1
4	15	HOLD	

Excess primers, salt and nts were removed by ethanol precipitation of the sequencing products. For each sequencing reaction a 0.5 mL tube containing a solution of 92.6% ethanol, 111.1 mM sodium acetate and 4.6 mM EDTA was prepared. Sequencing products were added, mixed by pipetting and incubated on ice for 20 min. Tubes were microcentrifuged at 20,800 *g* for 30 min at room temperature, supernatant discarded and pellets rinsed in 125 µL 80% ethanol. Finally, the tubes were centrifuged for 5 min at 20,800 *g*, supernatant removed and pellet dried under vacuum (SpeedVac Concentrator, Savant). Sequencing reactions were electrophoresed and interpreted by Francis Brigg (State Agricultural Biotechnology Centre sequencing facility, Murdoch University). Briefly; samples were resuspended in 2.25 µL loading dye [1:5 (50 mM EDTA, 50 mg/mL blue dextran): Hi Di Foramide], 0.85 µL of the resuspended sample was loaded into the 5.25% PAGE Plus acrylamide (6 M Urea) gel, and electrophoresed at 2400 Volts, 50 mA, 200 watts, gel temperature 51°C for 13 hours with laser power 40 mW, CCD offset 250, CCD Gain 2 in the sequencer 377XL (Applied Biosystems, Australia)

and gels interpreted with the ABI PRISM 377XL Data Collection version 2.6 and Sequencing Analysis version 3.4.1 (both Applied Biosystems, Australia). Finally, sequences were edited using seqEd v1.0.3 (Applied Biosystems, Australia). All edited sequences were imported into BioManager (www.biomanager.angis.org.au) and analysed using the programmes blastn v2.0 to 2.29 and translate.

2.10 Optimisation of protein expression and cell lysis

Proteins expressed in bacterial systems may be bound up in IBs, termed insoluble protein, thus complicating protein purification. The following experiments were carried out to achieve optimal yield of expressed soluble protein and to determine the best method of lysing cells for achieving the highest overall yield of released protein for purification.

2.10.1 Optimization of induction of full length and C-terminal truncated recombinant protein expression

Cells were transferred using a flame sterilized loop from glycerol stocks to 10 mL of 2×YT broth containing 100 µg/mL ampicillin. Tubes were incubated overnight with shaking (225 rpm) at 37°C. Overnight cultures were then diluted 1:20 with 2×YT containing ampicillin (100 µg/mL) and 2 µM d-Biotin (Sigma, Australia). Cultures were incubated at 37°C with shaking at 225 rpm and induced at optical densities measured at wavelength 600nm (OD_{600nm}) 0.32, 0.62, 0.90 and 1.19 for full length and OD_{600nm} 0.32, 0.59, 0.94 and 1.17 for C-terminal truncated protein with isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma, Australia) at a final concentration of 0.1 mM and culture simultaneously supplemented with d-Biotin to a final concentration of 4 µM. Samples were taken every 30 min for 5.5 hours post induction and additionally

overnight with C-terminal truncated protein. Samples were centrifuged at 20,800 *g* for 5 min and supernatant discarded.

Pellets were further resuspended in cell lysis buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl and 5% v/v glycerol) at a ratio of 0.1 mL buffer per mL induction culture. Tubes were centrifuged at 20,800 *g* for 7 min and supernatant discarded. The pellet was resuspended as before and phenylmethylsylphonyl fluoride (PMSF) (Roche, Germany) and lysozyme (Sigma, Australia) added to final concentrations of 1 mM and 1 mg/mL, respectively. Samples were rocked at room temperature for 10 min. Tubes were freeze-thawed using liquid nitrogen and a 42°C water bath 4 times. Triton X-100 was added at a final concentration of 1% (v/v) and tubes vortexed to mix. To separate soluble protein and insoluble IB protein, samples were centrifuged at 20,800 *g* at 4°C for 20 min. Soluble fraction (supernatant) was then transferred to a clean tube and the insoluble fraction (pellet) was re-suspended in 0.1 mL cell lysis buffer by vortexing. Optimal cell density and length of induction was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western immunoblot as described in sections 2.11 and 2.12, respectively.

All cell densities and lengths of induction resulted in production of insoluble protein, however it was found that at OD_{600nm} 0.62 and 2.5 hours of induction, full length expressing *E. coli* JM109 gave a low level of expressed soluble protein in addition to the stronger fraction of insoluble protein expressed. For C-terminal truncated protein expression, soluble protein could not be produced, however a strong insoluble fraction was found at OD_{600nm} 0.94 with 5 hours induction.

2.10.2 Optimization of cell lysis

Three procedures for cell lysis; lysozyme, sonication and freeze-thaw; were tried in order to determine the most efficient method for lysing JM109 *E. coli* JM109. Full length expressing, C-terminal truncated expressing, non-recombinant JM109 and Jembrana Disease Virus capsid protein expressing (termed Jca) cells were grown up overnight as previously described. These cultures were diluted 1:20 with 2×YT broth supplemented with 100mg/mL ampicillin (except for in the case of *non-recombinant E. coli* JM109), incubated for 1.5 hours at 37°C, d-Biotin added (final concentration 2 µM) and cultures induced at OD_{600nm} 0.81 for full length, 0.72 for 3' truncated, 0.53 for Jca and 0.95 for *non-recombinant* JM109 with IPTG (final concentration 100 µM) overnight. Cultures were centrifuged at 2,000 *g* for 7 min at 4°C, supernatant discarded and pellets resuspended in 1 mL cell lysis buffer per 10 mL induced culture by pipetting up and down on ice. PMSF was added to a final concentration of 1mM.

Lysis by lysozyme was performed by adding lysozyme to the resuspended cells at a final concentration of 1 mg/mL. The solution was then rocked for 2 hours at room temperature. Lysis by sonication was performed with sonicator XL2015 (Unimed, Australia) using 50% pulse cycles of 2×2 min at 3.5 microtip. For freeze-thaw lysis, lysozyme was added to a final concentration of 1 mg/mL. Tubes were submerged in liquid nitrogen until solidified and then thawed in a 42°C water bath. Cycle was repeated 10 times. Efficiency of lysis treatment was determined by microscope (Olympus microscope CH-2) examination at 400× magnification. Sonication was found to be the most efficient method for lysing full length and C-terminal truncated protein expressing *E. col* JM109*i*. However, *non-recombinant* JM109 cells and Jca expressing JM109 cells were more efficiently lysed by the freeze-thaw method.

2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDS-PAGE was carried out in vertical gels with 12.43% acrylamide (BioRad, Australia). Protein suspensions were made up with 1×loading dye (62.5 mM Tris-HCl, 10% v/v glycerol, 2% w/v sodium dodecyl sulphate, 5% v/v mercaptoethanol, 0.5% w/v bromophenol blue), loaded into the SDS-PAGE gel and electrophoresed for 1 hour at 200 Volts in 1×running buffer [3.47 mM SDS (BDH, Australia), 191.8 mM Glycine (BDH, Australia), 24.8 mM Tris-HCl (Progen Biosciences, Australia)] using the BioRad gel system. The size of separated proteins was determined by comparing with Precision Plus Protein Standards Dual colour (BioRad, Australia). To visualize protein bands, gels were stained with Coomassie Blue (45% methanol, 10% glacial acetic acid, 0.1% w/v Coomassie Brilliant Blue) overnight and excess stain removed by soaking in de-stain (40% methanol, 10% glacial acetic acid, 50% distilled water) overnight. Imaging was performed using a Minolta Dimage Xt digital camera and image contrast and brightness enhanced with Paint Shop Pro 6.

2.12 Western immunoblot

Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Australia) using a transblot apparatus (BioRad, Australia) or using a Transblot SD semidry transfer cell (BioRad, Australia). Nitrocellulose membrane, SDS-PAGE gel and Whatman filters were equilibriated for 20 min in western transfer buffer [20% methanol, 25 mM Tris (Progen Biosciences, Australia), 192 mM glycine (BDH, Australia)] prior to transfer. Transfer was carried out overnight at 40V in western transfer buffer when using transblot apparatus and 30 min and 15 Volts with the semi dry apparatus. Membranes were incubated in blocking solution [1×TBS, 0.05% v/v Tween (Sigma, Australia), 5% w/v skim milk powder] overnight at 4°C or for 1 hour at 37°C. Blocking solution was removed, and bound protein reacted with streptavidin-

alkaline phosphatase, chicken-anti-BFDV sera or cockatoo-anti-BFDV as described in sections 2.12.1, 2.12.2 and 2.12.3, respectively. Imaging was performed using a Nikon digital camera and image contrast and brightness were enhanced with Paint Shop Pro 6.

2.12.1 Streptavidin-alkaline phosphatase

The nitrocellulose membrane was incubated in blocking solution containing streptavidin-alkaline phosphatase (Promega, Australia) at a dilution of 1:5000 or 1:2000 with blocking solution for 1 hour at 37°C, washed 2×10 min with 1×TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% v/v Tween 20) and 10 min with 1×TBS (10 mM Tris-HCl, 150 mM NaCl) before adding Western Blue (Promega, Australia)-the substrate for alkaline phosphatase.

2.12.2 Chicken-anti-BFDV sera

Chicken-anti-BFDV sera with an HI titre of 5120 HI units (HIU) was provided by Dr Shane Raidal. The membrane was incubated with chicken-anti-BFDV sera at a dilution of 1:200 in 1×TBST overnight at room temperature and washed 2×10 min in 1×TBST and 10 min with 1×TBS to remove excess antibody. Horseradish peroxidase (HRP) conjugated rabbit-anti-chicken IgG (MP Biomedicals, Inc) was diluted 1:2000 with TBST and incubated with the membrane for 2 hours at room temperature, membrane washed as described previously and bound antibody visualized by adding HRP substrate [0.5 mg per mL HRP colour development reagent (BioRad, Australia), 16.7% v/v Methanol, 83.3% v/v TBS, 0.015% v/v H_2O_2].

2.12.3 Cockatoo-anti-BFDV sera

Cockatoo-anti-BFDV sera with an HI titre of 5120 HIU was provided by Dr Shane Raidal. The membrane was incubated with cockatoo-anti-BFDV sera at a dilution of 1:200 in TBST overnight at room temperature and washed 2×10 min in 1×TBST and 10

min with 1×TBS to remove excess antibody. Further, the membrane was incubated with goat-anti-cockatoo sera (supplied by Dr Shane Raidal) diluted 1:200 in 1×TBST for 2 hours at room temperature and then washed 2×10 min in 1×TBST and 10 min in 1×TBS. HRP conjugated rabbit-anti-goat IgG (MP Biomedicals, Inc) was diluted 1:2000 with 1×TBST and incubated with the membrane for 2 hours at room temperature. Membrane was washed as described previously and bound antibody visualized by adding HRP substrate.

2.13 Purification of full length recombinant BFDV protein from *E. coli* JM109

As mentioned, foreign proteins expressed in bacterial systems commonly become tied up in inclusion bodies thus resulting in an insoluble protein fraction. Optimization experiments resulted in proteins being expressed as both a soluble form and an insoluble form of the expressed full-length recombinant protein. Thus the purpose of the following purification experiments was to gain pure soluble protein and to purify and solubilize IB protein.

2.13.1 Soluble fraction

Soluble fraction was attempted to be purified by both batch capture and column capture methods. In both instances, 500 µL Softlink™ Soft Release Avidin Resin (Promega, Australia) was used per 20 mL soluble fraction obtained. Resin was prepared before use by pre-adsorbing nonreversible binding sites with 2 resin bed volumes of cell lysis buffer containing 5 mM d-Biotin. The buffer/resin mix was left at room temperature for 15 min, buffer removed and resin regenerated by washing in 8 resin-bed volumes of 10% acetic acid, and then in 8 resin bed volumes of 100 mM phosphate buffer (pH 7.0) until the solution reached pH 6.8. Protein was allowed to bind overnight at 4°C on a vertical rotor.

2.13.1.1 Batch capture

All steps were performed at 4°C. The suspension of overnight binding was centrifuged at 500 *g* for 10 min at 4°C to collect resin at the bottom of the tube and supernatant removed by aspiration. The supernatant was kept to determine the proportion of unbound protein by SDS-PAGE and western immunoblot. Resin was washed in 1 mL ice cold cell lysis buffer per 25 µL of resin by inverting 10 times and then centrifuging the sample at 500 *g* for 10 min at 4°C. The supernatant was aspirated and the wash process repeated 2 more times in 0.5 mL ice cold cell lysis buffer per 25µL resin. All 3 washes were kept to determine, using SDS-PAGE, whether the wash steps removed the recombinant protein or just contaminants. One millilitre elution solution (cell lysis buffer supplemented with 5 mM d-Biotin) was added per 250 µL resin and eluted at 4°C for 1 hour on a vertical rotor (elution 1). The elution was repeated a second time for approximately 5 hours (elution 2) and then finally overnight (elution 3). All 3 elutions were stored as they were expected to contain pure recombinant protein to be used in vaccination and were analysed by SDS-PAGE to determine such.

2.13.1.2 Column capture

All steps were performed at 4°C. The suspension from overnight binding was loaded into a sterile 0.8×4 cm Poly-prep chromatography column (BioRad, Australia) and allowed to flow-through. Flow-through was passed through the column 3 more times. The flow-through was stored and analysed by SDS-PAGE and western immunoblot to determine the amount of unbound recombinant protein. Resin was washed by adding 1 mL ice cold cell lysis buffer per 25 µL resin and inverting 10 times to completely disrupt the resin bead bed. The buffer was allowed to flow through, 0.5 mL fresh ice cold buffer added per 25 µL resin, and column left to allow for flow-through. Resin was washed a third time as for the second time. All washes were analysed for presence of

recombinant protein by SDS-PAGE. Elution solution was added at a ratio of 1 mL elution solution per 25 µL resin, allowed resin to settle for 1 hour and elutant collected in 0.5 mL fractions (elution 1 and elution 2). One millilitre elution solution was added and collected immediately in 0.5 mL fractions (elution 3 and elution 4). Finally, the resin was left in 1 mL elution solution per 25 µL resin overnight and then allowed to flow-through (elution O/N). All elutions were stored and analysed by SDS-PAGE.

Both the column and the batch method proved useless for purification of soluble protein as the biotinylated protein did not bind to the resin.

2.13.2 Insoluble fraction

Two methods were found useful for the purification and solubilization of IB protein; B-PER and Urea/DTT method. The latter however, was considered the most practical and time efficient.

2.13.2.1 B-PER

All steps were performed on ice. Pellet of 400 mL lysed culture was resuspended in 5 mL of cell lysis buffer and then added 20 mL of 1:10 dilution of B-PER (wash 1). Tube was centrifuged (Beckman centrifuge J-30 I) at 16,000 *g* for 30 min in a 4°C pre cooled rotor (Beckman rotor JA 25-50). Supernatant was taken off and pellet resuspended in 1:20 diluted B-PER (wash 2) and centrifuged as before, supernatant taken off and pellet resuspended in 1 mL of cell lysis buffer, then 9 mL buffer added (wash 3) and centrifuged again. The pellet was finally resuspended in 1 mL 1×PBS. All washes and the final product were stored and analysed by SDS-PAGE.

2.13.2.2 Urea/DTT solubilisation

The insoluble fraction used in this procedure was obtained as for the B-PER procedure, with the exception of French press being used for cell lysis in place of sonication, due to

sonicator being out of order. Three millilitres of wash buffer (10 mM Tris-HCl, 5 mM EDTA, 1% v/v Triton X-100) was added per 40 ml pelleted lysate and resuspended by vortexing, shaking, pipetting and using the vertical rotor at 4°C (wash 1). The suspension was centrifuged at 10,000 *g* for 15 min at 4°C (pre cooled Beckman rotor JA 25-50), and supernatant taken off. Three millilitres of wash buffer and 10 µg deoxyribonuclease 1 (Sigma, Australia) was added and the pellet resuspended as before (wash 2). Tubes were centrifuged again, supernatant taken off and pellet resuspended in 3 mL wash buffer (wash 3), centrifuged (10,000 *g*, 15 min) and supernatant taken off. Finally, the pellet was resuspended in 10 mL solubilization buffer [2 M Tris-HCl, 2M Urea, 0.4mM dithiothreitol (Progen, Australia), 1 mM PMSF, pH 12] per 40 mL pelleted lysate. All wash fractions and the final product were analysed by SDS-PAGE.

2.14 Inoculation of sheep with recombinant protein

Two sheep were inoculated with recombinant protein. Inoculum was prepared immediately before injection by homogenizing Urea/DTT solubilised full-length protein (prepared as described above) with Freund's incomplete adjuvant at a ratio of 1:1 using 2 sterile glass syringes connected to each other with a sterile steel tube. Both animals were bled from the jugular vein prior to injection. Prepared inoculum was injected subcutaneously into the left gluteal area and the dorsal neck with 2 mL vaccine in each site for animal number 761 and 1.5 mL for animal number 5.

Twenty one days post injection; both animals were bled and then boosted with 2.5 mL inoculum subcutaneously in both the left rump and the dorsal neck. Both animals were bled 27 days post boosting and then boosted again at the same sites as before with 3 mL for sheep 761 and 2.5 mL for sheep 5. Both animals were bled a last time at 16 days after the second boost. All bloods were collected into sterile plain glass containers and

allowed to clot. Serum was collected and heat inactivated at 57°C for 30 min and then stored at –20°C. Antibody responses were detected by western immunoblot and IHC as described in sections 2.16.1 and 2.16.2, respectively.

2.15 Vaccination of psittacine birds

Inoculum was prepared as described for sheep and 0.1-0.2 mL was injected into the pectoral muscle mass of 12 psittacine birds. Of these birds, 1 was boosted twice, 3 boosted once and the remaining 9 only received primary vaccination. Blood samples were collected onto filter paper (Whatman No. 3) before injection, 11 or 14 days post injection, 32 or 46 days post the first boost and 14 days post the second boost. Antibody response was detected as per Riddoch and Raidal (1996) and outlined in section 2.16.3.

2.16 Detection of antibody-response to recombinant BFDV protein by western immunoblot, IHC and HI

Detection of antibody response in sheep and birds was attempted by HI as described by Riddoch *et al.* (1996), however this did not give any results for the sheep samples because the sheep sera caused low levels of auto-agglutination interfering with the principles of the assay. Due to this complication, western immunoblot and IHC was employed for analysing sheep sera.

2.16.1 Western immunoblot detection of antibodies to full length recombinant protein in sheep sera

One hundred and fifty microliters of urea/DTT solubilised IB protein was electrophoresed as described under section 2.9 and transferred to a nitrocellulose membrane as described under section 2.10. Sera collected in section 2.13 was diluted 1:200 with 1×TBST and incubated with the membrane over nigh at room temperature, washed 2×5 min in 1×TBST and 10 min in 1×TBS and then incubated with HRP

conjugated rabbit-anti-sheep IgG diluted 1:2000 with 1×TBST. Membrane was washed as before and HRP colour development was carried out as described under section 2.12.2. Imaging was performed using a Nikon digital camera. Image contrast and brightness were enhanced with Paint Shop Pro 6.

2.16.2 IHC detection of antibodies to full length recombinant protein

Sections of formalin-fixed and paraffin embedded skin tissue from a chronically infected cockatoo were cut to a thickness of 5µm using a Leica RM 2135 microtome and placed onto a glass slide. The sample was then dewaxed 3 times in xylene for 3 minutes and further rehydrated using decreasing ethanol concentrations [100%, 95% and 70% (v/v)] for 3 min in each solution. Sections were then washed for 3 min in Tris buffer twice. Endogenous peroxides were removed by submerging the slides in a solution of 0.3% (v/v) hydrogen peroxide in methanol for 5 min and then washed 3×3 min in Tris buffer. Slides were further incubated in blocking solution (DAKO® Protein Block) for 30 min at room temperature in a humidified environment. Sheep sera diluted 1:50 in antibody diluent (DAKO®) was incubated with the slides at room temperature and unbound antibody was then removed by washing 3×3 min in Tris buffer before incubating with biotin conjugated rabbit-anti-sheep diluted 1:1000 in a humidified environment at room temperature for 15 min. The slide was washed as before and then incubated with HRP-conjugated streptavidin for 10 min at room temperature in a humidified environment before washing as previously described and antigen-antibody complexes visualised with HRP colour development as described in section 2.12.2. Imaging was performed using an Olympus BX 13 microscope and digital camera accessory. Image contrast and brightness were enhanced with Shop Pro 6.

2.16.3 HI detection of antibodies to full length recombinant protein in psittacine bird sera

A hole punch was used to clip out a piece of filter paper with blood, which was dropped into 100 µL 5% acid washed kaolin and left to elute for 1 hour at room temperature or overnight at 4°C. The sample was briefly centrifuged to pellet the kaolin and paper. Fifty microliters of supernatant was transferred to a new tube and mixed with 50 µL 10% galah erythrocytes and allowed to haemadsorb overnight at 4°C. Further, 50 µL PBS was added to every well of a microtiter plate and then 50 µL sample added to the first lane and serially diluted 1:2 across the lane. The microtiter plate was then incubated at 37°C for 60 min and 50 µL erythrocytes added to each well and incubated for 60 min at 37°C. Imaging was performed using a Nikon digital camera and image contrast and brightness enhanced with Paint Shop Pro 6.