The Development of a Flagellin Surface Display Expression System in the Gram-Positive Bacterium, *Bacillus halodurans* Alk36.

By

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Declaration

I, the undersigned hereby declare that the thesis submitted herewith for the degree *Philosophiae Doctor* to the University of Cape Town, contains my own independent work and hitherto has not been submitted for any degree at any university or faculty.

signature removed

MC Crampton
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PUBLICATION AND CONFERENCE PRESENTATIONS OF WORK DONE WITHIN THIS DISSERTATION

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Development of a Gram-positive cell-surface expression system for the over-expression and display of heterologous proteins and peptides. (**M. Crampton**, E. Berger, E. du Plessis, L. Joubert, and M.E. Louw)

CONFERENCE: **Paper presentation:** 10TH International Symposium on the Genetics of Industrial Microorganisms in Prague (June 2006).

A Cell Surface Expression System for the Display of Heterologous Gene Products Using Chimeric Flagellin Fusions of a *Bacillus halodurans* Isolate. (**M.C. Crampton**, E. Berger, E. du Plessis, and M.E. Louw).

CONFERENCE: **Poster Presentation:** The 4th Recombinant Protein Production Meeting: a comparative view on host physiology. Barcelona, Spain. 21–23 September 2006.

Recombinant lipase immobilised in the cell wall of *Bacillus halodurans* Alk36 exploiting the FliC protein. (**Michael Crampton**, Erika du Plessis, Santosh Ramchuran, Eldie Berger and Maureen Louw)

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ABSTRACT

This study relates to the development of an alkaliphilic, thermo-tolerant, Gram-positive isolate, *Bacillus halodurans* Alk36, for the over-production and surface display of chimeric gene products. This bacterium harbors the endogenous genetic background to over-produce flagellin protein continuously. In order to harness this ability, key genetic tools, such as gene targeted inactivation, were developed for this strain. The *hag* gene which codes for flagellin was inactivated on the chromosome giving rise to the *B. halodurans* BhFC01 mutant. This strain was non-motile as determined on motility plates and confirmed by PCR analysis. Motility was, however, restored through complementation of the expression vector carrying a functional *hag* gene.

Polylinkers were inserted as in-frame, chimeric, flagellin sandwich fusions in order to identify the permissive insertion sites corresponding to the variable regions of the flagellin protein. Flagellin expression and motility were evaluated for these constructs. Two sites were identified for possible peptide insertion in the flagellin gene, one of which produced functional flagella and was able to restore the motility phenotype to a non-motile mutant.

Peptides encoding a poly-histidine peptide and the HIV-1 clade C gp120 epitope were respectively incorporated into both of the permissive sites as in-frame fusions and found to be successfully displayed on the cell surface. The poly-His peptide was shown to be functional through metal binding and affinity purification studies. The display of the HIV-1 subtype C gp120 V3 loop was also shown to be functional through immunological studies using peptide specific antibodies. Surface display of the poly-His and HIV-1 epitope was shown to have improved metal binding and enhanced expression levels of the chimeric flagellin when the peptides were inserted at amino acid position 180 (pSECNC6). This specific site is the only insertion point that falls within the re-defined variable domain of the FliC protein from *B. halodurans* Alk36.

A lipase from *Geobacillus thermoleovorans* was also shown to be successfully displayed within both the permissive sites within the FliC protein. Unlike the peptides, the display of the lipase did not result in the formation of flagella on the cell surface. The FliC::lipase fusion did result in the successful targeting of the chimera to the cell surface as determined by whole cell lipase assays.

ABBREVIATIONS

Aa	amino acids
Amp	Ampicillin
AP	alkaline phosphatase
bp	base pair
BSA	bovine serum albumin
C-	carboxy terminal (end of protein)
Cm	chloramphenicol
CS	cell surface
CW	cell wall
°C	degrees celcius
dNTP	deoxynuceotide triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
EtBr	ethidium bromide
g	standard gravitational acceleration
gp	glycoprotein
GRAS	generally regarded as safe
h	hour (s)
His	histide
kDa	kilodaltons
kb	kilobase pairs
LB	Luria-Bertani broth
min	minutes
ml	milliliters
M	molar
mg	milligram
mM	millimolar
N-	amino terminal (end of protein)
nm	nano-metres
Ni	nickel
nt	nucleotides
nm	nanometre

OD_{540}	optical density at 540 nm
OD_{410}	optical density at 410 nm
OD_{405}	optical density at 405 nm
ORF	open reading frame
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
pNPP	*p*-nitrophenyl palmitate
SDS	sodium dodecyl sulfate
TAE	tris-acetate EDTA buffer
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
U	units of enzyme activity
µg	micrograms
µl	microlitre
UV	ultraviolet
w/v	weight per volume
::	novel joint (fusion)
α	alpha
β	beta
σ	sigma
µ	micro
Δ	delta

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Chapter 1

1.1 INTRODUCTION

A number of methods are currently available for the display of heterologous proteins on bacterial cell surfaces and these have been covered extensively in a number of current reviews (Chen and Georgiou, 2002, Samuelson *et al.*, 2002, Wernerus *et al.*, 2002, Lee *et al.*, 2003, Desvaux *et al.*, 2006).

Microbial cell surface display is the expression of a heterologous protein or peptide (passenger) as a fusion to a cell surface associated protein (carrier). The passenger protein or peptide can be fused to the carrier protein at either the C-terminal or N-terminal end, or inserted in the middle of the protein as a sandwich fusion. This area of research has gained enormous interest over the past twenty years and has potential in a number of areas including, immunology, applied microbiology and biotechnology (Fig. 1.1).

Lee *et al.* (2003) described 4 basic requirements which are essential for the successful display of heterologous proteins and peptides. These include an efficient signal peptide for transport through the membrane, a strong anchoring structure, compatibility with foreign proteins, and being protease resistant. Although many carrier proteins have been identified, each have unique characteristics and are therefore suitable for specific functions or applications. The location of the passenger protein within the carrier protein also determines the successful display of the heterologous protein. For example, it is necessary to identify the area of the carrier protein that is exposed to the external milieu. In some cases sequences and 3D structures are available and can be used to determine surface exposed sites, in some instances this may not be the case. In these instances the insertion of small reporter peptides can be useful to elucidate a suitable insertion site for the passenger protein.

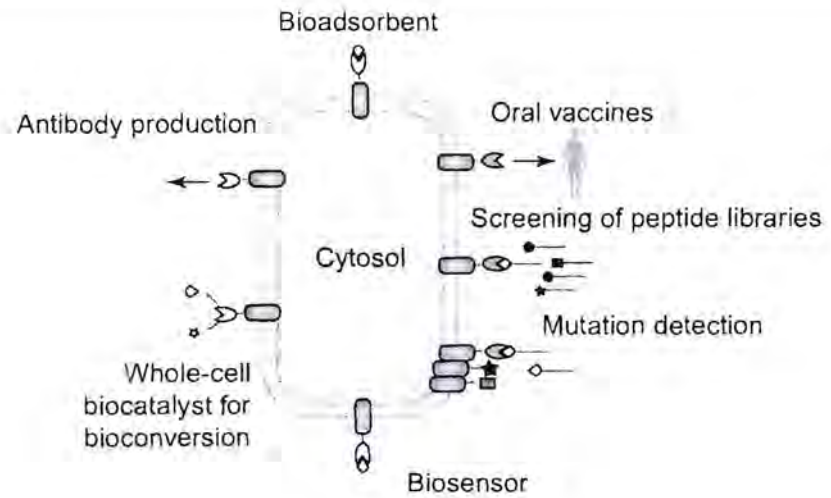


Figure 1.1 Potential applications for microbial cell surface display (Lee *et al.*, 2003).

The successful display of a heterologous protein or peptide is influenced by the passenger protein itself. Some of these influences include folding prior to translocation across the periplasmic membrane (Maurer *et al.*, 1997) or the insertion of charged residues resulting in inefficient secretion in bacteria (Nguyen *et al.*, 1995). Although altering the sequence of the passenger protein may be an option, the subsequent deletion or replacement of the amino acid residues will potentially change the function of the passenger protein.

Many new display systems are being developed using novel surface proteins in a wide range of bacterial species. The natural ability of cells to display functional proteins is critical for a number of biological processes which include adhesion, colonization, biofilm formation, cell-cell recognition, signal transduction, and immunoreactions (Kjaergaard *et al.*, 2002). This literature review will therefore focus on the most common display systems and list other systems in the tables within each section.

1.1 Gram-negative display systems

Gram-negative bacteria have a complex cell envelope structure and require that the fusion proteins pass through the cytoplasmic membrane, periplasm and outer membrane (OM) for successful display. Although this is the norm, Hoischen *et al.* (2002) were able to create recombinant proteins on the cytoplasmic membrane using L-form cells of *Escherichia coli* and *Proteus mirabilis* but will not be discussed in the following section. Three gene fusion strategies have been employed for the display of heterologous proteins. These include N-terminal, C-terminal and sandwich fusions to carrier proteins.

1.1.1 N-terminal fusions

The N-terminal fusion approach is suitable when the carrier protein has a C-terminal cell surface directed and anchoring motif.

Autotransporters

One such system makes use of the autotransporter secretion pathway. Autotransporters form part of the type V secretion pathway of Gram-negative bacteria. The other secretion pathways all make use of a complex combination of polypeptide machinery for successful secretion (3-20 different polypeptides) (Lee and Schneewind, 2001). Autotransporters on the other hand, contain all the necessary information for its successful translocation across the OM (Henderson *et al.*, 1998). All autotransporter proteins consist of an amino- terminal leader peptide, the secreted mature protein and a C-terminal portion that is capable of forming a pore within the outer membrane (OM) of Gram-negative bacteria. Most autotransporters are virulence determinants and have been reviewed by Henderson and Natro (2001) and Jose (2006). The pores that are created by the autotransporters consist of an embedded closed barrel of β-sheets with a hydrophobic exterior (N-terminal region) and a more hydrophilic interior (Maurer *et al.*, 1999, Oomen *et al.*, 2004).

This simplistic secretory pathway makes these proteins highly desirable for cell surface display. By removing the secreted protein and fusing a heterologous protein to the C-terminal region one can use this system to successfully transport a fusion protein through the OM (Jose *et al.*, 1996). However, one disadvantage of this system lies in the channel width (~ 2nm) of the OM located β-barrel ring (pore) (Fig. 1.2). The size of the pore is large enough to allow for the passage of some folded proteins, but the general consensus suggests that efficient translocation to the cell surface occurs only when the passenger proteins are devoid of any secondary structure or disulphide bonds (Klauser *et al.*, 1992, Jose *et al.*, 1996).

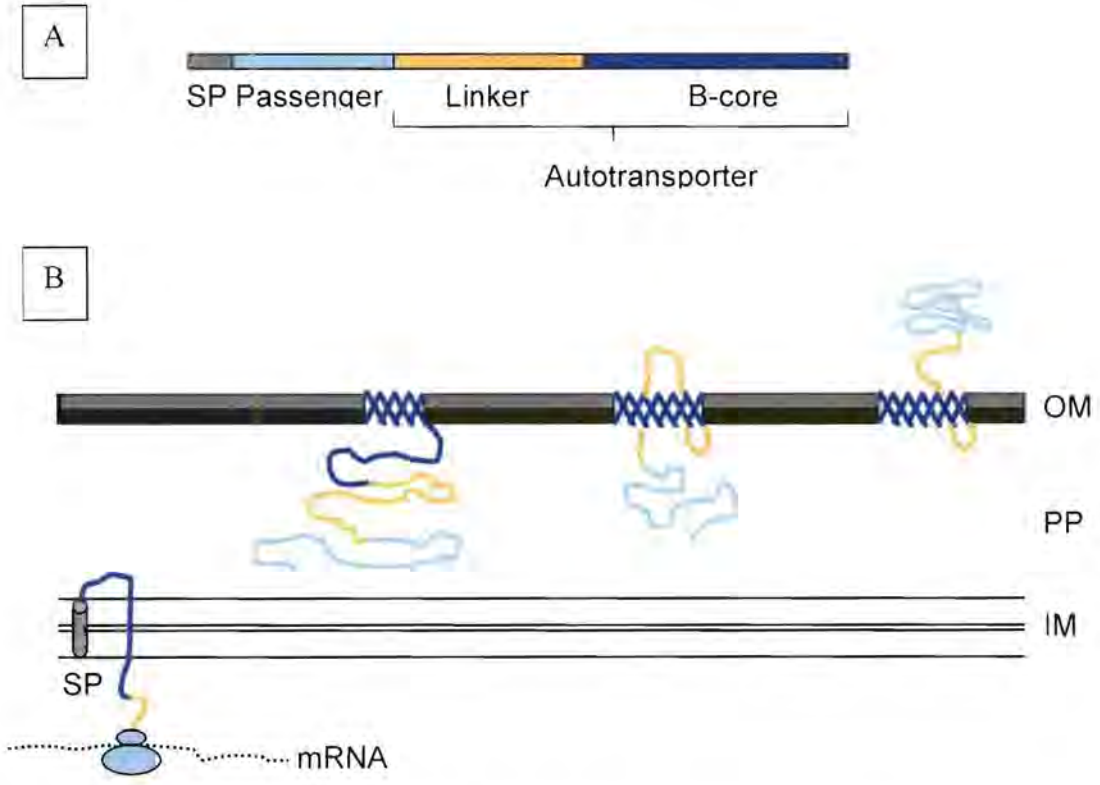


Figure 1.2. Secretion mechanism of the autotransporter proteins. A, Structure of the polyprotein precursor. B, Transport of the recombinant passenger. Through the use of a typical signal peptide (SP), a precursor protein is transported across the inner membrane (IM). Arriving at the periplasm (PP), the C-terminal part of the precursor folds as a porin-like structure, the so-called β-barrel within the outer membrane (OM) and the passenger is transmitted to the cell surface (Schultheiss *et al.*, 2002, Jose, 2006).

There are a number of examples of the successful display of heterologous proteins using autotransporters (Table 1.1). The autotransporter polyprotein precursor of the immunoglobulin A1 (IgA1) protease-like protein family are excellent candidates for cell surface display in Gram-negative bacteria (Maurer *et al.*, 1997). This IgA1 protease was thought to be an important virulence factor involved in infection of humans (Pohlner *et al.*, 1987), however, mutations within the IgA1 protease did not inhibit the ability of *Neisseria gonorrhoeae* to infect humans (Johannsen *et al.*, 1999). The *iga* gene is translated into a large 170 kDa precursor protein in which the mature protease (106 kDa) is flanked by an N-terminal signal peptide directing the export to the periplasm. At the C-terminal end, a 45 kDa IgAP domain from Val 1124 is

Table 1.1 Selected examples of N-terminal fusion display systems for expression in Gram-negative bacteria

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
Neisseria gonorrhoeae IgA 1 protease (45 kDa)	CtxB (13 kDa)	*E. coli (ompT-)*	Vaccines	Jose *et al.*, 1996
	Bovine adrenodoxin	*E. coli*	Steroid conversion	Jose *et al.*, 2001
	Mouse metallothionein (7 kDa)	*Ralstonia eutropha*	Bioremediation	Valls *et al.*, 2000
	Immunoglobulin	*E. coli*	Antibody production	Veiga *et al.*, 2004
E. coli adhesin (AIDA-I) (51.5 kDa)	CtxB (13 kDa)	*E. coli (ompT-)*	Vaccines	Maurer *et al.*, 1997
	Dimeric Adx (14.4 kDa)	*E. coli*	Whole-cell steroid synthesis	Jose *et al.*, 2002
	B-lactamase	*E. coli*	Biocatalysis	Lattemann *et al.*, 2000
	Esterase	*E. coli*	Biotransformation	Schultheiss *et al.*, 2002
	Hsp60	*E. coli*	Antigenic determinant	Kramer *et al.*, 2003
Shigella VirG (37 kDa)	*E. coli* MalE, PhoA (471 aa)	*E. coli (ompT-)*	Biocatalysis	Suzuki *et al.*, 1995
E. coli PAL (173 aa)	Anti-atrazine antibody fragment (252 aa)	*E. coli*	Biosensor	Dhillon *et al.*, 1999
E. coli PAL	Single chain antibodies	*E. coli*	Screening libraries of scFv antibidies	Fuchs *et al.*, 1991, 1996

responsible for the formation of the pore in the OM and is required for the translocation of the protease domain to the bacterial cell surface (Fig. 1.2). The β-barrel (pore) consists of 14 amphipathic β-sheets within the OM. By attaching passenger proteins to the C-terminus of the carrier, heterologous proteins can be translocated to the cell surface (Fig. 1.2) (Pohlner *et al.*, 1987).

Since IgA1 protease was one of the first autotransporters to be classified it is also one of the best studied proteins for surface display of heterologous proteins and peptides (Table 1.1). Although one of the disadvantages of autotransporters is the size of the pore, Veiga *et al.* (2004) showed that the IgA1 protease pore could tolerate the folding of two or three immunoglobulins, each with a folded diameter of 2nm. This was in contrast to previous studies where the cytotoxin B peptide could not be transported to the cell surface due to disuphide bond formation. This problem was overcome by creating a *dsbA* mutant (Jose *et al.*, 1996). This gene is responsible for the formation of disulphide bonds but is not critical for the function of the autotransporter. It is possible to use this system for the display of proteins which have some degree of folding as long as the diameter of the folded protein does not exceed 2nm. It must be noted that the IgA1 has an autoproteolytic domain releasing the N-terminal domain into the external environment (Klauser *et al.*, 1993). To inhibit release of the displayed peptide or protein it is critical to remove or alter this site.

The VirG protein (1102 aa) is derived from *Shigella*, the causative agent for shigellosis (a bloody diarrhoea in humans) and is responsible for the inter/intracellular spreading of *Shigella* cells (Suzuki *et al.*, 1995). Suzuki and co-workers (1995) used VirG for successful display of the MalE protein (an *E. coli* periplasmic protein) and PhoA (alkaline phosphatase). In both instances folding prior to transport across the OM resulted in diminished export. These results are consistent with the display of CtxB using IgAI protease. Improved export was noted when a *dsbA* mutant was used.

The autocatalytic feature is not present in all autotransporter systems. One such system is the AIDA-I autotransporter protein. AIDA-I is an *E. coli* adhesion protein involved in adherence to HeLa cells (Benz and Schmidt, 1989). The *E. coli* AIDA-I adhesion protein was used as an anchoring motif to display a number of antigenic

epitopes and enzymes (Table 1.1). All the heterologous proteins and peptides were successfully displayed and the esterase and β-lactamase were active on the cell surface.

Peptidoglycan associated lipoproteins

Peptidoglycan-associated outer membrane proteins (OMPs) as well as other OMPs help link the outer membrane to peptidoglycan through covalent and noncovalent forces (Fortney *et al.*, 2000). Peptidoglycan associated lipoprotein (PAL) is anchored to the inner face of the outer membrane and strongly binds to the cell wall polysaccharides with its N-terminal and C-terminal domains respectively (Dhillon *et al.*, 1999). The PAL protein interacts with a number of components including TolA, TolB, OmpA, the major lipoprotein and the murein layer. Interaction between the Tol and PAL proteins were shown to be important for outer membrane integrity (Cascales and Lloubes, 2004). A deletion of 40 aa at the N-terminal region was shown to have no deleterious effects on cell growth. The peptidoglycan binding sequence of PAL from *E. coli* is located between residues 97-114 (Cascales and Lloubes, 2004) and is critical for the successful display of peptides and proteins. It is feasible to bind a heterologous peptide or protein at the N-terminal side of the PAL protein and successfully display the chimera on the cell surface (Table 1.1).

Single chain recombinant antibodies were directed to the cell surface of *E. coli* using the pectate lyase signal sequence and the peptidoglycan associated lipoprotein component of PAL (Fuchs *et al.*, 1991, 1996). Although successful display was achieved using PAL, stability of the cell wall was reduced and resulted in "leaky" cells. Dhillon *et al.* (1999) were also able to show the successful display of an anti-atrazine scFv. Further studies are needed to determine the true potential of the PAL protein in surface display.

1.1.2 C-terminal fusion

Lipoproteins

In Gram-negative bacteria, the lipoproteins are anchored to either the inner or the outer membrane mainly via a covalently attached lipid moiety and hence the name lipoprotein. The location of the lipoprotein is thought to be determined by a single amino acid in position +2 (Seydel *et al.*, 1999). Bacterial lipoproteins have a wide range of functions such as antibiotic resistance, substrate binding, sensory systems and many more (Li *et al.*, 2003). Characteristic features of lipoproteins include a signal sequence in the N-terminal end followed by a cysteine residue. It is this cysteine residue that is modified by the addition of fatty acids that anchor the protein in the cell membrane (Wu, 1996).

The ice-nucleation protein (INP) is an outer membrane bound lipoprotein and is involved in active ice nucleation in plant pathogenic bacteria such as *Pseudomonas*, *Xanthomonas* and *Erwinia* (Wolber *et al.*, 1986). INPs are monomeric proteins of around 1200 aa with a deduced molecular weight of 118 kDa (Green and Warren, 1985). The N-terminal region (191 aa) interacts with the phospholipids moiety in the outer membrane via a glycosylphosphatidylinositol (GPI) anchor. This method of anchoring is unique since this motif is normally a characteristic found in eukaryotic lipoproteins (Samuelson *et al.*, 2002). The central region consists of a series of repeated amino acid sequences of 8, 16, and 48 residues which act as templates for ice crystal formation (Warren and Corrotto, 1989). The C-terminal region (49 aa) is highly hydrophilic and is exposed to the external milieu. INP also has two inherent advantages when considering its use for surface display. Firstly, it is stably expressed in stationary phase, and secondly, it can be expressed and displayed in numerous Gram-negative bacteria including *E. coli* (Orser *et al.*, 1985).

The first reported use of INP as a surface display system was by Jung *et al.* (1998a). They were able to successfully display carboxymethylcellulase (CMCase) and levansucrase (Jung *et al.*, 1998b). The CMCase was displayed on the surface using both the full length INP and a truncated INP with the central repeating domain removed. This truncated INP consists of the N-terminal specific domain including the

first two repeating units (221 aa) and the C-terminal region including the last three repeating units (97 aa). In both instances the CMCase was successfully displayed on the cell surface with full activity up to and including stationary phase (Jung *et al.*, 1998a, 1998b). Subsequently, INP has been used extensively for directed evolution of displayed proteins for improved activity (Table 1.2) (Kim *et al.*, 2000).

Shimazu *et al.* (2003) also demonstrated the successful display of INP-OPH (organophosphorous hydrolase) fusion in *Pseudomonas putida* KT2440. *P. putida* is a soil-borne bacterium known to survive in contaminated environments. The expression of enzymes on the surface of environmentally isolated bacteria should allow for successful applications in bioremediation. Similarly, *Moraxella* sp. a soil organism able to degrade p-nitrophenol (PNP) was also genetically engineered for the simultaneous degradation of organophosphorous pesticides and PNP using INP (Shimazu *et al.*, 2001). The display of eukaryotic enzymes using INP has also been demonstated (Jeong *et al.*, 2001). INP has further been used to display antigens, single chain antibodies, heavy metal binding proteins and biosorbents (Table 1.2).

In all the above examples INP proteins InaK or InaV from *Pseudomonas syringae* have been used for surface display. All displayed proteins made use of either full length sequences or truncated portions containing both the N and C-domains (INP-NC) with the addition of a number of internal repeating units. Jung *et al.* (1998b) demonstrated that the use of the truncated InaK gave better activity of the displayed CMCase than the full InaK. Since InaK gave better activity with the truncated form (including both the N and C-terminal domains), Li *et al.* (2003) investigated the use of the N-terminal domain (184 aa) as the sole anchoring motif for the reporter gene, green fluorescence protein (GFP). The N-terminal domain was found to display the GFP fusion protein as efficiently as INP-NC, demonstrating that the N-terminal region can direct foreign proteins to the cell surface. GFP was also replaced with OPH and showed similar results confirming the N-terminal regions ability to act as a display motif.

Table 1.2 Selected examples of C-terminal fusion display systems for expression in Gram-negative bacteria.

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
P. syringae INP (36 kDa)	CMCase (33 kDa)	*E. coli*	Whole-cell biocatalysts	Jung *et al.*, 1998a
	Levansucrase (424 aa)	*E. coli*	Utilization of levan	Jung *et al.*, 1998b
	CMCase mutant library (33 kDa)	*E. coli*	Development of protein, screening	Kim *et al.*, 2000
	HbsAg (168 aa)	*S. typhiTy21a*	Oral vaccines	Lee *et al.*, 2000
	Salmobin (26 kDa)	*E. coli*	Display of eukaryotic protein	Jeong *et al.*, 2001
	Synthetic phytochelatin (40 aa)	*E. coli*	Bioadsorption	Bae *et al.*, 2002
	OPH (365 aa)	*E. coli*	Screening of OPH variants	Cho *et al.*, 2002
	OPH (365 aa)	*Moraxella* sp	Biocatalysts	Shimazu *et al.*, 2001
	OPH (365 aa)	*P. putida*	Biocatalysts	Shimazu *et al.*, 2003
	MerR	*E. coli*	Biosorbtion	Bae *et al.*, 2003
	Thermostable lipase (TliA)	*E. coli*	Biocatalyst	Jung *et al.*, 2003
	ScFvs (c-myc oncoprotein) (30 kDa)	*E. coli*	Antibody production	Bassi *et al.*, 2000
	HIV 1 gp120	*E. coli*	Vaccine	Kwak *et al.*, 1999
	HCV core antigen	*E. coli*	Epitope mapping	Kang *et al.*, 2003
	GFP/OPH	*P. putida*	N-terminal display characterization	Li *et al.*, 2003
	TliA		Biotransformation	Jung *et al.*, 2006
E. coli TraT (26.5 kDa)	C3 epitope Polio virus	*E. coli*	Structure and Function (Vaccine)	Taylor *et al.*, 1990
	HbsAg.	*E. coli*	Vaccines	Chang *et al.*, 1999
	RHO (72 aa)	*E. coli*	Bacteria-host interaction study	Chang *et al.*, 1999
E.coli Intimin EaeA (659 aa)	EETI-II. interleukin 4, Bence-Jones protein REI (128 aa)	*E. coli*	Protein translocation study	Wentzel *et al.*, 2001
E. coli, FadL	Lipase	*E. coli*	Biotransformation	Lee *et al.*, 2004
E. coli Lpp-OmpA (123 aa)	β-lactamase	*E. coli*	Biotransformation	Georgiou *et al.*, 1996
	Organophosphorus hydrolase (365 aa)	*E. coli*	Biodegradation	Richins *et al.*, 1997

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
	Pytochelains (40 aa)	*E. coli*	Adsorption (bioaccumulation)	Bae *et al.*, 2000
	PE Dill antgen, extracellular domain of human ErbB2 and IL2-Ra (237 aa)	*E. coli*	Selection of phage antibody	Benhar *et al.*, 2000
Pseudomonas aeruginosa	Lipase (triacylglycerol acylhydrolase)(49.9kDa)	*E. coli*	Biotransformation	Lee *et al.*, 2005
E. coli Lpp-OmpA (123 aa)	GFP (239 aa)	*E. coli*	Improve efficiency reporter gene	Shi and Su, 2001
Lpp-OmpA-EETI-II	Sendai virus L-protein (13 aa) and Human bone Gla-protein epitope (17aa)	*E. coli*	Peptide libraries	Christmann *et al.*, 1999

The TraT lipoprotein (220 aa) from *E. coli* is also a well known carrier for the display of peptides on bacterial cell surfaces (Sukupolvi and Connor, 1990, Chang *et al.*, 1999, Chang and Lo, 2000). The TraT protein is encoded by the IncF plasmid and is responsible for surface exclusion activity (Aguero *et al.*, 1984). TraT prevents unproductive mating between cells bearing closely related plasmids by inhibiting the formation of stable mating aggregates (Sukupolvi and Connor, 1990) and is responsible for bactericidal serum resistance (Moll *et al.*, 1980). Five sites were chosen for the insertion of a C3 Polio virus antigenic determinant within the TraT protein in order to determine structure and function of this protein. Three of the insertion sites resulted in the successful assembly of TraT (Taylor *et al.*, 1990) and display of the C3 epitope. These initial studies demonstrated the possible application of TraT as a surface display system of foreign antigenic determinants. Chang *et al.* (1999) did not make use of these insertion sites but fused the HBV surface antigen and the snake venom rhodostomin (RHO) to the C-terminal end. The C-terminal end has been shown to be exposed to the external surface of the OM and could possibly be used for surface display (Taylor *et al.*, 1990). Both the above peptides were successfully displayed on the cell surface of *E. coli*. Since TraT is present at 20 000 to 30 000 copies per cell and in some mutants up 200 000 (Manning *et al.*, 1982), surface displayed proteins could potentially occur at high levels.

Intimins

Pathogenic Gram-negative bacteria display a number of binding domains which interact with receptors on the host cell surface (Klemm and Schembri, 2000a). Intimins and invasins form part of a protein family which interact with eukaryotic surface receptors resulting in bacterial adherence and invasion (Vallance and Finlay, 2000). A number of intimins have been described for *E. coli* and they are integrated into the OM via their N-terminal domains while the C-terminal domains (~280 aa) are exposed at the cell surface (Frankel *et al.*, 1994). One such intimin (EaeA) is derived from the enteropathogenic *E. coli* O157:H7. The N-terminal domain (550 aa) is thought to form a porin-like structure and is folded into antiparallel β-sheets while the C-terminal domain is responsible for the cell binding activity (Frankel *et al.*, 1994, Luo *et al.*, 2000). The receptor is situated at the end of a rigid extracellular arm which is bound to the N-terminal transmembrane domain through a flexible hinge. The exposed external domain consists of 3 immunoglobulin-like domains and the receptor (Luo *et al.*, 2000). The receptor domain and a single immunoglobulin domain were removed in order to allow the fusion of heterologous proteins at the C-terminal end. The specific proteins used for translocation to the cell surface using this system were the trypsin inhibitor (EETI-II), interleukin 4, and the Bence-Jones protein REI (Wentzel *et al.*, 2001). The successful display of these heterologous proteins demonstrated that the truncated intimin could be used as an anchor for foreign proteins on the cell surface of *E. coli*.

Truncated Omp and Lpp-OmpA hybrid systems

Omps (outer membrane proteins) are generally used as sandwich fusions with the inserted peptide being located in various permissive sites within the Omp. The use of Omps as display systems will be discussed in the following section. Lpp-OmpA is a hybrid display system which makes use of the first 9 aa of the major *E. coli* lipoprotein (Lpp) and aa 46-159 of the OmpA protein (Fig. 1.3). Lpp is unique in that the first 9 aa act as both the signal sequence and are also capable of directing the localization into the OM (Francisco *et al.*, 1992). Studies have also shown that aa 154-180 play a role in the correct localization of the OmpA protein (Klose *et al.*, 1988). Omps which have insertions greater than 60 aa, such as the alkaline phosphatase were

unable to be directed to the OM (Murphy and Klebba, 1989). The combination of the Lpp N-terminal region and aa 46-159 of the OmpA protein fused together could overcome these constraints. These two components were fused to the

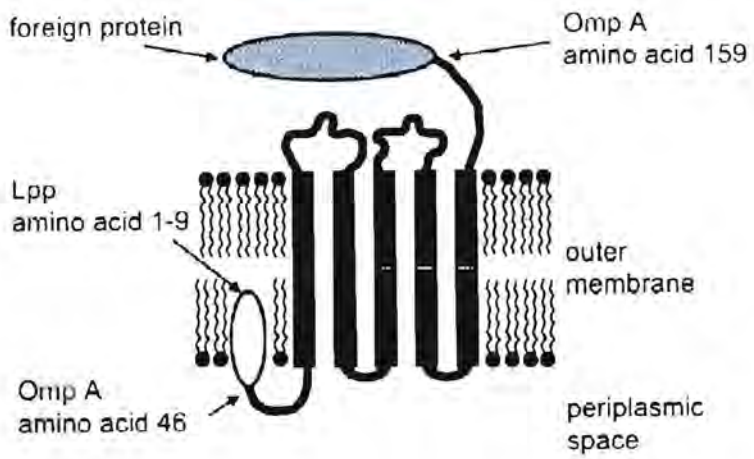


Figure 1.3. A schematic representation of the Lpp-OmpA display system (Samuelson *et al.*, 2002)

mature β-lactamase protein and were found to be predominantly located in the OM (Francisco *et al.*, 1992). Stathopoulos *et al.* (1996) attempted to display a bacterial alkaline phosphatase (PhoA), a periplasmic dimeric enzyme, on the cell surface of *E. coli* using the Lpp-OmpA system. Although the PhoA was directed to the OM it was not exposed at the cell surface. Disulphide bond formation and periplasmic folding inhibited the fusion in reaching the OM and was also lethal when expressed in high copy number vectors (Stathopoulos *et al.*, 1996). It therefore seems to indicate that the system is sensitive to secondary and tertiary structures of the passenger protein.

Georgiou *et al.* (1996) made use of various versions of the Lpp-OmpA system with different transmembrane regions in order to determine heterologous protein display efficacy, OM integrity and protease stability. A second OmpA segment consisting of aa 46-66 was found to mediate the display of β-lactamase on the cell surface. It was also noted that the display of a periplasmic protein resulted in unavoidable changes in

permeability of the OM of *E. coli*. Christmann *et al.* (1999) also made use of this truncated version of the LPP-OmpA display system in combination with the added fusion of EETI-II as a scaffold, to display small conformationally constrained peptides like Sendai virus L-protein and a 17 aa human bone Gla-protein epitope on *E. coli* cell surface. The potential of this fusion system for the rapid isolation of small peptide molecules was demonstrated from peptide libraries which bound acceptor molecules (Christmann *et al.*, 1999). This Lpp-OmpA system has also been used to display phytochelatins for bioremediation (Bae *et al.*, 2000), single chain antibody fragments (Benhar *et al.*, 2000) and GFP (Shi and Su, 2001).

Other outer membrane proteins

Although Omps are generally used in sandwich fusions (discussed in more detail in section 1.1.3.), a recent study done by Lee *et al.*, (2004) made use of C-terminal deletion mutants of the outer membrane FadL from *E. coli* to display a *B. subtilis* lipase (44.5 kDa) on the surface of *E. coli*. A C-terminal deletion in the ninth loop of the FadL was used as the fusion site (Lee *et al.*, 2004). Similarly, amino acid 164 and 188 of protein OprF were used for the successful display of the lipase on the outer membrane (Lee *et al.*, 2005). This area corresponds to the fifth external loop of the OprF protein. These are the first two studies reporting the use of a porin in a C-terminal fusion display system.

1.1.3 **Sandwich fusions**

Sandwich fusions are the most often used strategy for the display of heterologous proteins or peptides in Gram-negative bacteria. These fusions are associated with three classes of carrier proteins which include the outer membrane proteins, protein monomers of extracellular appendages (flagella and pilli), and S-layer proteins.

Outer membrane protein based systems

Porin proteins (e.g. OmpC) have two characteristics which differ from other outer membrane proteins. Firstly, they contain mainly polar amino acids compared to water soluble proteins and secondly, they contain β-structures and not α-helices like many

other membrane proteins (Lang, 2000). Porin proteins exist as trimers in the outer membrane and each monomer is generally composed of 250-450 amino acids. The β-strands which form the β-barrel are connected to each other via an extracellular loop structure and by β-turns in the periplasm (Lang, 2000). It is the flexible extracellular loops which are the targets for the display of heterologous peptides and proteins. The main porins of Gram-negative bacteria include OmpC, OmpS, PhoE (phosphate inducible porin) and LamB (maltoporin) and as such they have been used most extensively as surface display systems. Other porins used are listed in Table 1.3.

Xu and Lee (1999) were one of the first to make use of the OmpC protein from *E. coli* as a surface display system. OmpC is one of the most abundant OMPs in *E. coli* (1 x 10^5/cell) and consists of 16 transmembrane antiparallel β-strands constituting the β-barrel structure. Each strand is connected by 7 internal loops and 8 external loops (Nikaido, 1996). Since the external loops are not conserved they are thought to be more amenable to base deletions and insertions. The external loop 7 was chosen as the site for the insertion of a polyhistidine peptide (with several hexahistidine [6His] linkers) (Xu and Lee, 1999). By joining different numbers of the 6His linkers the permissive size of the polypeptide fused to the external loop could also be examined. In this study Xu and Lee (1999) were able to construct OmpC::His fusions containing 19-240 aa inserts in loop 7. Each fusion was easily detected with PAGE analysis but OmpC-$(6His)_{12}$ (162 aa) could only be detected when induced for an extended period of time. Best yields from this display system were obtained when 19-84 amino acids were inserted into OmpC. It is clear that by using the OmpC system, size constraints must be taken into consideration. Although the chimeric protein yield decreased as insert size increased the relative amount of adsorbed Cd^{2+} increased and reached 32.0 μmol of Cd^{2+} per g (dry weight) of cells. Although cells successfully displayed $(6His)_{12}$ the effect on cell membrane integrity needed to be considered. Xu and Lee (1999) exposed recombinant *E. coli* cells to EDTA and SDS and found that only cells carrying 1 or 2 copies of the 6His linker were resistant to treatment. It is thus only feasible to use a single or double copy of the 6 His linker for bioremediation.

Table 1.3. Selected examples of sandwich fusion display systems for Gram-negative bacteria.

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
E. coli OmpC (367 aa)	Poly-His peptides (162 aa)	*E. coli*	Bioremediation	Xu and Lee, (1999)
Salmonella typhimurium	Rota virus Vp4 epitope (18 aa)	*S. typhimurium*	Vaccine	Puente *et al.*, 1995
E. coli, OmpA	Hexa-His	*E. coli*	Bioremediation	Majare *et al.*, 1998
P. aeruginosa, OprF	Malarial antigen (80 aa)	*S. typhimurium*	Vaccine	Haddad *et al.*, 1995
E. coli FliC	Adhesive domains of staphylococcal FnBPA and *Yersinia enterolitica* YadA (115 aa)	*E. coli*	Expression of adhesive peptides	Westerlund-Wikström *et al.*, 1997
	Egg white epitope		Vaccine development	Kuwajima *et al.*, 1988
	SlpA, FnBpP (81 aa)		Characterisation of the adhesive epitope	Tanskanen *et al.*, 2000
	scFv (249 aa), GFP (236 aa), Alkaline phosphatase (471 aa)		Antibody, Biotransformation	Ezaki *et al.*, 1998
	Thioredoxin protein, peptide libary		Affinity detection, biosensor, bioremediation	Lu *et al.*, 1995, Tripp *et al.*, 2001, Thai *et al.*, 2004
S. typhimurium, FliC	CTP3, CTP1	*S. typhimurium*	Vaccine	Newton *et al.*, 1989
	HBY surface antigen, S and preS antigenic regions	*S. dublin*	Vaccine	Wu *et al.*, 1989
	Gp120 V3 loop (14 aa)	*S. typhimurium*	Vaccine	Cattazzo *et al.*, 1997
	M Protein epitope (15 aa)		Vaccine	Newton *et al.*, 1991b
	Influenza epitope		Vaccine	Levi and Arnon, 1996
S. muenchen, FliC	HIV-1 IIIB env Ag epitope (17 aa)	*E. coli*	Vaccine	Newton *et al.*, 1995
E. coli, FliD, FliC	SlpA (150 aa)	*E. coli*	Multiple binding motif	Majander *et al.*, 2005a

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
E. coli FimH	Random peptide library (33 aa)	*E. coli*	Screening of binding motif (bioremediation)	Kjaegaard *et al.*, 2000, Kjaergaard *et al.*, 2001.
	PreS (50 aa), CTB epitope, Hepatitis B virus surface Ag	*E. coli*	Vaccine	Palleson *et al.*, 1995
E. coli FimA (180 aa)	CtxB epitopes (34 aa)	*E. coli*	Vaccine	Stentebjerg-Olesen *et al.*, 1997
	Hepatitus Ag, FMDV, Polio virus		Vaccine	Hedegaard and Klemm, 1989
E. coli FelA Fimbriae	GnRH	*E. coli*	Pharmaceutical peptide	Van Die *et al.*, 1988, van Die *et al.*, 1990
E. coli 987P Fimbriae	HSV-I, glycoprotein D, virus spike protein Ag.	*E. coli*	Vaccine	Rani *et al.*, 1999
	TGEV		Vaccine	Chen and Schifferli, 2003
E. coli Dr Fimbriae	HSV gpD	*E. coli*	Vaccine	Zalewska *et al.*, 2003
E. coli F pilin (7.5 kDa)	Peptide (15 aa)	*E. coli*	Selective phage infection	Malmborg *et al.*, 1997
E. coli Pilin K88	Influenza hemaglutinin peptide, somastatin	*E. coli*	Vaccine	Thiry *et al.*, 1989
Caulobacter crescentus RsaA (1073 aa)	Fragment from *P. aeruginosa* K pilin (12 aa)	*C. crescentus*	Not indicated	Bingle *et al.*, 1997
E. coli, FepA		*E. coli*		Armstrong and McIntosh, 1995
E. coli, BtuB, FhuA	myc epitope	*E. coli*	Ligand screening	Etz *et al.*, 2001
E. coli LamB (446 aa)	HMT, YMT (66 aa)	*E. coli, S. typhimurium*	Bioremediation	Sousa *et al.*, 1998
	Poly-histidine		Bioremediation	Sousa et al., 1996
	HbsAg		Antibody production	Charbit *et al.*, 1986
	preS (14 aa, 19 aa)		Vaccine	Charbit *et al.*, 1987
	Shiga toxin		Vaccine	Su *et al.*, 1992
	Protein A		Vaccine	Steidler *et al.*, 1993
	Evolution variants for selection		Screening of variants	Patel *et al.*, 2001
E. coli, PhoE	FMD virus VP1 (30-50 aa)	*E. coli*	Vaccine	Agterberg *et al.*, 1990, Agterberg *et al.*,1987
	HP, CP		Vaccine	Kotbra *et al.*, 1999
Vibrio cholerae OmpS (390 aa)	Epitopes from staphylococcal FnBPA *E. coli* PapG (115 aa), C3 epitope Polio virus (11 aa), CTB (15 aa).	*E. coli*	Vaccine	Lang *et al.*, 2000 Lang and Korhonen, 1997
E. coli TraT (26.5 kDa)	Epitope from polio virus.	*E. coli*	Peptide	Taylor *et al.*, 1990

S. typhi OmpC protein was used as a display system to display a viral epitope on the cell surface (Puente *et al.*, 1995). The VP4 epitope (18 aa) was inserted into loops 4 and 6. In both instances, the chimeric OmpC was successfully isolated from the outer membrane and shown to cross react with antibodies specific to the displayed epitope. However, cross reactivity was reduced in loop 6 compared to loop 4. This variation could have been a result of secondary structure constraints within that region. Nonetheless, the display of antigenic epitopes using OmpC was successful and is thus a useful tool for vaccine development.

The LamB porins are involved in the transport of malto-oligosacharides (maltose and maltodextrins) across the outer membrane (Samuelson *et al.*, 2002). These porins are induced when maltose is added to the medium and found across a wide range of Gram-negative bacteria including *E. coli*, *S. typhimurium*, *Klebsiella pneumoniae*, *Aeromonas salmonicida*, *Vibrio cholerae*, *Vibrio. parahaemolyticus*, *Actinobacillus* and *Yersinia enterolitica* (Lang, 2000). All these proteins are highly conserved and have the same basic structure. Charbit *et al.* (1986) was one of the first researchers able to locate permissive sites for the display of heterologous peptides on the surface of *E. coli* using LamB. These first successful insertion sites (amino acid positions 153 and 374) were used to display the C3 epitope of the VP1 coat protein from the type 1 polio virus (11 aa). A further two epitopes from the preS2 region of the hepatitis B virus (14 and 19 aa respectively) were also successfully displayed using the permissive LamB insertion sites (Charbit *et al.*, 1987). One of these epitopes (preS2A) was shown to stimulate an immunogenic response in mice and the isolated antibodies cross reacted with the viral particles. Su *et al.* (1992) noted that stronger promoters were deleterious to the host cells while moderate to weak promoters resulted in high cell viability and cell densities. Although the general rule of size limitation within the LamB display system stands true, Steidler *et al.* (1993) did manage to display the staphylococcal protein A (232 aa) within the LamB 153 aa site.

The LamB system has been mainly used as an antigen delivery system (Table 3). The diversity of applications has also been extended to include enhanced metalloadsorption for possible bioremediation processes (Sousa *et al.*, 1996, Sousa *et al.*, 1998, Kotbra *et al.*, 1999). Eukaryotes respond to heavy metal pollution by producing metallothioneins (MTs). These are low molecular weight Cys-rich proteins

that are able to bind metal ions and subsequently render them inactive (Schmidt and Hamer, 1986). A class II MT from *Saccharomyces cerevisiae*, CUP1 (66 aa), which is responsible for copper tolerance, was inserted into the LamB 153 and 183 permissive sites (Sousa *et al.*, 1998). Previous attempts to produce MTs in bacterial cells were poor or unsuccessful due to the interference of the Cys-rich protein with redox pathways in the cytosol (Pugsley, 1992). Other examples of metal binding can be seen in Table 1.3.

OmpS is a maltoporin (43kDa) like LamB and is induced in the presence of maltose (Lang *et al.*, 2000). This protein has 18 membrane spanning regions and 9 externally exposed loops. Lang and Korhonen (1997) managed to successfully display the 11 aa C3 epitope from type 1 polio virus, a 15 aa cholera toxin B subunit epitope and one, two and three fibronectin binding D-repeats (38 aa) of FnBPA of *S. aureus*. Lang *et al.* (2000) were also able to insert 53 and 186 aa of the P-fimbrial adhesion class II PapG globoside binding protein of *E. coli* into loop 4 of the OmpS of *V. cholerae*. *E. coli* expressing the OmpS::PapG fusion was able to display the same phenotypic characters as PapG for *E. coli* strains carrying the papG gene and could bind to globoside. By displaying the PapG, as well as truncated versions of PapG, Lang *et al* (2000) were able to characterise the binding domain of this protein.

Like LamB, PhoE is an inducible Omp found in *E. coli* and is expressed under phosphate deficient conditions. Each subunit has the standard β-strand configuration with 8 external loops (Mizuno *et al.*, 1983). Agterberg *et al.* (1987) made use of the fifth external loop for the display of a synthetic antigenic determinant corresponding to the C-terminal part of the VP1 protein from the foot and mouth disease virus. Multiple insertions of varying regions of the VP1 protein were also inserted into loops 2 or 8 (Agterber *et al.*, 1990) which results in a total insert size of between 30-50 aa. Two observed limiting factors included reduced translocation due to the insertion of hydrophobic residues and charged residues resulting in the accumulation of precursor proteins in the cytoplasm. Other lesser used examples of OMP as display systems include FhuA (involved in ferrichrome uptake), BtuB (uptake of vitamin B_{12}) (Etz *et al.*, 2001), and FepA (Armstrong and McIntosh, 1995).

All of the above outer membrane proteins form part of a group of outer membrane proteins known as porins which exist as trimers in the outer membrane. OmpA exists as a monomer but has the same general structure as the other porins. OmpA from *E.coli* was the first OMP to be used as a surface display system and was able to carry a 15 aa peptide in loop 4 (Freudl *et al.*, 1986) (Fig. 1.4). Freudl (1989) subsequently made use of loop 2 and 3 to display peptides and proteins on the cell surface. He was also able to incorporate peptides at two sites simultaneously without having any detrimental effect on OmpA assembly and stability. A hybrid OmpA was created from the *ompA* genes from *E. coli* and *Shigella dysenteriae* which accepted peptide insertions in loops 3 and 4 individually as well as simultaneously (Schorr *et al.*, 1991). Haddad *et al.* (1995) made use of this hybrid system to display a malarial antigen (80 aa) on the cell surface of *S. typhimurium* and *E. coli*.

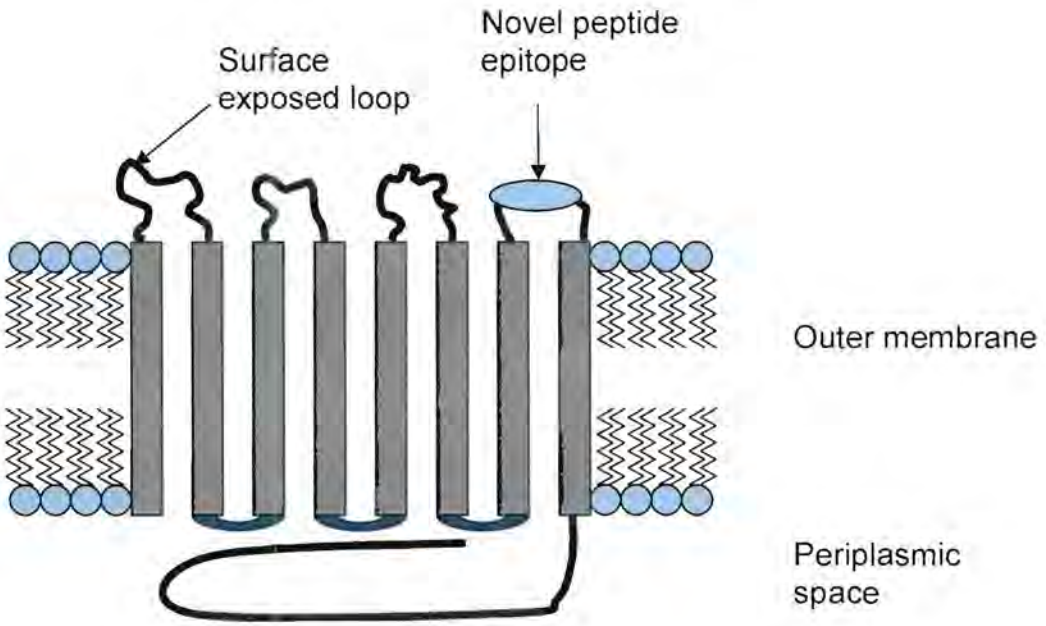


Figure 1.4 Schematic presentation of *E. coli* OmpA with a novel epitope expressed in a surface-exposed loop. Rectangles represent membrane-spanning β-strands of OmpA (Samuelson *et al.*, 2002).

All the above examples have focussed on the use of OMPs from *S. typhimurium* and *E. coli*. Other OMPs such as OprF and OmpS from *P. aeruginosa* and *V. cholera* respectively (Wong *et al.*, 1993, Wong *et al.*, 1995, Lang and Korhonen, 1997, Lang

et al. 2000, Lee *et al.*, 2005) have also been used. OprF (porin) is the major Omp in *P. aeruginosa* and also plays a role in the structural integrity of the outer membrane. In a previous study, insertion mutation analysis was done using a 12 bp linker carrying a *Pst*I site (Wong *et al.*, 1993). Ten permissive sites were identified within the OprF protein. These permissive sites carrying the linker were then used to insert a malarial epitope into the *Pst*I sites. The antigenicity of the malarial epitope differed depending on the site of insertion. Insertion sites at positions aa 188 and 196 were most antigenic as determined by ELISA and Western blot analysis. Variations in antigenicity were also detected when using whole cell and purified OM fractions. The extraction procedure could also play a role in the antigenicity of the displayed epitope.

Fimbriae (Pili)

Fimbriae are organelles which allow bacteria to adhere to target host tissue and in so doing assist in bacterial colonization. They occur as thread like structures up to 500 copies per cell (Klemm and Schembri, 2000b). Fimbriae are polymeric structures in which hundreds of subunits are held together by non-covalent interactions. Type 1 fimbriae are found across most *Enterobacteriaceae* and are generally 7 nm wide and 1μm long (Klemm and Schembri, 2000b). They consist of approximately 1000 copies of the major subunit, FimA, and occur as a stacked helical cylinder (Brinton, 1965). The fimbriae also have a minor subunit, FimH, which exists at the tip of the organelle and is scattered within the FimA. FimH is responsible for receptor binding to mannose containing structures (Krogfelt *et al.*, 1990). Both of these subunits have been used to display foreign peptides.

Fimbriae are good immunogens and have been used extensively as potential vaccines (Levine *et al.*, 1994). This, in combination with their high copy number, makes them favourable targets for use in surface display of foreign epitopes. In addition, fimbriae are easily detached from the bacteria and allow for rapid and easy purification.

Initial surface display studies were done by Hedegaard and Klemm (1989) who inserted heterologous peptides (Hepatitis B surface antigen, foot and mouth disease virus, and poliovirus) into naturally occurring restriction sites within the *fimA* gene. The results indicated that fusions of foreign epitopes within FimA yielded fully

functional type 1 fimbriae. Antibodies against the full length antigen could cross react with FimA hybrid proteins indicating that these epitopes were displayed correctly and elucidated permissible insertion sites within the FimA protein. The CTB epitope was also inserted into 4 sites within the Fim A protein (Stentebjerg-Olesen *et al.*, 1997). Only three of the four insertion sites were confirmed to display the foreign epitope. Antibodies isolated from immunized animals injected with the FimA-CTB fusion were able to react with native antigen.

FimH is a 300 aa protein which, upon processing, exists as a 279 aa mature protein. As with FimA, FimH was probed by linker insertion mutagenesis to identify permissive sites for heterologous peptide insertions (Schembri *et al.*, 1996). Two potential insert positions at aa 225 and 259 of the FimH protein were identified. Palleson *et al.* (1995) made use of these sites to display a 52 aa peptide of the preS region of the hepatitis B virus surface antigen as well as the CTB epitope. Both peptides were successfully displayed and the chimeric fimbriae were either fully or partially capable of binding mannose.

FimH has also been used to display random peptide libraries (Schembri and Klemm, 1998, Kjaergaard *et al.*, 2001) for the selection of metal binding peptides. Recombinant cells using FimH as a carrier were shown to be heterobifunctional and bound to both metals and saccharides. This demonstrates the ability of the chimeric FimH to bind to two ligands independently (Schembri and Klemm, 1998). An advantage of this binary system would be the ability to immobilize the bacterial cells using one adhesive domain while allowing for the adsorption of heavy metals in bioremediation.

Other fimbriae have also been used for the display of foreign peptides (Table 3). These examples include the insertion of a number of foreign epitopes in the hypervariable region of the FelA of F11 fimbriae (a type P fimbriae) (van Die *et al.*, 1988, van Die *et al.*, 1990). This display system was used to produce the brain peptide gonadotropin releasing hormone (GnRH) for the development of a contraceptive vaccine for fertility control in domestic animals (van der Zee *et al.*, 1995). This peptide was expressed successfully on the cell surface of *E. coli* and was shown to

induce serological and pharmacological effects that altered the reproductive characteristics of female rats and young bulls.

The enteroadhesive 987P fimbriae of *E.coli* was used to display HSV-1, glycoprotein D and transmissible gastroenteritis virus spike protein antigens at amino acid position 29. This position elicited high levels of foreign epitope antibodies in rabbits immunized parenterally. Bacteria were still able to mediate cell surface attachment to intestinal receptors and thus have the potential beneficial effect of enteroadhesion needed for mucosal immunization (Rani *et al.*, 1999). Further enhancements were made by including the inducible *pagC* promoter which responds to low Mg^{2+} concentration resembling conditions within endocytic vacuoles of macrophages (Chen and Schifferli, 2001). The use of a multimeric display system including two immunogenic epitopes of TGEV was also incorporated into this display system. These changes elicited significantly higher mucosal and systemic immune responses to the displayed antigen. The removal of the major protease gene, *pgtE*, increased the expression of intact hybrid fimbriae (Chen and Schifferli, 2003). The replacement of the promoter driving the expression of these hybrid fimbriae to an inducible *spiC* promoter was also examined. This promoter is induced in *Salmonella* within vacuoles of antigen-presenting cells such as macrophages. Both the incorporation of a new inducible promoter and the deletion of the major cell wall protease from *Salmonella* resulted in the expression of intact hybrid fimbriae and increased TGEV neutralizing activity.

The enterotoxigenic *E. coli* K88 pili are responsible for host-specific adhesion to epithelial cells and allow for the colonization of the intestine of porcine (Klemm, 1985). K88 itself acts as an excellent immunogen and was the first recombinant vaccine against porcine diarrhoea (Klemm and Schembri, 2000b). A comparison of the amino acid sequences of pilins resulted in the identification of conserved and variable regions. The conserved regions were reported to be essential for the production of pili while variable regions were not involved in secretion or binding processes (Klemm, 1985). Two of the variable regions (K88ac and K88ad) corresponding to aa 164-171 and aa 208-221 respectively were replaced by a DNA polylinker sequence (Thiry *et al.*, 1989). Three synthetic peptides (two influenza hemagglutinin peptides and the hormone somatostatin) were inserted into these

polylinker sites. The deletion of both antigenic sites still allowed for the production of pilin and confirms that these variable regions are not essential. There was however, a reduction in the expression levels of pilin when the K88ac epitope was replaced with the DNA polylinker. The polylinkers were designed to carry three restriction sites (*Kpn*I, *Xba*I and *Hind*III) which allowed for easy cloning of foreign peptides within the K88 pilin (see Table 1.3 for examples).

Flagella

Flagella are the organelles associated with locomotion in bacteria. Motility allows bacteria to disperse as well as infect their hosts in the case of pathogenic bacteria (Morgan and Khan, 2001). The flagellar structure has been well characterised and is highly conserved between bacterial species (Beatson *et al.*, 2006). The flagellum are composed of up to 20 000 copies of the major flagellin protein (FliC), a pentamer (FliD) at the tip and FlgL and FlgK which connect the flagellum to the hook structure (Fig. 1.5) (Wilson and Beveridge, 1993, Morgan and Khan 2001). The function of the hook is to couple the flagellar motor to the rigid flagellum filament. The hook structure is also composed of several copies of a single protein (hook protein) which forms a flexible helical filament. The hook is connected to the basal body which is composed of a number of components which have been extensively characterised for enteric bacteria (Fig. 1.5) (Morgan and Khan, 2001).

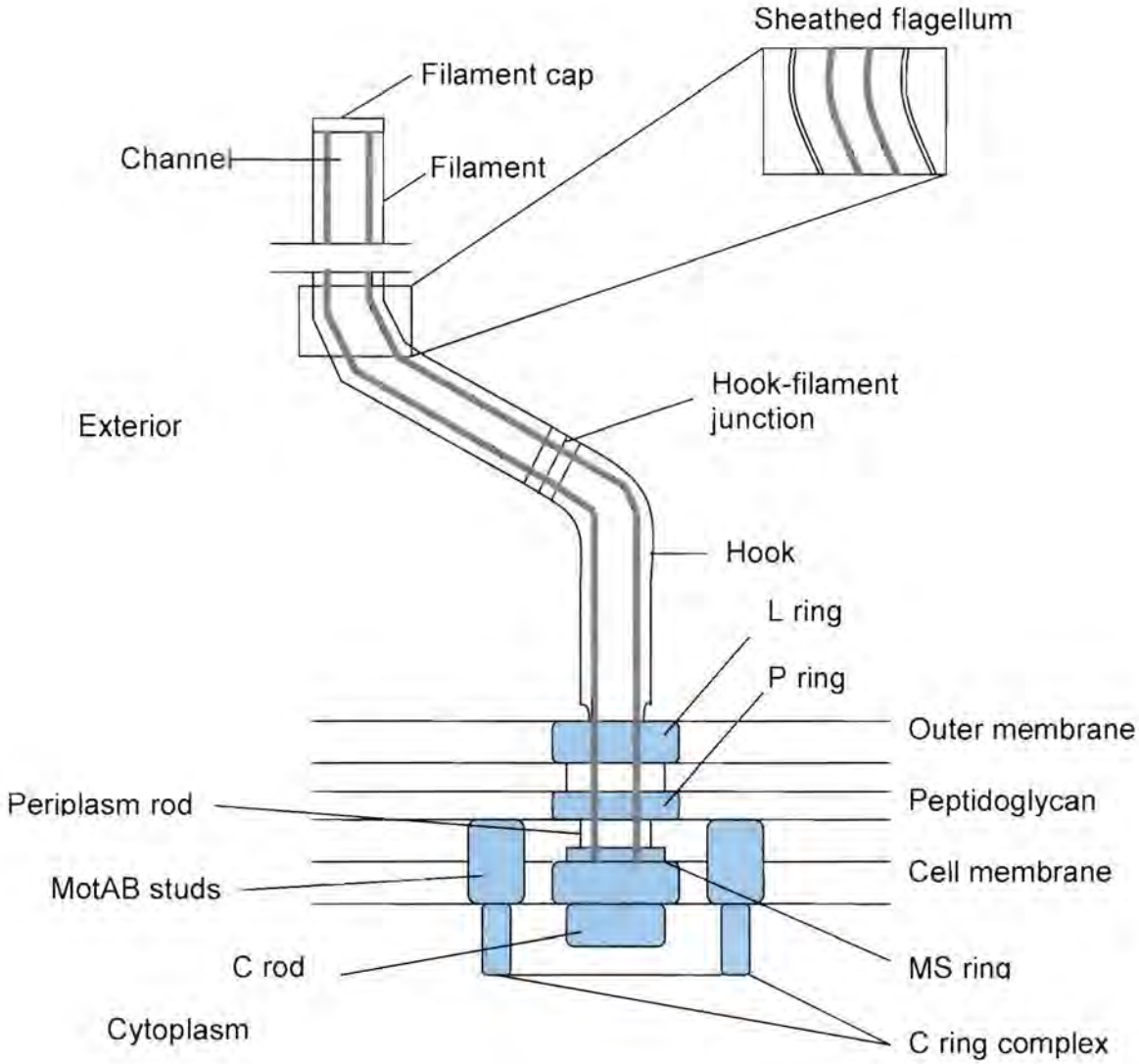


Figure 1.5 Schematic diagramme of the bacterial flagellin base. The diagramme is based on the *S. typhimurium* flagellin, while the inset indicates how a sheathed filament would appear. The MS (membrane/supramembrane), L (lipopolysaccharide) and P (peptidoglycan) rings anchor the structure into the cell wall. The MotA MotB studs represent the site of the motor complexes which drive flagellar rotation. The C (cytoplasmic) ring complex is involved in controlling the direction of flagellar rotation. The C (cytoplasmic) rod may control export of flagellar proteins. The filament, cap (FliD) and hook-filament junction sites consists of minor proteins (Morgan and Khan, 2001).

Comparison of the FliC proteins from different bacteria and strains showed a large amount of sequence similarity at the N and C termini while the central domain is highly variable in both sequence and length (Samatey *et al.*, 2001, Beatson *et al.*, 2006).

The antigenic domain (variable domain) can be removed (187 aa) from the *E. coli* FliC without the loss of flagellar polymerization or function (Kuwajima, 1988). Similarly a 105 bp region of the *fliC* gene from the *Salmonella* variable region can be removed without alteration of flagellar function (Newton *et al.*, 1991a). This suggests that this region might be amenable to peptide insertions. Flagellin secretion makes use of the Type III secretory apparatus and thus bypasses the periplasmic space. The effect of this is a lack of disulphide bond formation and thus restricts the type of foreign epitopes that can be displayed (Macnab, 1995, Westerlund-Wikström, 2000). One advantage of using flagella for display is the ease with which flagella can be isolated and purified. Most of the examples that will be discussed are related to the display of antigenic epitopes, peptide libraries, metal binding peptides and adhesive peptides. One added advantage of using flagella as a carrier of antigenic determinants is its inherent ability to induce an immune response (Cuadros *et al.*, 2004) including $CD4^+$ T cells *in vivo* (McSorley *et al.*, 2002).

The first use of the FliC system for display was carried out by Kuwajima *et al.* (1988) where an eleven amino acid epitope from the egg-white lysozyme was displayed on the surface of *E. coli*. Unfortunately, antibodies generated using the egg white epitope did not result in the production of antibodies capable of recognizing the native protein or epitope *in vitro*.

Flagellin from a variety of *Salmonella* spp. was also used in the 1980's and 1990's as carriers for immunogenic epitopes (Newton *et al.*, 1989, Wu *et al.*, 1989, Stocker and Newton, 1994). *S. muenchen* is a nonvirulent bacterial species ideal for vaccine development. The *Salmonella* gene encoding the flagellin protein was found to contain a 723 bp central variable region (Beatson *et al.*, 2006). When a 48 bp *Eco*RV-*Eco*RV fragment within the variable region was removed and replaced with a cholera toxin epitope (15 aa), the epitope was still immunogenic (Newton *et al.*, 1989). This 48 bp deletion reduced but did not abolish flagellar function. The added insertion of

the CTP epitope also did not prevent flagellar filament assembly and function, as cells were still motile. The foreign epitope was shown to be exposed at the flagellar surface. Unlike CTP3, CTP1 insertion into the *Eco*RV site resulted in a non-motile phenotype but still produced flagella as seen by electron microscopy (Stocker and Newton, 1994). The immune response to both non-motile and motile hybrid flagella was similar indicating that motility is not required for the induction of the immune response. The insertion of a single copy of the S-antigen from the hepatitis B virus resulted in no visible flagella. A clone with two copies of the S antigen lying tail to tail was also isolated and found to be motile indicating that a critical number of aa at the insertion site are important in maintaining motility (Stocker and Newton, 1994). Many antigenic determinants have been used and successfully displayed on the bacterial cell surface using FliC (Table 1.3).

In all instances where antigenic determinants were used to stimulate an immune response when coupled to the flagellin, the resulting antigen was inserted as a small peptide. The location, size and number of copies of these inserted antigenic determinants still need to be investigated in order to determine the optimal immune responses in mammals.

The flagellin display system has also been used to display peptides involved in bacterial adhesion. These molecular interactions provided information on the dynamics of cell-to-cell interactions between the pathogens and their hosts (Table 1.3) (Westerlund-Wikström *et al.*, 1997, Westerlund-Wikström, 2000). The FliC protein from *E. coli* used in this study had a deletion of 58 aa in the variable region and is also the site for the display of the adhesive polypeptides. Westerlund-Wikström and colleagues (1997) inserted varying size fragments of the YadA adhesion protein from *Yersinia enterolitica* ranging from 39 to 302 aa. It is generally acknowledged that the limiting size of inserted peptides is less than 60 aa. This was the first report of the display of a large polypepetide within the FliC protein. Prior to the insertion of the YadA peptides, the FnBPA (fibronectin binding protein) D repeats (115 aa) from *S. aureus* were inserted into the FliC protein. Initial results demonstrated the importance of all three D repeats being present for effective fibronetin binding and colonization by *S. aureus*. More importantly, this demonstrates the successful use of the *E. coli* FliC system for studies relating to cellular interactions. Tanskanen and coworkers

(2000) took the flagellin display one step further by constructing a multihybrid display system whereby flagellar filaments carried two foreign adhesive peptides. This would have far fetching biotechnological applications including the production of multivalent vaccines, construction of targeted effector molecules, and in histological localization of specific tissue domains for diagnostic purposes (Tanskanen *et al.*, 2000). Both the 115 aa D (15kDa) repeats of the FnBPA and YadA (302 aa, 33kDa) were displayed simultaneously on the flagellum filament. Results showed the co-expression of the hybrid flagellins at equal frequencies within the flagellum. These results confirm the possible use of this bihybrid display system in the applications mentioned above.

A multihybrid system was developed by Majander *et al.* (2005), where multiple foreign peptides were displayed using the FliD (Cap protein) and FliC (filament) proteins in *E. coli*. The cap protein exists as 5 FliD subunits located at the tip of the filament and promotes polymerization of FliC to form flagellin filaments. The highly conserved N-terminal 40 aa and C-terminal 50 aa legs of the FliD are essential for anchoring the cap to the tip of the filament. The central region that forms part of the cap plate is important for polymerization of FliD (Maki-Yonekura *et al.*, 2003). Sequence alignments of FliD sequences revealed 3 small variable domains which were examined as possible insertion sites for display by using the FnBPA D repeats of *S. aureus*. The variable region corresponding to amino acids 189-221 was able to tolerate a deletion of 14 aa and insertion of the D3 repeat from FnBPA (Majander *et al.*, 2005). FliD was also able to accept insertion of up to 150 aa (N-terminal region of the *L. brevis* SlpA protein). The addition of the FliD display system in combination with the previously described dihybrid display system using FliC enabled these authors to create a tri-hybrid display system. This system consisted of the FliD::D3, FliC::SlpA, and FliC::YadA fusion proteins and were all successfully displayed on the cell surface.

Lu *et al.* (1995) developed the FLITRX system which encompasses the use of the FliC from *E. coli* and a cytoplasmic thioredoxin protein. Thioredoxin's active site sequence –CGPC- forms a tight, disulphide constrained loop on the protein surface (Katti *et al.*, 1990). This site has also been shown to be highly permissive for the insertion of foreign peptide sequences (LaVallie *et al.*, 1993) without compromising

thioredoxin folding. The inserted sequences are conformationally constrained and are tethered at each end by tertiary folds within thioredoxin itself. The entire coding region of the *trxA* gene was inserted into the *fliC* gene from *E. coli*. The resulting construct, FLITRX, was used to display a random library of dodecapeptides. Ninety percent of the individual fusions retained the ability to assemble into functional flagella. The applicability of this system was determined by studying protein ligand interactions (Lu *et al.*, 1995). Westerlund-Wikström (2000) identified three advantages of this system; firstly, it is quick, easy and cheap to propagate *E. coli* (no phages required), secondly, displayed peptides have a constrained conformation and thirdly, peptides can be easily identified by biopanning processes as peptides are expressed as fusions directly to thioredoxin. Tripp *et al.* (2001) also made use of this library to identify peptides with pH or metal ion-sensitive mAbs (monoclonal antibodies) recognition, known as "switch epitopes".

It is more common for smaller peptides to be displayed using the flagellin display system. Although the YadA adhesive protein (302 aa) was successfully displayed, examples are rare (Westerlund-Wikström *et al.*, 1997). Ezaki *et al.* (1998) made use of a slightly modified FliC display system from *E. coli* to display a larger repertoire of peptides and proteins. They made use of the full FliC protein with a polylinker (15 aa) inserted into the centre of the variable region in order to display foreign peptides. These included the region C (110 aa) and sFv (249 aa) of the antiporphyrin antibody, green fluorescent protein (GFP, 236 aa) and an alkaline phosphatase (471 aa). Only the smaller region C was displayed at levels equal to the full FliC::polylinker hybrid flagella. The larger proteins, although successfully displayed, resulted in a dramatic reduction in hybrid flagellar production. Although no mention was made of the alkaline phosphatase activity, the GFP chimera was found to fluoresce when illuminated with blue light. The attempts at functional display of these larger proteins could broaden the biotechnological applicability of the flagellar display system to include antibody production and biotransformation.

Needle Complex

Type three secretion systems (TTSS) are proteinaceous structures found on the surface of Gram-negative pathogens and are used to inject virulence factors into target eukaryotic cells (Hueck, 1998). The TTSS channel is an assembly of proteins from 20 gene products and is termed the needle complex (Crepin *et al.*, 2005). The general structure of the needle complex resembles that found for the flagellum hook and basal body structure with needle being the equivalent of the hook structure. Enteropathogenic (EPEC) and enterohaemorrhagic (EHEC) bacteria have a unique extension to the needle complex called the EspA filament (Knutton *et al.*, 1998). The helical packing and symmetry of the EspA is similar to that of the flagellum filament and also has a central channel. Deletion mutation studies of two hypervariable domains located in the antigenic surface exposed loop were created. Only deletion of the 6 aa region corresponding to aa 123-129 was permissible and did not affect the polymerisation into functional EspA filaments. A larger deletion of 12 aa at position 117-129 resulted in deformed EspA filaments. Hydrophobicity and hydrophilicity studies of the variable domain were used to select a suitable insertion site for foreign peptides. A site between D117 and I118 was identified as suitable position for the display of a hydrophilic flag tag sequence (DYKDDDDK). Although biologically active filaments were observed they appeared shorter than wild type filaments. These results do demonstrate the successful display of a heterologous peptide but the insertion site may not be optimal and requires further investigation.

1.2 Gram-positive Display Systems

Initial studies investigating various display systems all focussed on Gram-negative bacteria, but more recently numerous strategies for surface display in Gram-positive bacteria have also been described and reviewed (Hansson *et al.*, 2001, Wernerus *et al.*, 2002, Desvaux *et al.*, 2006). Structurally, Gram-positive bacteria have a thicker cell wall which provides a more rigid structure for surface display which makes them suitable as whole-cell catalysts and whole-cell adsorbents (Lee *et al.*, 2003). The lack of an outer membrane should also simplify extracellular secretion of heterologous proteins to the cell surface. Gram-positive bacteria have the added advantage of

having GRAS (generally regarded as safe) status and originate in normal gut flora and are potential hosts for live oral vaccines. Various proteomic studies have identified known surface proteins for Gram-positive bacteria and it is conceivable that most of these proteins could possibly be developed as surface display systems (Ton-That *et al.*, 1997, Calvo *et al.*, 2005, Desvaux *et al.*, 2006). Many of these proteins have common features necessary for cell wall anchoring which has led to a diverse array of proteins as carriers for heterologous polypeptides.

1.2.1 N-terminal Fusions

Cell Wall Anchored Proteins

Pathogenic staphylococci and other Gram-positive bacteria require surface proteins to interact with specific host molecules or target cells in order to adhere, invade and evade host cell responses (Marraffini and Schneewind, 2005). Many of the cell wall anchored proteins have common mechanisms for cell wall attachment. In order for the cell surface proteins to be directed to the cell surface, the proteins require an N-terminal signal peptide and a C-terminal cell wall sorting signal (Schneewind *et al.*, 1992). The C-terminal sorting signal has been well characterised and consists of a conserved pentapeptide motif (LPXTG), a hydrophobic region of 15-22 aa and a short charged tail of 6-7 aa (Fischetti *et al.*, 1990, Schneewind *et al.*, 1993). Once the surface protein has been translocated across the membrane and the signal sequence has been cleaved, the C-terminal sorting region retains the cell bound protein within the cytoplasmic membrane (Schneewind *et al.*, 1992). A membrane anchored sortase A cleaves the sorting signal between the threonine and glycine residues, and subsequently results in the linkage of the threonine to a branched peptide, via the amino group of the pentaglycine crossbridge in the peptidoglycan layer (Schneewind *et al.*, 1995, Ton-That *et al.*, 1997, Navarre and Schneewind, 1999, Marraffini and Schneewind, 2005). The use of this anchoring domain as a display system is the one most frequently used in Gram- positive display system.

Probably the most well characterized Gram-positive cell wall anchoring protein is protein A from *Staphylococcus aureus* (SpA) (Fig. 1.6). SpA is a cell surface receptor of *S. aureus* and is capable of binding to immunoglobulins (Langone, 1982). SpA

consists of a N-terminal signal peptide (S), five immunoglobulin binding sites (A-E), a repetitive glycine and proline rich cell wall spanning region (X), and a C-terminal sorting sequence (M) (Hansson, *et al.*, 2001) (Fig. 1.6).

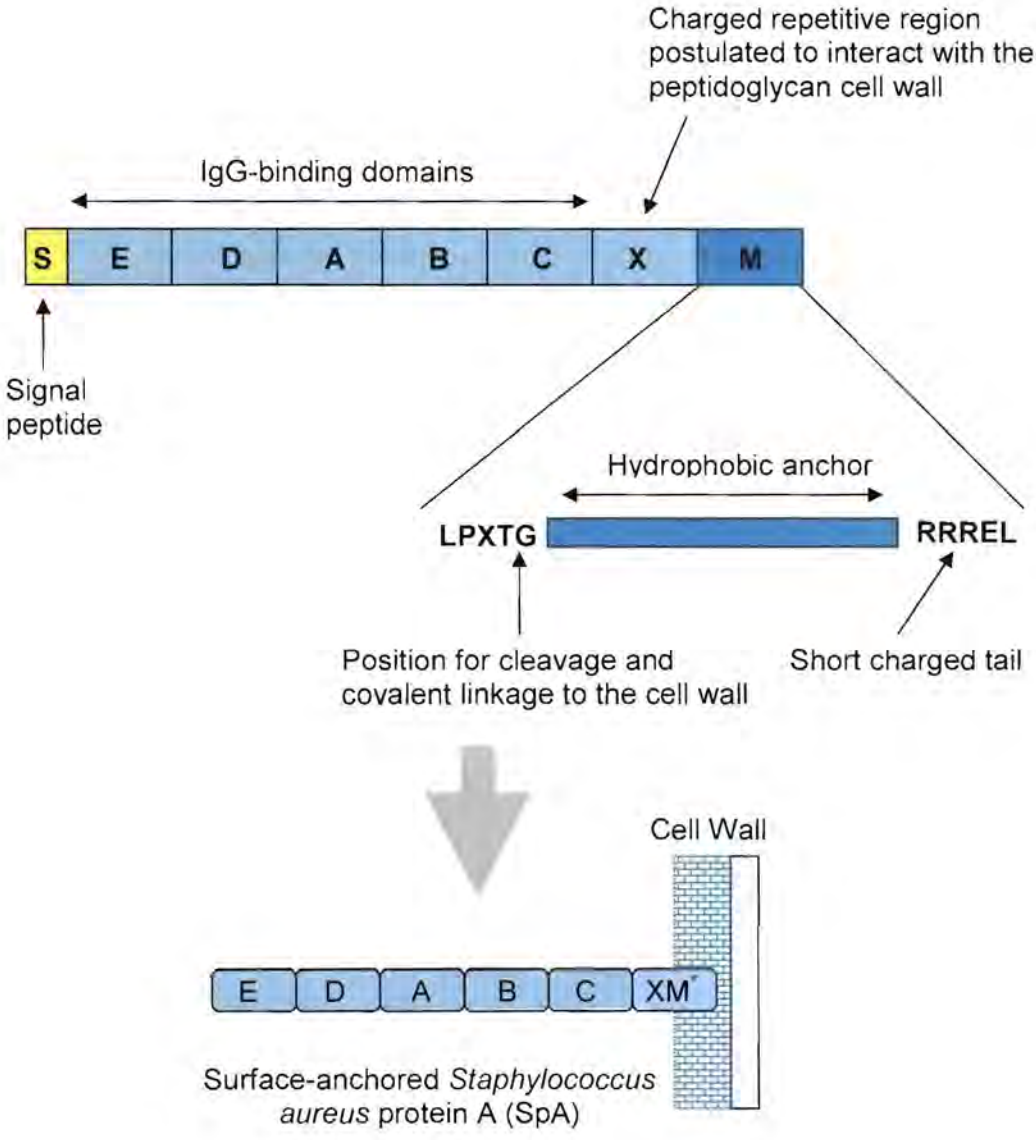


Figure 1.6. A schematic representation of the different regions of *S. aureus* protein A (SpA) and a simplistic illustration of how SpA is processed and anchored to the staphylococcal cell wall. The N-terminal signal peptide S and the C-terminal regions X and M are responsible for surface sorting. The M region contains the signals for proteolytic cleavage and covalent linkage of the surface protein to the bacterial cell wall. The M^* represents the processed cell wall bound version of the SpA M region (Hansson *et al.*, 2001).

The SpA protein's signal sequence (S) and surface attachment region (X and M) were used for the surface exposure of recombinant proteins on *S. xylosus* (Hansson *et al.*, 1992). The recombinant gene fragments encoding the albumin binding peptide from the streptococcal protein G (BB) and the peptide M3, derived from the C-terminal part of the *Plasmodium falciparum* malaria antigen, were inserted into the expression vectors between the signal sequence and C-terminal sorting domain. These proteins were expressed in *S. xylosus* and the recombinant proteins were exposed on the cell surface (Fig. 1.7). Although these peptides were exposed at the cell surface the BB and M3 were only detected in 40-50% and 10-15% of the cells examined. This was hypothesised to be a result of steric hindrance caused by differential structures on the cell wall. The addition of protein spacers may make these heterologous peptides more accessible on the cell surface and result in less interference from structures on the cell wall.

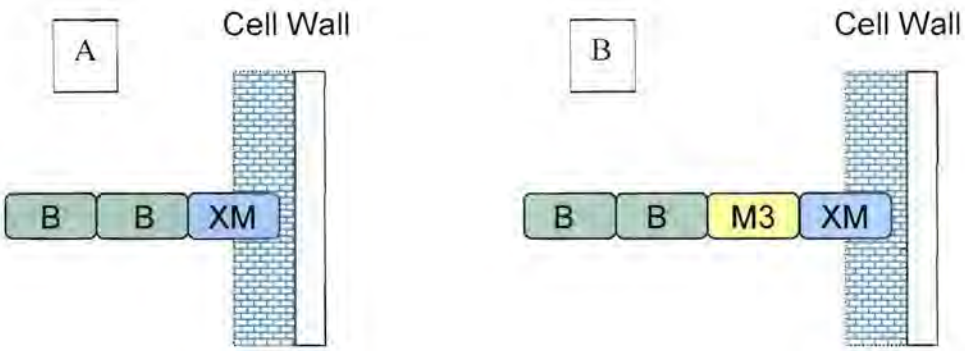


Figure 1.7. Schematic diagram showing the processed and ecoded gene products BBXM (A) and BBM3XM (B) displayed on the cell surface of *S. xylosus* using the SpA signal sequence and C-terminal sorting domain (X and M) (Hansson *et al.*, 1992).

The system described above was modified for the expression of the M3 peptide in *S. carnosus* (Samuelson *et al.*, 1995). In this instance the signal sequence was replaced with the promoter, signal sequence and propeptide (PP) from the lipase gene from *S. hyicus* (Gotz, 1990) but retained the SpA cell surface attachment motif. The promoter of this lipase has been previously modified for overexpression and was used for lipase production in *S. carnosus* (Lechner *et al.*, 1988). A 198 aa region from the albumin binding protein (ABP) was expressed adjacent to the cell wall binding motif in order

Table 1.4. Selected examples of N-terminal fusion display systems in Gram-positive bacteria.

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
S. aureus, SpA	Albumin binding protein G (BB), M3 Antigen (80 aa)	*S. xylosus*	Vaccine	Hanson *et al.*, 1992
	M3 antigen	*S. carnosus*	Vaccine	Samuelson *et al.*, 1995
	CTB (103 aa)	*S. carnosus and S. xylosus*	Vaccine	Liljeqvist *et al.*, 1997
	CTB_p epitope (25 aa)		Vaccine	Cano *et al.*, 1999
	RSV G protein		Vaccine	Cano *et al.*, 2000
	scFv		Antibody	Gunneriusson *et al.*, 1996
	SpA	*S. carnosus*	Antibody purification	Gunneriusson *et al.*, 1999
	Poly-His, CBD (36 aa)		Bioremediation	Samuelson *et al.*, 2000, Wernerus *et al.*, 2001, Lehtio *et al.*, 2001
	Strepavidin	*Lactococcus lactis*		Steidler *et al.*, 1998
S. pyogenes, M6	HPV (98 aa)	*S. gordonii*	Vaccine	Pozzi *et al.*, 1992
	Ag 5.2 (98 aa)		Vaccine	Medaglini *et al.*, 1995
	E7 HPV		Vaccine	Medaglini et al., 1997
	HIV-1 gp 120 V3 loop		Vaccine	Fabio *et al.*, 1998, Oggioni *et al.*, 1999b
	LTB		Vaccine	Ricci *et al.*, 2000
L. fermentum, Rlp	Poly-His, CFTR (10 aa)	*L. fermentum*	Bioremediation	Turner *et al.*, 2003
S. aureus, FnBP	Lipase, β-lactamase	*S. carnosus*	Biotransformation	Strauss and Gotz, 1996
	α-amylase	*B. subtilis*	Biotransformation	Nguyen and Schumann., 2005
L. lactis, AcmA	SlpA	*L. lactis*	Cell targeting	Åvall-Jääskeläinen *et al.*, 2003
	VP1 Antigen		Vaccine	Raha *et al.*, 2005

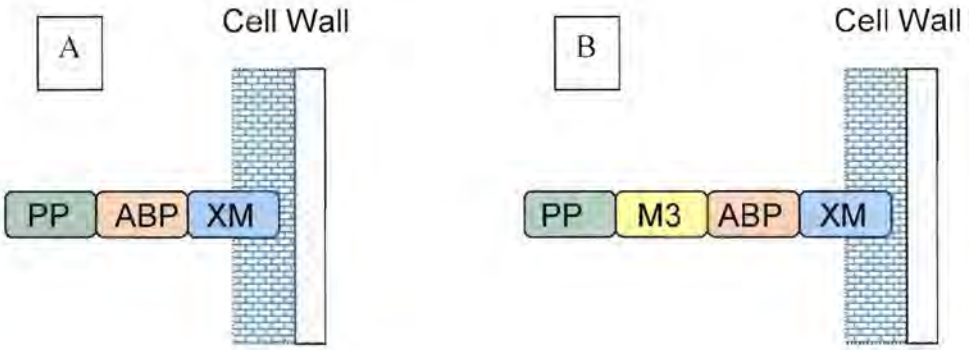


Figure 1.8. Schematic diagram showing the processed and encoded gene products PPABPXM (A) and PPM3ABPXM (B) displayed on the cell surface of *S. carnosus* using the SpA signal sequence and C-terminal sorting domain (XM) (Samuelson *et al.*, 1995).

to increase accessibility of the displayed M3 peptide (Fig. 1.8). The ABP spacer acts as a reporter molecule and also increases the accessibility of the 80 aa M3 malarial antigen on the cell surface. Other antigens have also been successfully displayed using SpA (Table 1.4). Live delivery of the staphylococci carrying the antigenic epitopes elicited significant IgG responses as opposed to purified hybrid flagella (Cano *et al.*, 1999, Cano *et al.*, 2000).

Both systems used in *S. xylosus* and *S. carnosus* were also utilised for the production of single chain Fv antibody fragments (Gunneriusson *et al.*, 1996) (Table 1.4). This was the first report of the successful production and display of a scFv antibody in Gram-positive bacteria and the fact that it was also functional is of great significance for future scFv production.

The importance of displaying metal binding peptides for bioremediation has been extensively discussed for Gram-negative bacteria. The general applications of displaying these metal binding peptides are no different for Gram-positive bacteria. One added advantage of using Gram-positive bacteria for bioremediation is their inherent ability to bind metals due to the thick peptidoglycan layer (Mullen *et al.*, 1989). The staphylococci display systems were employed to display polyhistidine peptides for the capture of divalent cations (Samuelson *et al.*, 2000) (Table 1.4).

For successful surface display of peptides involving biotransformation or bioremediation a mechanism is needed for immobilisation of the recombinant bacteria. Lehtio *et al.* (2001) made use of a CBD (cellulose binding domain) derived from *Trichoderma reesi* cellulase Cel6A, displayed in its native form, on *S. carnosus.* The recombinant staphylococci were found to be functional as they bound efficiently to cotton fibres. The co-display of this CBD together with biologically active enzymes or metal binding peptides could provide a potential method for the immobilization of recombinant bacteria to cotton fibres for use in biotransformation and bioremediation respectively.

The SpA system was also functional in *Lactococcus lactis* (Steidler *et al.*, 1998). This is not surprising since the mechanism of sorting in Gram-positive bacteria is highly conserved (Goward *et al.*, 1993). The fusion protein created for *L. lactis* differed from that mentioned for the staphylococci in that it contained the lactococcal USP45 signal sequence, a strepavidin monomer, and the SpA anchor. Strepavidin was used as a tool for detection but can also be useful for immobilization of the recombinant *L. lactis* on solid surfaces. The alkaline phosphatase conjugated biotin was immobilized on polystyrene beads and were shown to bind recombinant cells under the light microscope thus confirming the usefulness of this system for immobilization of recombinant *L. lactis* (Steidler *et al.*, 1998).

The fibrillar M6 protein is an α-helical coiled surface protein from *Streptococcus pyogenes*, with the typical cell wall anchoring features described for SpA. These proteins are found in all group A streptococci and are responsible for the anti-phagocytic properties of the bacteria. The insertion and display of foreign polypeptides was initially developed by Pozzi *et al.* (1992). Foreign epitopes were subsequently inserted into a polylinker MCS (multiple cloning site) within the M6 protein. This M6 derivative had a deletion of 180 aa of the B and C repeats (Fischetti, 1989). Insertions of foreign peptides within the MCS resulted in an N-terminal fusion with the M6 protein still having its own A repeat region and anchoring domain. This display system was subsequently modified (Oggioni and Pozzi, 1996) to include the M6 signal peptide, a polylinker MCS, the full C repeat region and the anchor domain, but no A repeat region. This resulted in an N-terminal display system similar to that described for SpA. Since both of these M6 display systems follow similar principles

and make use of a C-terminal anchoring domain, all examples using these two similar systems will be dealt with in this section.

Both the above expression systems are able to integrate into the host chromosome and express the M6 gene fusions. The first example carried a M6::E7 protein fusion (Pozzi *et al.*, 1992). After integration into the *Streptococcus gordonii* chromosome the M6::E7 fusion protein was shown to be present on the cell surface and ellicit an immune response in mice. Streptococcal species colonize the mucosal surfaces of humans and most are non-pathogenic which makes them ideal candidates to express heterologous antigens and in so doing, stimulate local immune responses. The induction of systemic and mucosal immune responses using this vector system was confirmed for many antigens (Table 1.4). Although the V3 loop of HIV-1 gp120 is immunogenic, the antigenic response is relatively poor. Heat-labile toxin B subunit (LTB) is a known mucosal adjuvant and possesses immunomodulating properties (Rappuoli *et al.*, 1999). Maggi and co-workers (2002) made use of both the expression vectors constructed by Oggioni *et al.* (1999a) and Ricci *et al.* (2000) to simultaneously express and display both the V3 loop antigen and the LTB. Subcutaneously immunized mice using recombinant streptococci displaying both antigens resulted in a 4 fold increase in IgG titres for the V3 loop antigen compared to just the M6::V3 loop fusion alone. The co-expression of adjuvants is able to facilitate the antibody response towards a specific antigen which is highly advantages in recombinant vaccine design.

The M6 protein has also been expressed in *L. lactis*, *Lactobacillus fermentum*, *Lactobacillus sake* and *Streptococcus thermophilus* (Piard *et al.*, 1997). This would suggest an attachment mechanism that is highly conserved for these proteins. However, differences in anchoring efficiencies were observed with *S. pyogenes*, *L. lactis*, and *S. thermophilus* having detectable levels in the supernatant which suggest incomplete attachment to the cell wall. *L. sake* and *L. fermentum* did show complete attachment to the cell surface. These results indicate the potential of the M6 display system to be utilised across a broad range of Gram-positive bacterial species. This was successfully demonstrated by Cortes-Perez *et al.* (2005) using a system composed of the Usp45 signal sequence, E7mm (engineered E7 protein), and the *S. pyogenes* M6 cell wall anchoring domain. In this instance successful cell wall anchoring was

only observed for *L. lactis*. *Lactobacillus plantarum* produced the hybrid protein but it was not attached to the cell wall. The M6 anchoring motif was replaced by a *L. plantarum* cell wall anchoring protein, which subsequently allowed efficient anchoring of the E7mm antigen to the cell surface.

The use of probiotic bacteria (eg. *Lactobacillus* sp.) as delivery systems for heterologous proteins to specific mucosal sites is of great benefit in vaccine development. *Lactobacillus fermentum* is one such example. Turner *et al.* (2003) identified two novel proteins carrying the cell wall anchoring motif LPXTG from *L. fermentum*, namely Rlp and Mlp. The Rlp cell sorting domain was used to display a poly-His epitope and the human cystic fibrosis transmembrane regulator (CFTR, 10 aa) while Mlp was used to secrete large proteins. It is thus possible to use covalently cell wall anchored proteins for both display and secretory purposes depending on the application required.

Strauss and Gotz (1996) used the C-terminal sorting sequence of the *S. aureus* fibronectin binding protein B (FnBPB) to immobilize the *S. hyicus* lipase to the cell wall of *S. carnosus*. The hybrid lipase bound to the cell wall gave 80% activity when compared to the unmodified form, but subsequent lysostaphin treatment (releasing the lipase) restored full activity. The importance of the spacer region was also evaluated and a minimal length of 90 aa is required for maximum enzyme activity and correct folding. The subsequent replacement of the lipase with an *E. coli* β-lactamase yielded similar results. Immobilization and maintenance of enzyme catalytic activity on Gram-positive bacterial cell surfaces provides a useful tool for biotransformation processes in various industrial applications. Nguyen and Schumann (2006) attempted to develop *B. subtilis* cells into cellular chips and made use of the FnBPB of *S. aureus*. *B. subtilis* provides a number of advantages over other Gram-positive bacteria which include; GRAS status (generally regarded as safe) organism; has an efficient secretion capacity; large scale fermentation techniques are available and the genetic tools are well developed. In addition to the FnBP fusion protein, the *srtA* gene from *Listeria monocytogenes* was also transformed into *B. subtilis*. This provided the cleavage machinery needed for cell wall anchoring of the FnBP LPXTG binding motif. The FnBPB cell wall sorting region was fused to the *Bacillus*

amyloliquefaciens α-amylase and shown to be cell wall associated with a predicted 240 000 molecules of active enzyme on the cell surface (Nguyen and Schuman., 2006). This equates to a 24 fold increase in the number of cell wall anchored molecules compared to the previously described display of a lipase in *S. carnosus*. Optimal spacer length was also shown to be slightly longer for *B. subtilus* (123 aa) as opposed to *S. carnosus* (90 aa).

In order for the successful display of heterologous proteins or peptides on the surface of Gram-positive bacteria using cell wall anchoring proteins, a number of factors need to be taken into consideration and in some cases optimised. These include, ensuring that the relevant sortases are present for cell wall anchoring, selection of the optimal cell wall anchoring motif and ensuring the optimal spacer length for the display of an enzymatically active protein.

Cell Membrane Anchored Proteins

Autolysins

Autolysins are cell wall hydrolases and have been used as surface display systems in both N- and C-terminal fusions. Autolysins are required for cell separation and are responsible for cell lysis during stationary phase (Buist *et al.*, 1995). N-terminal fusions have made use mainly of the AcmA autolysin from *L. lactis*. This autolysin consists of an N-terminal signal sequence, a central active domain and a C-terminal membrane anchor. The C-terminal domain consists of three repeats of 44 aa and are involved in cell wall binding (Buist *et al.*, 1997). However, only a single repeat is required for cell wall anchoring.

As already mentioned, *L. lactis* also has GRAS status and is capable of surviving passage though the intestinal tract (Klijn *et al.*, 1995). For this reason, *L. lactis* could be used as a safer alternative than attenuated pathogens for the delivery of antigens for intranasal and oral immunisation. Åvall-Jääskeläinen and colleagues (2003) made use of the AcmA anchor region to determine the ability of an S-layer protein (SlpA) from *Lactobacillus brevis,* to adhere to human intestinal cells and to endow a non-adhesive

L. lactis with cell binding capabilities. The area responsible for cell binding has already been characterised (flagellin display, section 1.1.3) and is known to be located in the N-terminal region of SlpA (Hynonen *et al.*, 2002). However, flagellin display did not result in the detection of SlpA using antibodies. The current display cassette consists of the AcmA anchor, a PrtP spacer and N-terminal located SlpA binding domain. In vitro adhesion assays of recombinant *L. lactis* to human intestinal epithelial cells demonstrated the inheritance of cell binding capabilities. The transfer of receptor-binding regions between bacteria provides a tool for directing recombinant antigenic bacteria to target areas on epithelial cells and in so doing enhance immunological responses. Raha *et al.* (2005) were able to make use of the AcmA display system using a single binding domain to deliver two viral capsid antigens from the VP1 protein (Table 1.4). Both antigens were stably maintained on the cell surface for 5 days and shown to be located on the cell surface using whole cell immunological assays. Unfortunately the study did not discuss the immune response to the recombinant live vaccine delivery system and the efficacy of this display as a live vaccine is still to be determined.

Cell surface display using attenuated L. lactis

Many examples have been discussed using the M6, SpA, and AcmA proteins as anchors. Ramasamy and co-workers (2006) made use of the covalently bound proteinase, PrtP and non-covalently bound AcmA from *L. lactis* to display a malarial parasite antigen (MSA2, 223 aa). Since the use of recombinant bacteria (especially *L. lactis*) is widespread, the transfer of genetic material poses an un-intentional environmental risk and thus a novel approach was adopted to display the malarial antigen by the authors. This display system makes use of a non-genetically modified *L. lactis* cellular support (Gram-positive enhancer matrix; GEM) bound to heterologous peptides fused to proteins capable of binding to the peptidoglycan layer (protein anchor). *L. lactis* cells are pre-treated with acid resulting in non-living particles (GEM) which are used to bind antigen-protein anchor fusions. In this way antigens can be presented to the immune system without any recombinant DNA being present. This system was only adopted for the AcmA display system where the hybrid protein was derived from an alternate source and bound to the GEM particles in vitro. This display system was compared to the covalently bound PrtP display system

carrying the same malarial antigen but generated using live *L. lactis* cells carrying the expression cassette. Both hydrid display proteins delivered as live cells (PrtP) and GEM particles (AcmA) yielded similar titres of serum antibodies when administered orally (Ramasamy *et al.*, 2006). It is thus possible to use non-genetically modified bacterial cells to deliver antigenic determinants and elicit a systemic immune response without possible release of recombinant DNA into the environment.

1.2.2 C-terminal fusions

Cell Membrane anchored proteins

Autolysins

Autolysins and the role they play have been discussed in section 1.2.1. *B. subtilis* cell wall hydrolase, CwlB, is a class II amidase (Tsuchiya *et al.*, 1999). CwlB (LytC) is the vegetative major autolysin of *B. subtilis* and consists of a N-terminal cell wall binding domain and a C-terminal catalytic domain (Kuroda and Sekiguchi, 1991). Tsuchiya and colleagues (1999) made use of the CwlB to localize a *B. subtilis* lipase (LipB) on its cell surface (Table 1.5). In industrial processes the lipase is immobilised prior to use as lipases generally occur extracellularly (Jaeger and Reetz, 1998). This method of display negates the need for artificial immobilisation on a solid support and also provides an easy purification method for the isolation of the CwlB::LipB fusion. However, the lipase was unstable and lypolytic activity was low. Mutations of a number of key proteases (NprE and AprA) did not significantly improve yields. The removal of the major cell wall binding protease, WprA did result in a minor increase in yields in stationary phase (Kobayashi *et al.*, 2000). A *sig D* mutant was also created and showed significantly higher yields of fusion protein. This could be a result of two factors namely, reduced cell surface competition between native CwlB and CwlB lipase fusion proteins and, possible reduction in various unknown proteases under the control of the SigD protein. Other lipases were also shown to be localised on the cell surface using this display system and retained lypolytic activity (Table 1.5).

Table 1.5 Selected examples of C-terminal fusion display systems in Gram-positive bacteria.

Carriers (size)	Passengers (size)	Hosts	Applications	Refs
B. subtilis, CwlB	LipB	*B. subtilis*	Biotransformation	Tsuchiya *et al.*,1999
	CutL		Biotransformation	Kobayashi *et al.*, 2002
B. anthracis, EA1, Sap	Levansucrase	*B anthracis*	Biotransformation	Mesnage *et al.*, 1999a
	Tet Toxin		Vaccine	Mesnage *et al.*, 1999b
B. sphaericus, SbpA	BetV1	*B. sphaericus*	Vaccine	Ilk *et al.*, 2002
B. subtilis, CotB	TTFC (459 aa)	*B. subtilis*	Vaccine	Isticato *et al.*, 2001
B. subtilis, CotC	TTFC, LTB (12 kDa)	*B. subtilis*	Vaccine	Mauriello *et al.*, 2004
B. thuringiensis, protoxin	Anti-phox, EGFP	*B. thuringiensis*	Antibody	Paerk *et al.*, 2004, Du *et al.*, 2005

A dual display system was subsequently developed using the C-terminal anchoring motif of the CwlC (N-terminal fusion) and CwlB system described above. Both lipases were shown to be displayed simultaneously. This provides a tool to degrade a wider variety of lipids since each lipase has specificity against a different range of fatty acid esters (Kobayashi *et al.*, 2002).

Cell Surface Associated Proteins

S-layer Proteins

S-layer proteins have been identified in many different species of bacteria across all major phylogenetic groups including the Archae bacteria (Sara and Sleytr, 2000). In Gram-negative bacteria the S-layer proteins are attached to components of the outer membrane while in Gram-positive bacteria they are attached to the peptidoglycan layer. Sara and Sleytr (2000) have extensively reviewed S-layer proteins.

S-layer proteins in general consist of conserved N-terminally associated S-layer homologous (SLH) motifs and a C-terminal cell associated exo-enzyme. Interaction between the S-layer proteins and the cell wall is non-covalent, and can account for 15% of total cell protein (Mesnage *et al.*, 1999a). S-layer proteins have three repeat motifs at the N-terminal end of about 50 amino acids. These repeats are called S-layer homology (SLH) motifs (Lupas *et al.*, 1994) and it is this region that is responsible for cell surface anchoring. *Bacillus anthracis* has two S-layer proteins Ea1 and Sap (Mesnage *et al.*, 1999a). By fusing the N-terminal repeat motifs of these S-layer proteins to levansucrase, Mesnage and colleagues (1999a) were able to demonstrate successful and stable display of this enzyme. Subsequently, a tetanus toxin fragment was also displayed on the cell surface of *B. anthracis* using this display system (Mesnage *et al.*, 1999b). The *Bacillus sphaericus* SbpA S-layer protein was screened for the best position for display by inserting an affinity tag, *Strep*-tag 1. Once this position was characterised it was exploited for the successful display of the major Birch pollen antigen (Bet v1) (Ilk *et al.*, 2002) (Table 1.5).

Surface Display on Spores

Spores possess a number of potential advantages which include high stability, good safety record as many spore producers have GRAS status, and are economical and simple to produce (Isticato *et al.*, 2001). *B. subtilis* spores are surrounded by a coat which is a proteinaceous structure organized into two distinct layers and composed of at least 25 polypeptides (Driks, 1999) (Fig. 1.9). The outer coat consists of five polypeptides, CotA (65 kDa), CotB (59 kDa), CotC (11 kDa), CotF (8 kDa) and CotG (24 kDa) (Ricca and Cutting, 2003).

Isticato *et al.* (2001) were able to confirm the location of CotB to be on the spore surface and subsequently used CotB to display a 459 amino acid C-terminal fragment of the tetanus toxin (46 kDa, TTFC) as a C-terminal, N-terminal and sandwich fusion.

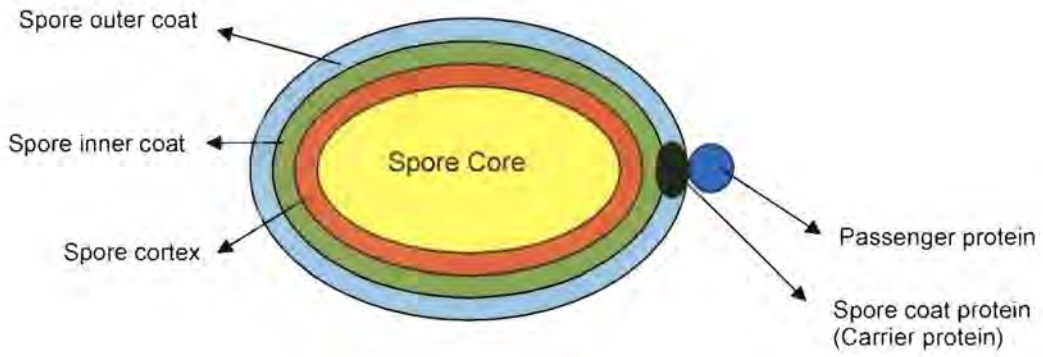


Figure 1.9. Spore-surface display using spore coat proteins. The *B. subtilis* spore is composed of an internal core surrounded by a peptidoglycan like cortex and proteinaceous coat sub-divided into an inner and outer coat. The fusion protein, composed of a carrier and a passenger is exposed on the spore surface (Ricca and Cutting, 2003)

In order to accomplish the C-terminal fusion, the three 27 amino acid repeats at the C-terminal end of CotB were removed. Failure to remove these repeats led to incomplete assembly of the hybrid protein. A C-terminal fusion of the heat labile toxin of the enterotoxigenic strain of *E. coli* (12 kDa, LTB), to the C-terminal end of the CotB protein was not correctly assembled. This resulted in poor sporulation and germination efficiencies.

CotC is present at much higher levels relative to the other Cot proteins. Both CotB and CotC are dispensable within the spore as mutants lacking these proteins were still properly formed and remained viable (Ricca and Cutting, 2003). In the case of CotC only C-terminal fusions have been used to display the tetanus toxin and the B subunit of the heat labile toxin of enterotoxigenic strain of *E. coli* (Mauriello *et al.*, 2004). In this instance both hybrid CotC products were correctly assembled and had no detrimental effect on sporulation and germination. Although CotC occurs at a much higher level than CotB the relative amounts of the chimeric proteins are similar. The importance of the C-terminal end in assembly into the spore coat requires further investigation which will yield improved methods for the display of heterologous proteins using CotC. Although both CotB and CotC were successfully used as display systems of heterologous proteins, only the CotB::TTFC fusion was tested for immune

response and protective capabilities in mice immunized via the oral and intranasal route. Both immunizations elicited an immune response and resulted in mice capable of surviving inoculation with the tetanus toxin (Duc *et al.*, 2003).

Bacillus thuringiensis spores have also been used for the successful display of EGFP (enhanced green fluorescence protein) (Park *et al.*, 2004, Du *et al.*, 2005) and a single chain antibody (anti-phOx) (Du *et al.*, 2005) (Table 1.5). In both instances the heterologous proteins were shown to be functional. The highly efficient display of heterologous peptides, the simplistic purification procedure and high stability makes this display system a prime candidate for surface expression of many bioactive molecules.

1.2.3 Sandwich fusions

The major surface protein antigen gene (*spaP*) from *Streptococcus mutans* is a cell wall bound protein (see section 1.2.1) and has been found on the surface of almost every oral streptococcal species (Navarre and Schneewind, 1999). These proteins are large (190 kDa) and contain multiple binding activities and repeat domains (Jenkinson and Demuth, 1997). These surface antigens have been reported to be able to bind salivary glycoproteins as well as other oral microbes (Navarre and Schneewind, 1999).

Lee *et al.* (1999) made use of the SpaP protein to display a recombinant pertussis toxin S1 subunit in *S. gordonii*. *S. gordonii* is an oral commensal bacterium and has the potential to be delivered as a live oral vaccine vehicle (Lee *et al.*, 1999). Insertion of the N-terminal 179 aa of the S1 toxin into the centre of the SpaP protein as an in frame fusion was done and confirmed to be located at the surface. Mice immunized with heat killed *S. gordonii* hybrids were found to be resistant to the toxic effects of the pertussis toxin. The use of cell wall bound proteins as N-terminal and sandwich fusions indicate the versatility of these proteins for a diverse array of proteins and peptides.

As our understanding of protein secretion, cell wall structure, and isolation of novel organisms increase so will the number of display systems. One challenge that does

still remain is to transfer the technology of surface display into a viable industrial application. Current display systems have not made this transition and remain an untapped technology.

1.3 Aims of the Project

Bacillus halodurans Alk36 was isolated from a soil sample in South Africa (Louw *et al.*, 1993) and originally classified as *Bacillus brevis* Alk36. 16s rDNA sequence analysis reclassified *B. brevis* Alk36 as *B. halodurans* Alk36. Work done on protein expression profiles showed that *B. halodurans* Alk36 was capable of constitutively expressing a cell surface protein of 34 kDa. This protein was identified as FliC (flagellin), the product of the *hag* gene (Crampton *et al.*, 2007) and was subsequently chosen for development as the anchoring motif for the display of heterologous peptides and proteins. The use of flagella to display heterologous peptides and proteins has as yet not been demonstrated in Gram-positive bacteria but has received much attention in Gram-negative bacteria (section 1.1.3). The advantages of a Gram-positive surface display system together with the high and continuous expression levels of the FliC protein is also unique since most *Bacillus* sp tend to sporulate in stationary phase and flagellin synthesis ceases. *B. halodurans* Alk36 is a moderate thermophile (grows at 30-55°C) and alkaliphilic (pH 7.5-10.5). The *B. halodurans* C-125 genome has been fully sequenced (Takami *et al.*, 2000) and genes aligned from *B. halodurans* Alk36 thus far are greater than 99.9 % homologous.

B. halodurans Alk36 posseses a number of unique characteristics and potentially has novel or enhanced applications for surface display. The continuous and high expression levels of the flagellin protein provide an exciting possibility for heterologous peptide and protein display. The wide range in growth conditions provides the potential for applications in unique environments, especially the expression and display of thermo tolerant enzymes. The main aim of this project was therefore to develop *B. halodurans* Alk36 as a surface display host utilizing the flagellin (FliC) as an anchoring motif in a sandwich fusion. To achieve these goals genetic systems had to be developed for gene targeted replacements (*hag* gene) for strain development and expression of the chimeric flagellin fusions. These systems will allow for the insertion of the chimeric flagellin into the chromosome thus

omitting the use of selectable antibiotic markers. After construction of the necessary bacterial strains, a region within the FliC protein had to be identified which permitted the insertion of heterologous peptides. For characterisation of the permissible insertion sites a number of peptides and a protein were inserted. These examples include a metal binding peptide (poly-His linker), HIV antigen, and a lipase. Each of these examples was found to be biologically active.

Chapter 2.

Construction of a *Bacillus halodurans* Alk36 Δ *hag* Mutant for Surface Display of Heterologous Peptides and Proteins.

2.1 INTRODUCTION

At the beginning of this project, this research group found the *B. halodurans* Alk36 strain continuously expressed a 34 kDa surface protein (Crampton *et al.*, 2007). Through N-terminal sequencing this protein was identified as flagellin, product of the *hag* gene. At the nucleotide level this gene as well as the up- and down- stream regions was found to show 100% identity with the corresponding gene from *B. halodurans* C-125. The genome of *B. halodurans* C-125 has been sequenced and is available on the DNA Data Bank of Japan (DDBJ; http://gib.genes.nig.ac.jp). *B. halodurans* C-125 has been shown to have numerous peritrichous flagella and to be highly motile (Takami and Horikoshi, 1999).

The *hag* gene from *B. subtilis* is transcribed by the σ^D form of RNA polymerase (LaVallie and Stahl, 1989, Mirel and Chamberlin, 1989) and upstream of the alkaliphilic *Bacillus* sp. C125 *hag* gene, a promoter sequence resembling that of a σ^D promoter was identified (Sakamoto *et al.*, 1992). Transcription of the *hag* gene in wild-type *B. subtilis* cells demonstrated a peak of expression as the cells entered stationary phase. The transcript levels then decreased to zero within 4 hours after the onset of sporulation (Mirel and Chamberlain, 1989). This is different to flagellin production by *B. halodurans* Alk36 where *hag* gene was found to be continuously expressed up to 144 hours. This indicates that, despite the presence of a σ^D promoter sequence in *B. halodurans* Alk36, the mechanism controlling flagellin expression differs from that of *B. subtilis*, therefore leading to continuous expression of this protein (Fig 2.1).

The inherent lack of flagellin repression in *B. halodurans* Alk36 presents an opportunity to harness the over-expression of the FliC protein as a carrier for surface

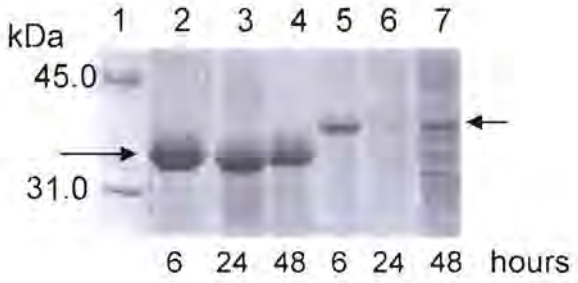


Figure. 2.1 SDS PAGE comparing cell surface protein profiles at different times between *B. halodurans* Alk36 (pH 8.5, 42°C) and *B. subtilis* 1A46 (pH 7.0, 37°C) grown in LB broth. Lanes 2-4, *B. halodurans* Alk36 and lanes 5-7, *B. subtilis* 1A46. Lane 1, low molecular mass marker. The arrows point to the FliC protein.

display of heterologous peptides and proteins. The wide range in growth conditions (Chapter 1) provides the potential for applications in unique environments, especially the expression and display of thermo-tolerant enzymes. However, the necessary genetic tools needed to be developed in order to create a Δ *hag* mutant and subsequently complement the phenotype on an expression vector. For this reason, a temperature sensitive integration vector which can also be used as a shuttle vector or expression vector for *B. halodurans* Alk36 was constructed. *B. halodurans* Alk36 is erythromycin resistant, hence the vector created would need to carry an alternative antibiotic marker.

Integration vectors for genetic manipulation have been developed for numerous Gram-negative and Gram-positive bacteria (Maguin *et al.*, 1992, Perego, 1993, Leenhouts *et al.*, 1998). One such method makes use of single cross-over events (Campbell-type) resulting in multiple copies of the vector to be inserted into the chromosome (Leenhouts *et al.*, 1998). Although this allows for increased copy numbers and higher gene dosage, these integrations are not stable and recombinant bacteria need to be grown under selectable conditions at all times. Although integration in Gram-negative bacteria is easy, due to high transformation efficiencies, Gram-positive bacteria are less amenable to transformation and in general have lower transformation efficiencies. One way to overcome this problem is to use mobilizable integration vectors which can be transferred from their host (usually *E. coli*) to a recipient through the process of conjugation (Schäfer *et al.*, 1994, de Muro and Priest,

2000). An alternative strategy is to develop a temperature sensitive integration system (Biswas *et al.*, 1993). Such a system was developed for *B. halodurans* Alk36 and in so doing negates the need for high transformation efficiencies needed for chromosomal integration and gene manipulation.

In this chapter the construction of a temperature sensitive integration vector (pSEC194) and its subsequent successful use to create a Δ *hag* mutant (BhFC01) will be discussed. The vector is a shuttle vector allowing genetic manipulation to be efficiently carried out in *E. coli*. The vector was also used for the expression of the *hag* gene and resultant complementation of the non-motile phenotype.

2.2. MATERIALS AND METHODS

2.2.1. Bacterial strains, plasmids and growth conditions.

A *Bacillus* species isolated from a soil sample in South Africa (Louw *et al.*, 1993) was identified as *B. halodurans* Alk36 using 16S rDNA sequence analysis (Weisburg *et al.*, 1991). Plasmid pE194 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). pSKBluescript (pSK) and pBCKS were obtained from Stratagene. All constructs were transformed into *E. coli* DH10B (F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) (Φ80d*lac*ZΔM15) Δ*lac*X74 *end*A1 *rec*A1*deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ^-). *E. coli* cultures were grown at 37°C in Luria-Bertani medium (LB) pH 7. *B. halodurans* Alk36 was grown in LB medium pH 8.5 at 42°C unless otherwise indicated. Transformants were selected using 100 μg/ml ampicillin and 10 μg/ml chloramphenicol respectively.

2.2.2. DNA techniques.

Plasmid DNA was isolated using a Plasmid Midi Kit (Qiagen). Restriction enzymes were used as specified by the manufacturer (Fermentas). All plasmid mini-preps were done using Perfectprep Plasmid Mini Kit (Eppendorf). All DNA manipulations were done in *E. coli* which was transformed using electroporation (Dower *et al.*, 1988). *Thermus aquaticus* DNA polymerase was used for PCR (polymerase chain reaction) as recommended by the supplier (BIOLINE). Primers used are outlined in Table 2.1.

Table 2.1 List of primers and their corresponding nucleotide sequences. Restriction enzyme sites used are underlined.

Primer Name	Nucleotide sequence	Restriction Enzyme Site
SigDKpn	5'CTCGGTACCCTCGCGTTACGCTCTTTCTGT 3'	*Kpn*I
DownRev	5' CGCTGCAGAAGAGGAACGTAAACG 3'	*Pst*I
UpFor	5' GCGGATCCGTGTGGTGACATTTGAC 3'	*Bam*HI
UpRev	5' GCTCTAGACGATGCGCATTCATTGCTGG 3'	*Xba*I
DownFor	5' GCTCTAGAGAGTCTCGTATCCGTG 3'	*Xba*I
M13F	5' TGACCGGCAGCAAAATG 3'	-
FliDNR2	5' CCAAGACCGGCAGAGTTAATGTC 3'	-
Up-Forward	5' GTGTGGTGACATTTGAC 3'	-

2.2.3. Construction of temperature sensitive shuttle vector pSEC194.

Plasmid pE194 (3.278 kb) was digested with *Taq*I. The 1.35 kb fragment containing the temperature sensitive *ori* was ligated to pSK digested with *Cla*I (2.959 kb) and transformed into *E. coli* DH10B to create pSE194 (4.313 kb, Figure 2.2). The pE194 *ori* cannot replicate above 43 °C (Villafane *et al.*, 1987). Plasmid pJM103 (Perego, 1993) was digested with *Bgl*II / *Pvu*II to obtain the chloramphenicol gene (1.2 kb) originally from pC194, (Lofdahl *et al.*, 1978). This fragment was ligated to pSE194 digested with *Bam*HI / *Sma*I and transformed into *E. coli* DH10B to create pSEC194 (Fig. 2.2).

2.2.4. Construction of pSECFliC and pSECFlg-

Plasmid pSEC194FliC carrying the full *hag* gene and the σ^D promoter was constructed using primers SigDKpn and DownRev (Table 2.1). PCR amplification was done using PWO DNA polymerase according to the manufacturer's instructions (Roche Diagnostics) with an annealing temperature of 56°C and an extension time of 1 minute using *B. halodurans* Alk36 genomic DNA as the template. The PCR product was digested with *Kpn*I only and ligated into pSEC194 (*Kpn*I/*Hind*II) to obtain pSECFliC (Fig. 2.3). Construction of pSECFlg- involved deleting most of the internal region

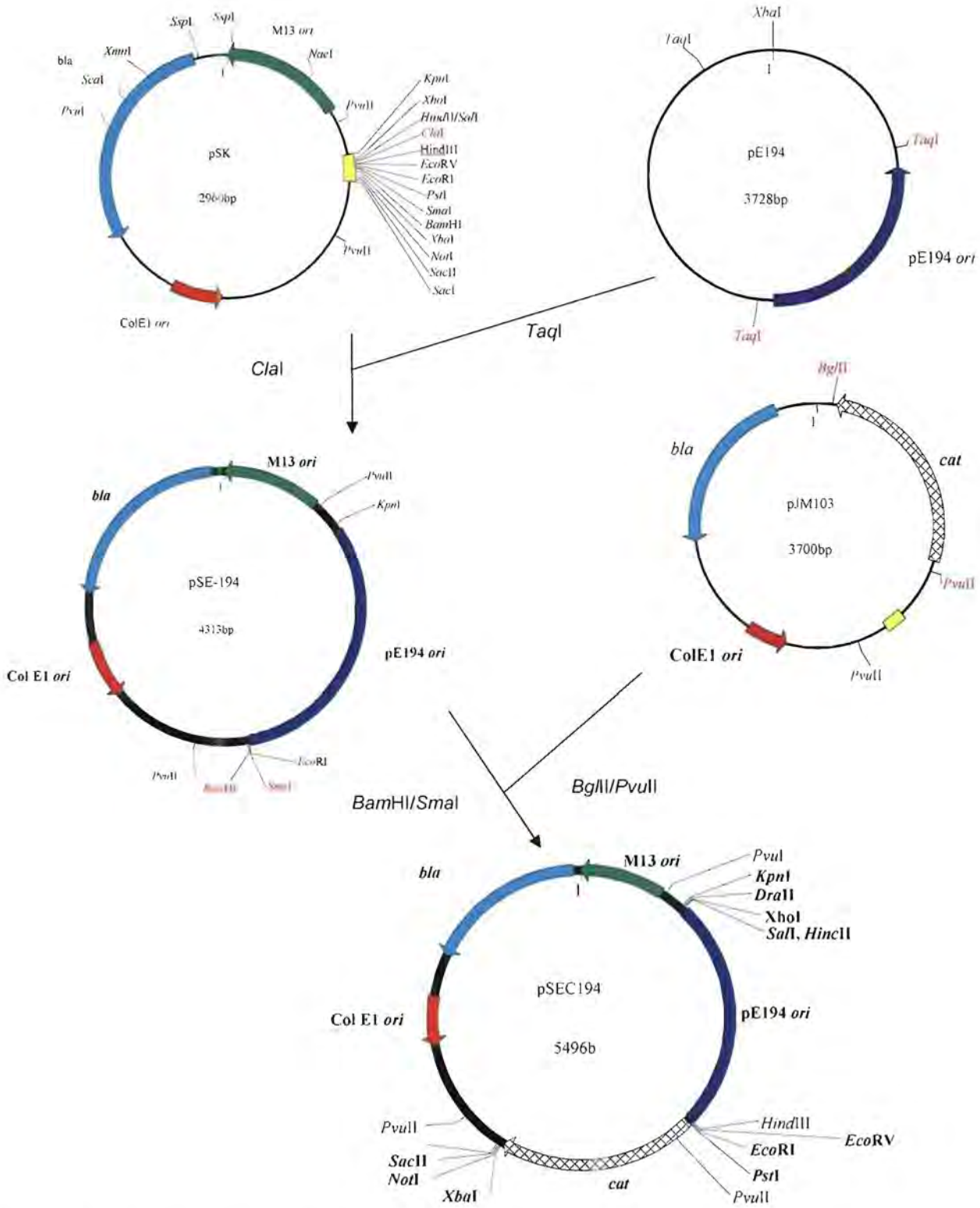


Figure 2.2 Construction of the integration/shuttle vector pSEC194. Restriction sites used for subcloning are in red. Restriction sites in bold can be used for cloning in pSEC194. Plasmid map created with DNAMAN, Version 4.1.

(641 bp) to obtain a defective *hag* gene. PCR primers were designed to obtain the upstream: (UpFor,UPRev) and downstream: (DownFor, DownRev) fragments needed for construction of the defective *hag* gene (Flg-). PCR products were digested with the appropriate restriction enzymes and ligated together into pBCKS digested with *Bam*HI/*Pst*I in a 3 way ligation to obtain the Flg- fragment (pBCFlg-). The truncated *hag* gene from pBCFlg- was removed with *Eco*RI and cloned into pSEC194 digested with *Eco*RI to obtain pSECFlg- (Fig. 2.3).

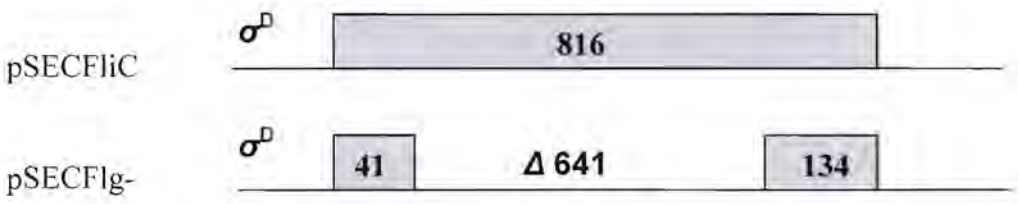


Figure 2.3. Schematic presentation of the different gene constructs in plasmid pSEC194. Lines represent untranslated DNA regions; filled bars (grey) the coding regions of the *hag* genes. The symbol *Δ* denotes deletions and the numbers inside the blocks denote the size in nucleotides of the *hag* fragments. σ^D represents the σ^D-dependent promoter of the *hag* gene.

2.2.5. Transformation, integration and inactivation of the *hag* gene on the chromosome of *B. halodurans* Alk36.

B. halodurans Alk36 was transformed using the modified protoplast method according to Kudo *et al.* (1990). Further modifications involved using polyethylene glycol (PEG) 4000 (final concentration, 22.5%) to introduce the plasmid DNA into the protoplasts. DM3 protoplast regeneration medium (pH 7.8) contained PEG (4000) at 1% final concentration, chloramphenicol (5 μg/ml) and no $CaCl_2$. Integration was a combination of two different methods by Biswas *et al.* (1993) and Poncet *et al.* (1997) (Fig. 2.4). *B. halodurans* Alk36 containing pSECFlg- was grown over night in LB

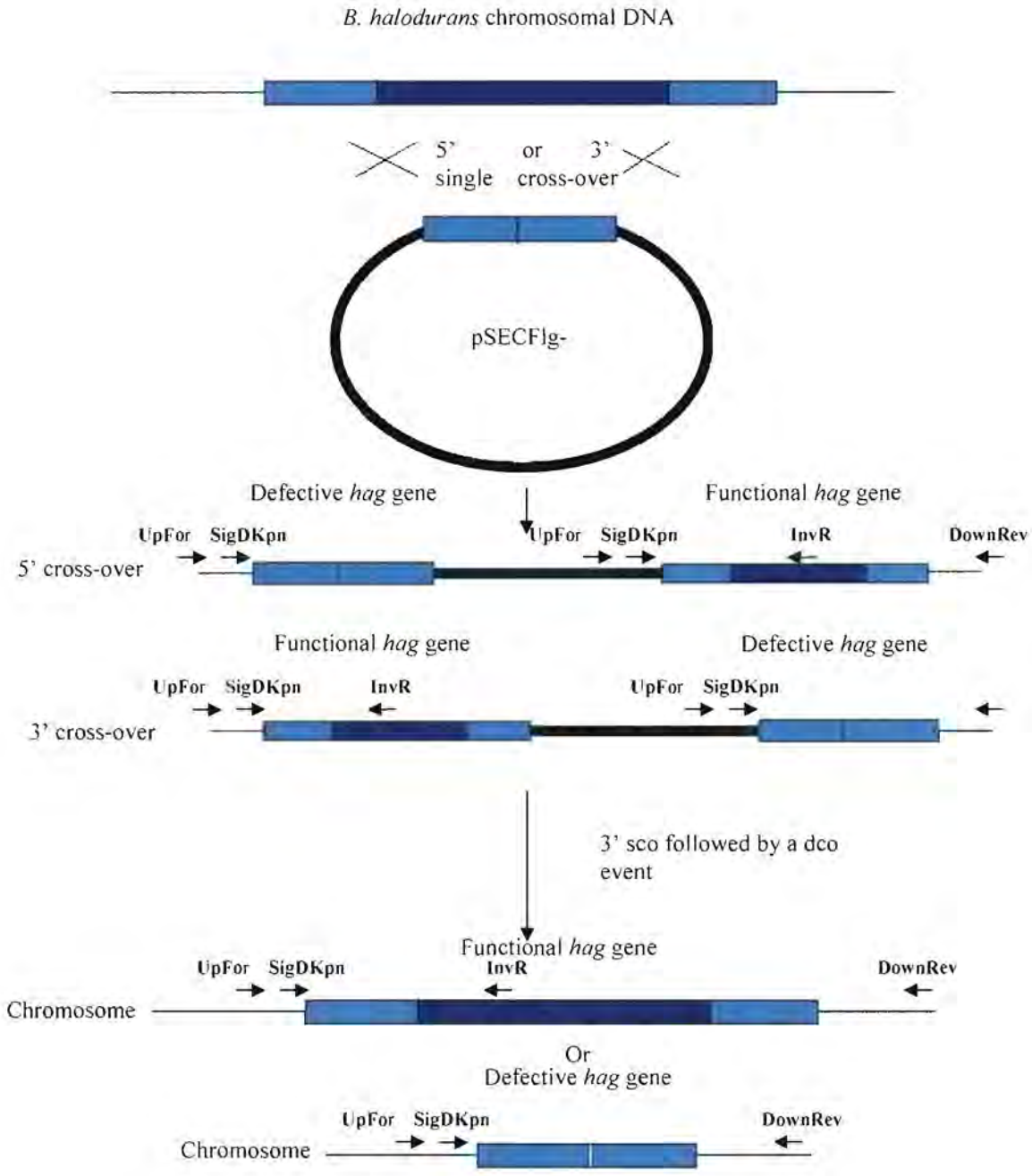


Figure 2.4. Schematic representation of the single cross-over (sco) event and subsequent double cross-over (dco) event resulting in an Δ *hag B. halodurans* Alk36 mutant strain (BhFC01). Primers used for sco and dco event confirmation are in bold.

(pH 8.5, chloramphenicol 10 µg/ml) at 30°C. A 1/100 dilution was made into 25 ml LB (pH 8.5, chloramphenicol 10 µg/ml) and grown at 52°C for approximately 20 generations to force a single crossover event (sco). Serial dilutions were plated onto LB (pH 8.5, chloramphenicol, 10 µg/ml) plates and incubated over night at 52°C. Sco events were confirmed with PCR using plasmid primer UpFor and DownRev (Table 2.1). A sco colony was re-inoculated into LB (pH 8.5) and grown without selection over night at 30°C. Serial dilutions were made and single colonies were screened on selective and non-selective plates. Colonies unable to grow on the chloramphenicol plates were transferred to motility plates (LB pH 8.5, 0.4% agar, 0.8% gelatin) to screen for the motility phenotype. Non-motile phenotype would indicate a double crossover event (dco) resulting in a flagellin minus (Δ *hag*) integration mutant (BhFC01) (Fig. 2.4). The dco event was confirmed with PCR using primers UpFor/DownRev and SigDKpn/InvR (Table 2.1).

2.2.6. Protein extraction and analysis.

Protein extracts from the different cell fractions were isolated as follows:

Extracellular protein fraction: Cells were grown at 30°C in 30 ml LB (pH 8.5) to stationary phase (16 -24 hours). The cells were centrifuged at 7000 X *g* for 10 minutes. The supernatant was collected and an equal volume of 5% TCA (trichloroacetic acid) was added and incubated at room temperature with shaking for 30 minutes. The precipitate was pelleted at 15 000 X *g* for 30 minutes. The pellet was then air dried for 30 minutes and resuspended in 300 µl phosphate buffer pH 7.5. This contained the extracellular protein fraction.

Cell surface protein fraction: The cell pellet was resuspended in 2.5 ml sterile water. An equal volume of 0.2 M NaOH was added and the cell solution was stirred vigorously with a stirrer bar for 30 minutes on ice. Cells were centrifuged at 8000 X *g* for 10 minutes to remove cell debris. Proteins were precipitated using an equal volume of 5% TCA and resuspended as described for the extracellular protein fraction.

Cell wall and intracellular protein fraction: The cell wall protein fraction was obtained by resuspending the cell debris in 5 ml phosphate buffer and lysed using a Sonoplus ultrasonic homogeniser at full power for 30 minutes (10 min intervals). Lysed cells were centrifuged at 12000 x *g* for 10 minutes. The cell pellet was rinsed with sterile water and resuspended in 500 µl phosphate buffer (cell wall proteins). The supernatant was also collected and constituted the intracellular protein fraction.

Protein concentrations were determined according to Bradford (1976). All proteins were run on a 10% SDS-PAGE as described by Laemmli (1970).

2.2.7. Western blot anaylsis.

Western blot analysis was carried out according to Gallagher *et al.* (1997). CAPS buffer (pH 10) was used for protein transfer to PVDF membrane (Millipore). Polyclonal rabbit anti-flagellin antibodies were generated using *B. halodurans* Alk36 flagellin protein excised from a SDS-PAGE containing cell surface extracts. Rabbits were injected with 50 µg of protein. Alkaline phosphatase conjugated goat anti-rabbit antibodies (Sigma) were used as the secondary antibody. Colorimetric detection was done using NBT/BCIP solution (Roche Diagnostics) according to the manufacturer.

2.3. RESULTS

2.3.1 Construction of temperature sensitive shuttle vector pSEC194

Plasmid pE194 was successfully digested with *Taq*I in order to obtain the pE194 temperature sensitive *ori* of replication. This temperature sensitive *ori* was ligated to pSK Bluescript to obtain pSE194 and confirmed to be correct by restriction digests (data not shown). The *cat* gene was isolated from pJM103 digested with *Pvu*II and *Bgl*II and successfully ligated to pSE194 to give pSEC194. This was again confirmed through restriction digests. This vector carries the selectable markers for both chloramphenicol (in *B. halodurans*) and ampicillin (in *E. coli*). The vector also contains the temperature sensitive *ori* of replication allowing for the integration of the vector into the *B. halodurans* chromosome. A schematic representation of the cloning process can be seen in Figure 2.2.

2.3.2 Construction of pSECFliC and pSECFlg-

In order to create the *B. halodurans* Alk36 non-motile mutant (BhFC01) plasmid pSECFlg- was created in a 3-way ligation and confirmed to be correct by PCR analysis using SigDKpn and FliCRev. Plasmid pSECFlg- was constructed as an out of frame truncated *hag* gene mutant containing the 960 bp upstream region and a 840 bp down stream region flanking the truncated *hag* gene (Fig 2.3). This will allow for efficient cross-over events for the integration of the defective *hag* gene into the *B. halodurans* Alk36 chromosome.

Insertion of the full *hag* gene and σ^D promoter PCR amplified from the *B. halodurans* Alk36 chromosome was inserted into pSEC194 to give pSECFliC (Fig. 2.3). The vector was confirmed to be correct by PCR analysis using primers SigDKpn and FliCRev (data not shown). This vector could then be used to restore the motile phenotype when transformed into the *B. halodurans* Alk36 non-motile mutant.

2.3.3 Creation of a *B. halodurans* Alk36 Δ*hag* mutant (BhFC01).

The defective *hag* gene constructed in 2.3.2 has 641 bp of the internal region deleted (Fig. 2.3). Prior to the transformation of plasmid pSECFlg-, the vector pSEC194 was used to optimise the transformation method of *B. halodurans* Alk36. Protoplast transformation yielded 1×10^1 to 1×10^3 transformants/µg DNA. The addition of 1% PEG 4000 to the DM3 plates led to an overall increase of 2.4×10^2 - 1.24×10^4 transformants/µg DNA. Plasmid pSECFlg- was transformed into *B. halodurans* Alk36 and clones were confirmed to be correct by performing restriction enzyme digests. Single crossover frequencies per cell occurred at 4.8×10^{-4} events at an OD_{540} of 1.04 after 7 hours at the non-permissive temperature, 52°C. Although non-specific integration of pE194 has been reported (Hofmeister *et al.*, 1983), PCR analysis using vector primer UpFor and chromosomal primer DownRev showed that of the 2 colonies screened, both were C-terminal cross-overs (Fig 2.5). A C-terminal cross over will result in a PCR product corresponding to 1.8 Kb.

The dco event to generate the deleted *hag* gene was encouraged by growing the sco strains without selection. Colonies unable to grow on selective plates could potentially be either motile revertants or nonmotile mutants depending on whether a C-terminal or N-terminal cross-over occurred. The dco event was confirmed by PCR analysis using both UpFor/DownRev and SigDKpn/InvR (Fig. 2.5). The lack of a PCR product using SigDKpn/InvR is confirmation that the dco event will result in a non-motile phenotype as the InvR primer region has been deleted during the construction of the pSECFlg-vector. The UpFor/DownRev will only amplify the 1.8 Kb fragment as seen in the sco PCR. Colonies were also placed on to motility plates and the absence of a motility halo confirmed that a single isolate was a Δ*hag* mutant (strain BhFC01) (Fig 2.6).

Figure 2.5. Agarose gel of PCR products confirming sco and dco events resulting in *B. halodurans* strain BhFC01. Lane 1 and 2, sco events; lane 3 dco event (BhFC01); lane 4, *B. halodurans* Alk36; and lane 5, DNA molecular marker III (Roche).

Figure 2.6. Motility plate showing *B. halodurans* Alk 36 and mutants. Colony 1, *B. halodurans* Alk36; colony 2, BhFC01; and colony 3, BhFC01 complemented with pSECFliC.

The cell surface protein extraction and Western blot analysis of strain BhFC01 demonstrated the absence of the flagellin protein confirming the deletion of the *hag* gene (Fig. 2.7).

2.3.4 Complementation of Δ*hag* mutant

Complementation of the *hag* gene in strain BhFC01 was carried out by transforming the plasmid pSECFliC into BhFC01. The motility haloes obtained with strain BhFC01 containing pSECFliC were similar to the haloes obtained with *B. halodurans* Alk36 (Fig. 2.6). The cell surface protein fraction was extracted and separated on a SDS–PAGE to demonstrate the reconstitution of the flagellin protein (Fig. 2.7 A). Analysis of this gel demonstrated the expression of the flagellin protein in the cell surface fraction of BhFC01 containing pSECFliC. This was confirmed by Western Blot analysis using rabbit anti-flagellin antibodies (Fig. 2.7 B).

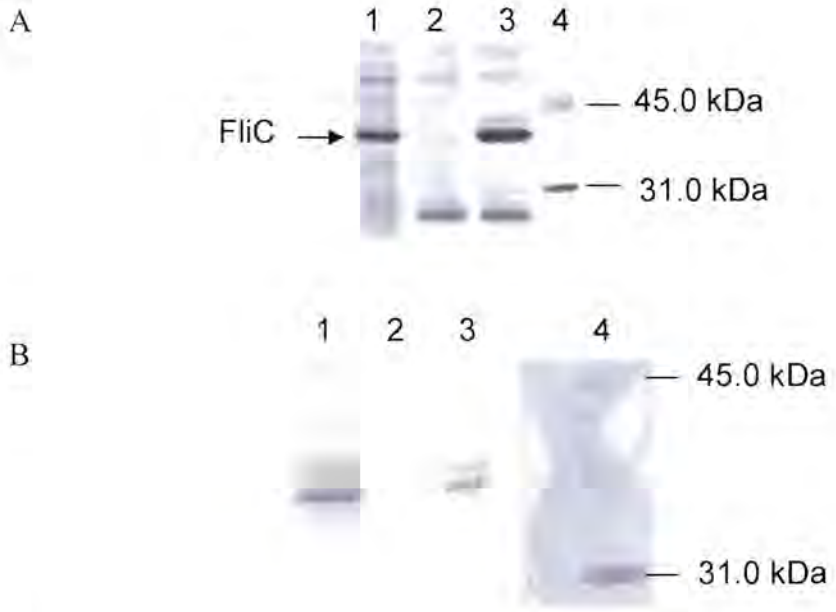


Figure 2.7. 10 % SDS-PAGE (A) and Western blot analysis using anti-flagellin antibodies (B) of the cell surface protein fractions demonstrating the absence of FliC protein in the BhFC01 strain as well as complementation of this phenotype in the presence of plasmid pSECFliC. Lane 1, BhFC01 containing plasmid pSECFliC; lane 2, BhFC01; lane 3, *B. halodurans* Alk36 and lane 4, molecular mass marker (BioRad). The arrow indicates the FliC protein band.

2.4. DISCUSSION

In order to exploit the continuous expression of flagellin by *B. halodurans* Alk36 as a surface display system, genetic techniques had to be developed for this strain. They included a transformation system, shuttle vectors and an integration system enabling gene-targeted inactivation. Transformation of various *B. halodurans* isolates has been reported using both protoplasting (Aono *et al.*, 1993, Kudo *et al.*, 1990) and a simple competence method (Martinez *et al.*, 1999). Transformations under these conditions yielded 4×10^5 (Martinez *et al.*, 1999) to 2×10^6 transformants/μg DNA (Kudo *et al.*, 1990). However, transformation of *B. halodurans* Alk36 using the temperature-sensitive plasmid, pSEC194, yielded no transformants using the competence method. The protoplast method used initially in this study also gave poor results ($1 \times 10^1 - 1 \times 10^3$ transformants/μg DNA). These results were much lower than previously mentioned for *B. halodurans* isolates (Kudo *et al.*, 1990). The subsequent addition of

PEG 4000 to the DM3 plates increased the overall transformation efficiency of *B. halodurans* Alk36 ten fold to 1.24×10^4 transformants/μg DNA. PEG binds tightly to negatively charged nucleic acid molecules which subsequently pass more readily through the bacterial membrane. The presence of PEG in the DM3 medium probably prolongs the adherence and uptake of DNA without any detrimental effects on cell regeneration and in so doing increases overall transformation efficiency. This has not been reported before.

Few Gram-positive bacteria allow for efficient integration of foreign DNA and these include *Streptococcus pneumoniae* (Pozii and Guild, 1985) and *B. subtilis* (Niaudet *et al.*, 1982). Most industrial and medical Gram-positive strains require extensive manipulations to integrate foreign DNA (Biswas *et al.*, 1993). Initial integration systems for Gram-positive bacteria were based on plasmids unable to replicate and required efficient transformation methods (Maguin *et al.*, 1992, Perego, 1993). This is not always feasible and alternative integration systems are needed. One such system was developed by Biswas *et al.*, (1993) who made use of a thermosensitive *ori* of replication from *Lactococcus lactis* subsp. *cremoris*. This *ori* of replication is similar to the *ori* located on plasmid pE194 derived from *S. aureus* (Gryczan *et al.*, 1982). By developing an efficient temperature sensitive integration system, one negates the need for high transformation efficiencies. The use of the pE194 *ori* to create the plasmid pSEC194 provides an inherently unstable vector that cannot replicate above 43°C. Therefore its use in the directional integration of the plasmid into the *B. halodurans* Alk36 chromosome was facilitated by increasing the growth temperature to 52°C. The plasmid constructed makes use of the rolling circle method of replication as determined by the pE194 *ori* of replication. This can be used to stimulate double cross-over recombination events which result in plasmid excision when grown at low temperatures (30°C). The initiation of replication of the integrated plasmid at this temperature should enhance the double cross-over event (Noirot *et al.*, 1987). Double cross-over frequencies were found to occur in *B. halodurans* Alk36 at a frequency of 0.2-0.4%. The addition of the *cat* gene from pC194 was needed in order to create a selectable marker for integrants as *B. halodurans* Alk36 is resistant to erythromycin, the selectable marker on pE194. Utilizing this integration system, we have shown successful inactivation of the *hag* gene on the chromosome of *B. halodurans* Alk36. The development of a genetic system allowing for chromosomal gene targeted

inactivation of *B. halodurans* Alk36 without the presence of residual vector or antibiotic markers will be an important tool in optimising this strain further as a heterologous expression system, specifically through the inactivation of key protease genes.

The construction of a multicopy vector system in *B. subtilis* carrying the complete *hag* gene and its σ^D promoter resulted in the formation of long filamentous cells and the accumulation of flagellin intracellularly (LaVallie and Stahl, 1989). However, complementation studies of the *B. halodurans* Alk36 BhFCO1 strain with the plasmid pSECFliC (carrying the complete *hag* gene and σ^D promoter from *B. halodurans* Alk36) did not result in filamentous cells or intracellular flagellin accumulation indicating a possible difference in the mechanisms of flagellin expression.

These results demonstrated the successful construction of genetic tools, an integration vector system based on a temperature-sensitive origin of replication and the first report of its use in developing a gene targeted inactivation system for *B. halodurans* Alk36 (BhFC01). This vector was also used for the expression of genes when used at a permissive temperature and was used for complementation of the Δ *hag* phenotype when carrying a functional *hag* gene and corresponding σ^D promoter.

Chapter 3

Characterisation of a Suitable Insertion Site for Heterologous Protein and Peptide Surface Display using *Bacillus halodurans* Alk36 FliC.

3.1. INTRODUCTION

Microbial cell surface expression can be defined as the display of a heterologous protein or peptide of interest (passenger protein) by attaching it to the bacterial cell wall through an anchoring motif (carrier) so that it is exposed to the outside of the bacterial cell. Proteins or peptides can be fused at either the C-terminal or N-terminal ends of the carrier protein or as a sandwich fusion. Sandwich fusions have mostly been associated with the display of heterologous proteins or peptides in Gram-negative bacteria (Lee *et al.*, 2003). These fusions are associated with two major classes of carrier proteins which include outer membrane proteins and protein monomers of extracellular appendages such as flagella and pili. Flagellin display systems thus far have only been successfully reported in Gram-negative bacteria. This involved the use of the flagellin protein (FliC) from either *E. coli* or *Salmonella* as a carrier protein which is able to display the passenger protein as an in-frame sandwich fusion (Cattozzo *et al.*, 1997, Ezaki *et al.*, 1998, Westerlund-Wikström, 1997). Comparison of the FliC proteins from different Gram-positive and Gram-negative bacteria showed high sequence homology at the N and C termini whereas the central domains were highly variable in size and sequence (Beatson *et al.*, 2006). Samatey *et al.* (2001) characterised the FliC protein structure of *S. typhimurium* and denoted 4 distinct domains, two of which are highly conserved (D0 and D1) while the central variable antigenic domains (D2 and D3) are surface exposed. It is this region which is therefore targeted as the site for insertion of foreign peptides in both *E. coli* and *Salmonella*.

The first heterologous peptide to be displayed in this manner was an eleven amino acid epitope from egg-white lysozyme (Kuwajima *et al.*, 1988b). Other examples utilising this system in Gram-negative bacteria include the display of peptide libraries

(Lu *et al.*, 1995), the development of vaccine delivery systems (Cattozzo *et al.*, 1997, Levi and Arnon 1996, Stocker and Newton, 1994) and determining the structure and function of adhesive epitopes (Hynonen *et al.*, 2002, Majander *et al.*, 2005a).

Bacillus halodurans Alk36 is able to continuously over-express a ~ 34 kDa cell surface protein (FliC). With the creation of a Δ*hag* mutant (BhFC01), stable maintenance of the expression vector carrying the *hag* gene and σ^D promotor (pSECFliC), provides the necessary tools for the characterisation of a suitable insertion site within the putative variable domain of the FliC protein. The insertion technique described by Ehrmann *et al.* (1990) was used to determine membrane topology in a precise manner. By inserting a reporter gene they were able to characterise various periplasmic and cytoplasmic loops within the membrane spanning protein, MalF. This technology works well when the protein being examined has no known secondary structure. However, *S. typhimurium* FliC protein has a well characterised structure (Beatson *et al.*, 2006) and the topology is well known. This allowed allow focussing of the insertional analysis to the variable domain of the *B. halodurans* Alk36 FliC protein. Thus the surface display site within the FliC protein was characterised through the insertion of small peptides at different regions within the variable domain and the subsequent monitoring of the expression and display of these chimeric flagella.

3.2. MATERIALS AND METHODS

3.2.1 Bacterial strains, plasmids and growth conditions.

The construction of *B. halodurans* BhFC01 (Δ *hag*) was described in Chapter 2. Vector pSEC194 was constructed in our laboratory (Chapter 2). All other bacterial strains and plasmids were as described in Chapter 2.

3.2.2 DNA techniques.

All DNA techniques were carried out as described in Chapter 2.

3.2.3 Identification of a display site within the FliC protein of *B. halodurans* Alk36.

pSECNC1 (NC1) was constructed by using pSECFliC as the template to obtain the N and C terminal regions for the construction of the truncated *hag* gene. pSECFliC was digested with *Kpn*I/*Eco*RI to obtain the N-terminal fragment (566 bp). PCR amplification using the FliCR and CterF primers (Table 3.1) obtained the C-terminal fragment (232 bp). The C-terminal PCR product was digested with *Eco*RI/*Dra*I and ligated with the N-terminal fragment (*Kpn*I/*Eco*RI) to pSEC194 digested with *Kpn*I/*Dra*I in a 3 way ligation to give pSECNC1 (Fig. 3.1).

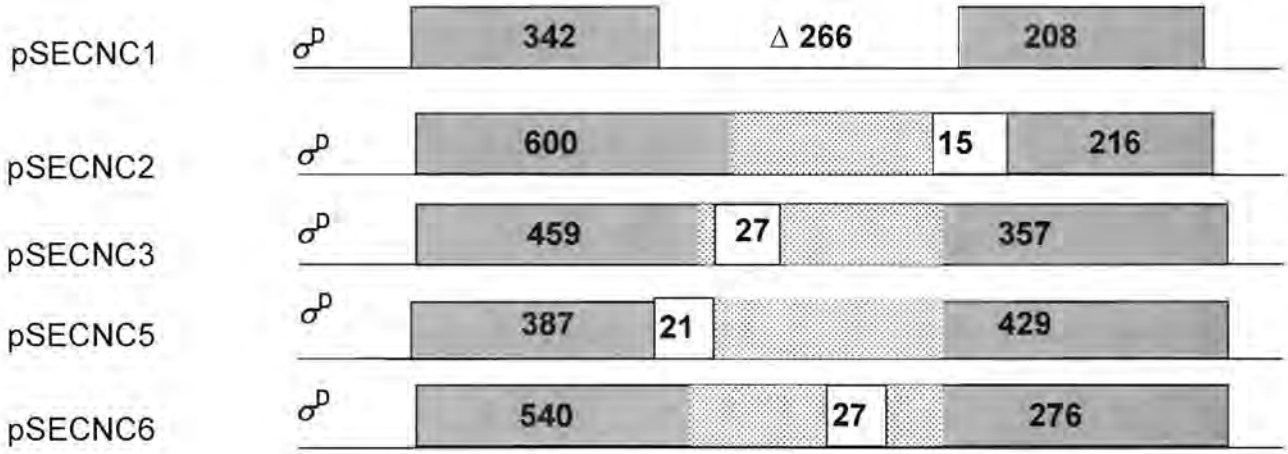


Figure 3.1 Schematic presentation of the different gene constructs in plasmid pSEC194. Lines represent untranslated DNA regions, filled bars (grey), the coding regions of the *hag* gene, the dotted region the putative variable region as defined from this study and white bars, inserted linkers. The symbol Δ denotes deletions and the numbers inside the blocks denote the size in nucleotides of the *hag* gene fragments. σ^D represents the σ^D-dependent promoter of the *hag* gene.

The template for all the NC reactions was *B. halodurans* Alk36 genomic DNA. Construct pSECNC2 (NC2) was obtained by inserting a 15 bp poly-linker at nucleotide position 600 of the open reading frame (Fig. 3.1). The N-terminal was PCR amplified using the primers SigDKpn and FliN-terRev (840 bp) and the C-terminal using the primers CterF2 and DownRev (800 bp) (Table 3.1). The N-terminal fragment was digested with *Xho*I/*Kpn*I and the C-terminal fragment with *Xho*I/*Ssp*I.

pSEC194 was digested with *Kpn*I/*Hinc*II and a 3 way ligation resulted in pSECNC2 (Fig. 3.1).

Table 3.1. List of primers and their corresponding nucleotide sequences. Restriction enzyme sites used are underlined.

Primer name	Nucleotide sequence	Restriction digest site
FliCR	5' CAA CAA AGT AAC GGT TGA GCG 3'	-
CterF	5' CGC GAA TTC CTA GGA GCT ATG CAA AAC C 3'	*EcoR*I
FliN-terRev	5'CTC CTC GAG CGA CCT TCT GAA ACA GC 3'	*Xho*I
SigDKpn	5' CTC GGT ACC CTC GCG TTA CGC TCT TTC TGT 3'	*Kpn*I
CterF2	5' CAC GAA TTC TCG AGC CCG GGA TCC TCT TAC CTA GGA GCT ATG CAA AAC 3'	*EcoR*I
VNR2	5' CGG CAG CTG TTC ACC AGA ATT AGC ACC AAC 3'	-
VCF	5' CAC GTC GAC TCG AGC CCG GGA TCC TTA ATT GAA CTT GAT TTA ACA AAA G 3'	*Sal*I
NC5F	5' CAC GTC GAC TCG AGC CCG GGA TCC TTT AAT ACG CAA AAA TTA CTC 3'	*Xho*I
NC5RX	5' CAC CTC GAG TGA GTT GTA TCT TTG ATT C 3'	*Xho*I
VCF6	5' CAC GTC GAC TCG AGC CCG GGA TGG ATC CAG AAT GCA CAA TCA GCT ATT GAC 3'	*Sal*I
VNR6	5' GAC GTC GAC AGT GTG GTC AGT AAT ATC CTC 3'	*Sal*I

Construct pSECNC3 (NC3) was obtained by inserting a 27 bp poly-linker at nucleotide position 459 of the open reading frame (ORF). The N-terminal (694 bp) was PCR amplified using the primers SigDKpn and VNR2. The C-terminal (1.048 kb) was PCR amplified using primers VCF and DownRev (Table 3.1). *B. halodurans* genomic DNA was used as the template for all PCR reactions. The C-terminal product

was digested with *Sal*I and *Pst*I and ligated into pSK digested with the same two restriction enzymes to create pSKCter. The N-terminal fragment was digested only with *Kpn*I (the other end was left blunt) and then ligated to pSKCter digested with *Hinc*II and *Kpn*I to create pSKNC3. This construct was digested with *Kpn*I and *Ssp*I to liberate the pSECNC3 fragment which was then ligated to pSEC194 digested with *Kpn*I and *Hinc*II to obtain pSECNC3 (Fig. 3.1).

The insertion site of construct pSECNC5 (NC5) is at nucleotide position 387 of the ORF and the size of the polylinker was 21 bp. The N-terminal (614bp) region was PCR amplified using primers SigDKpn and NC5RX and the C-terminal (1133bp) with primers DownRev and NC5F. The N-terminal product was digested with *KpnI/XhoI* and the C-terminal with *XhoI/SspI*. These fragments were ligated to pSEC194 digested with *KpnI/Hinc*II in a three way ligation to give pSECNC5 (Fig. 3.1).

pSECNC6 (NC6) insertion site is at nucleotide position 540 with an insert size of 27 bp. The N-terminal (767 bp) region was PCR amplified using primers SigDKpn and VNR6 and the C-terminal (349 bp) with primers DownRev and VCF6. The C-terminal product was digested with *SalI/Pst*I and ligated into pSK digested with *SalI/Pst*I to give pSKCter2. pSKCter2 was digested with *KpnI/Sal*I and the N-terminal product digested with *KpnI/Sal*I. These two products were ligated together to give pSKNC6. The NC6 fragment from pSKNC6 was PCR amplified (PWO *taq*) using primers SigDKpn and FliCR to give a blunt ended PCR product, digested with *Kpn*I and ligated into pSEC194 (*KpnI/Hinc*II) to give pSECNC6 (Fig. 3.1). All polylinker and inserted amino acid sequences can be seen in Table 3.2.

Table 3.2. Nucleotide and amino acid sequences of inserted polylinkers.

Construct Name	Polylinker nucleotide sequence	Amino Acid sequence
pSECNC2	5' TCG AGC CCG GGA TCC 3'	Ser-Ser-Pro-Gly-Ser
pSECNC3	5' CAG CTG CCG GAC TCG AGC CCG GGA TCC 3'	Gln-Leu-Pro-Val-Ser-Ser-Pro- Gly- Ser
pSECNC5	5' GTC GAC TCG AGC CCG GGA TCC 3'	Val-Asp-Ser-Ser-Pro-Gly-Ser
pSECNC6	5' GTC GAC TCG AGC CCG GGA TGG ATC CAG 3'	Val-Asp-Ser-Ser-Pro-Gly-Trp- Ile-Gln

3.2.4. Protein extraction and analysis.

Proteins were extracted and analysed as described in Chapter 2.

3.2.5. Western blot anaylsis.

Western blot analysis was carried out according to (Gallagher *et al.*, 1997) using anti-flagellin antibodies as described in Chapter 2.

3.3 RESULTS

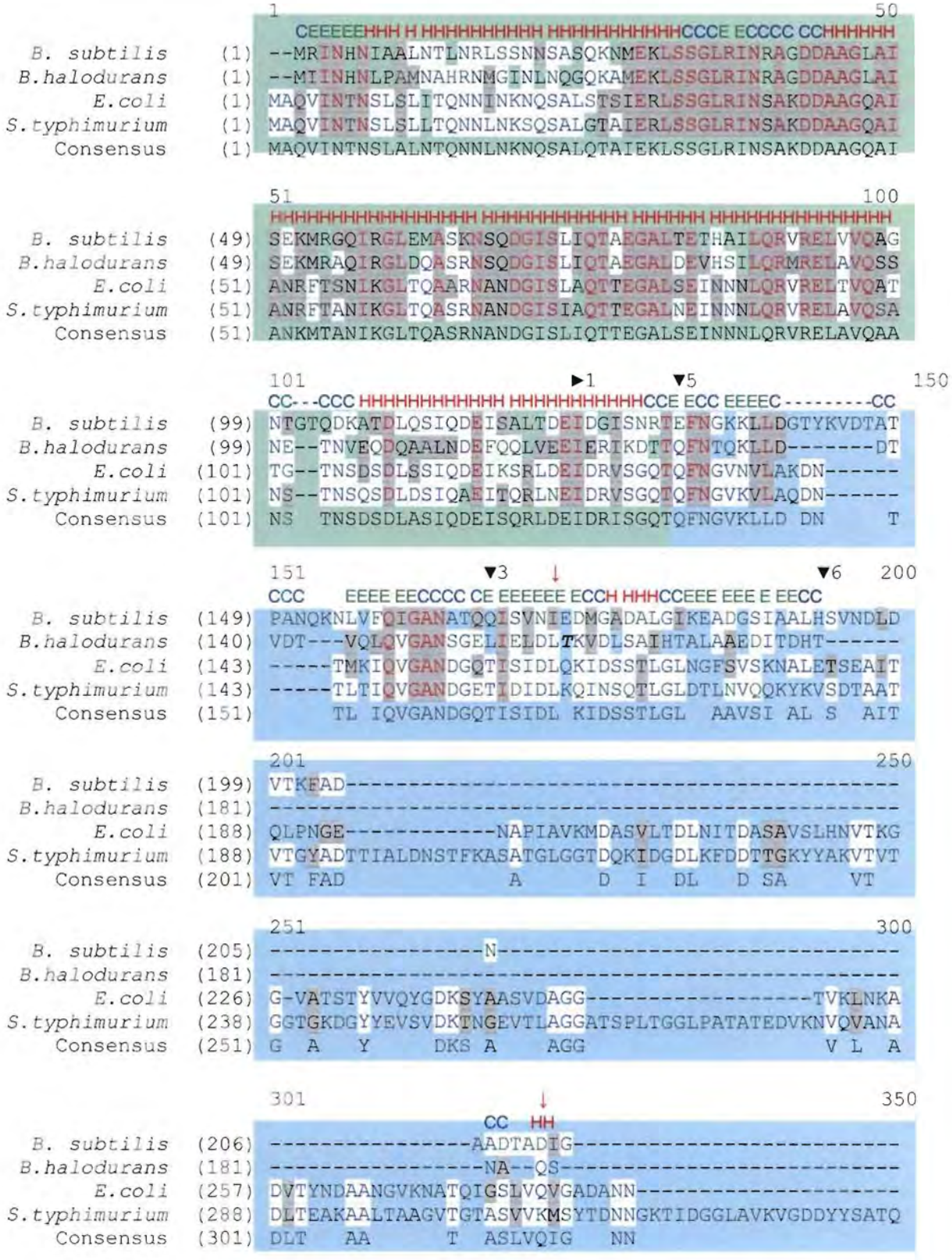
3.3.1. Bioinformatic analysis of the *B. halodurans* Alk36 FliC protein.

The *B. halodurans* Alk36 flagellin is significantly smaller than that of *E. coli* O157 (30 kDa versus ~52 kDa) with a correspondingly small variable region (Fig. 3.2). It must however be mentioned that there is considerable variation in FliC size and the corresponding variable domain of different *E. coli* strains. However, the predicted conserved N- and C-termini of the *B. halodurans* Alk36 FliC shows a high level of identity to *S. typhumurium* (45%), *E. coli* O157 (40%) and *B. subtilis* 168 (55%) FliC proteins both in length and amino acid sequence (Fig. 3.2). It was therefore important to determine whether it was possible to disrupt the variable region either by deletion or insertion of small peptides and still obtain over-expression of functional flagellum. From these alignments and those described by Sakamoto *et al.* (1992) and subsequent secondary structure analysis (using 3D-PSSM software) the putative variable domain was defined to incorporate amino acids 127-200 (Fig. 3.2).

3.3.2 Characterisation of an insertion site for peptide display

In order to identify the ideal site for the insertion of heterologous peptides and proteins, poly-linkers (5-9 amino acids) were inserted as in-frame fusions in four different sites within the putative variable region based on sequence alignments between *B. subtilis*, *Salmonella*, *E. coli* and *B. halodurans hag* genes. A single deletion was also created (Fig. 3.1). All constructs were verified with sequencing.

A putative variable domain was predicted and consisted of amino acid 127-200 for the *B. halodurans* Alk36 FliC protein (Fig. 3.2). For this reason the single deletion



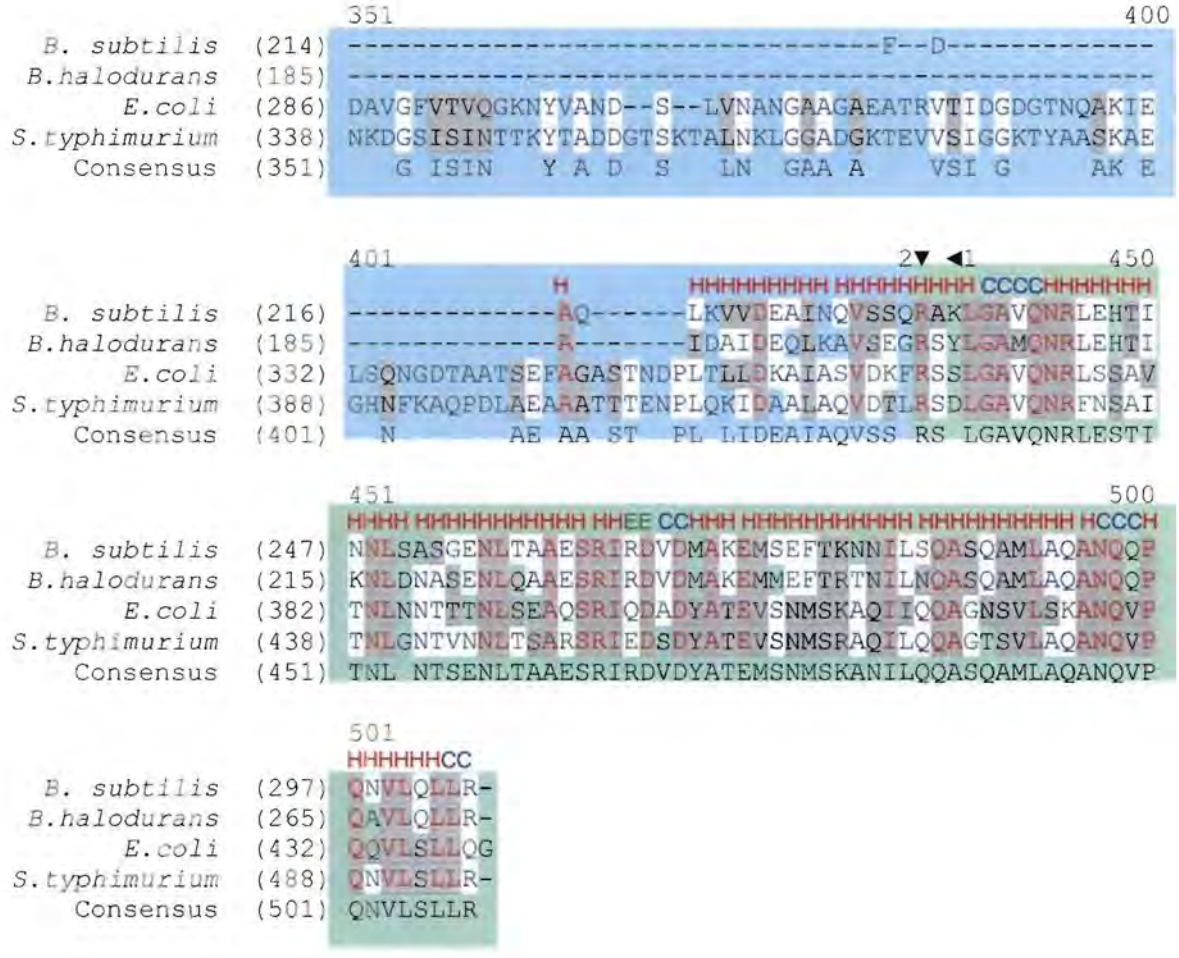


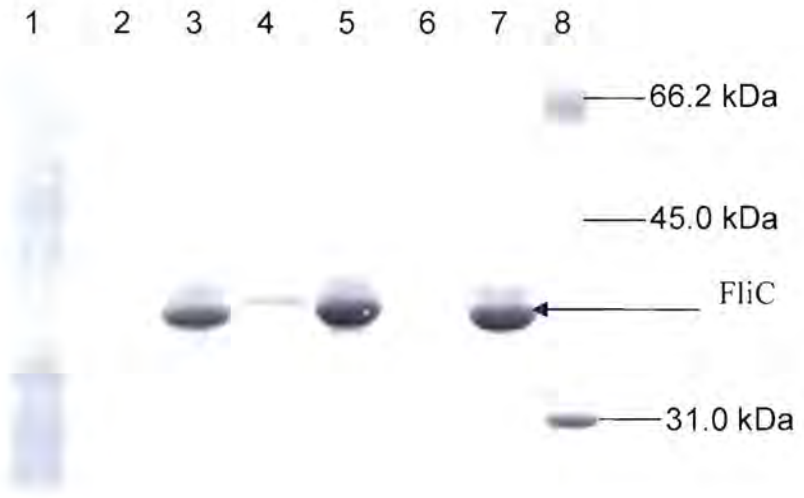
Figure 3.2. Multiple alignment of flagellin homologues of *E. coli* O157 (accession number, AAQ22690), *S. typhimurium* (accession number, AAR10677), *B. subtilis* 168 (accession number, NP391416) and *B. halodurans* C125 (accession number, NP244483). Initial variable domain boundary analysis as defined by Sakamoto *et al.* (1992), sequence alignments, and secondary structural analysis using 3-D PSSM (Kelley *et al.*, 2000). Conserved N and C-terminal domains in green and variable domain in blue. ▼ indicate the insertion sites (2,3,5,6) of the polylinkers and ►◄ (1) defines the area which was deleted to create pSECNC1. ↓ indicates the revised variable domain according to sequence alignments and secondary structural analysis described by Beatson *et al.* (2006) and confirmed by our findings for *B. halodurans* Alk36 flagellin amino acid sequence.

construct pSECNC1 (NC1) was designed to remove this variable domain and surrounding areas. Polylinkers were inserted at four sites within the predicted variable domain boundaries and within the variable domain itself and designated pSECNC2, pSECNC3, pSECNC5 and pSECNC6 (Fig. 3.2). These constructs, carrying between 5 and 9 amino acids, were all transformed successfully into BhFC01. All the constructs were shown to be stably maintained through mini-prep analysis. Transformants containing different constructs were transferred onto a motility plate (Fig. 3.3). No motility haloes were observed for constructs pSECNC1, pSECNC2, pSECNC3 or pSECNC5. However, pSECNC6 was shown to be motile indicating that in this construct the individual chimeric flagellin monomers were polymerized to form functional flagellum.

Figure 3.3. Motility plate showing the presence or absence of motility haloes of the various constructs. Colony 1, *B. halodurans* Alk36; 2, BhFC01; 3, BhFC01 (pSECNC2); 4, BhFC01 (pSECNC3); 5, BhFC01 (pSECNC5); 6, BhFC01 (pSECNC6) and 7, BhFC01 (pSEC194).

Cell surface protein fractions were isolated and analysed on a SDS-PAGE. There were no detectable flagellin protein bands of the correct size for constructs pSECNC1 or pSECNC2 protein fractions (Fig. 3.4 A). However, there was a band slightly larger than that of the normal FliC protein present in the cell surface protein fractions of

A



B

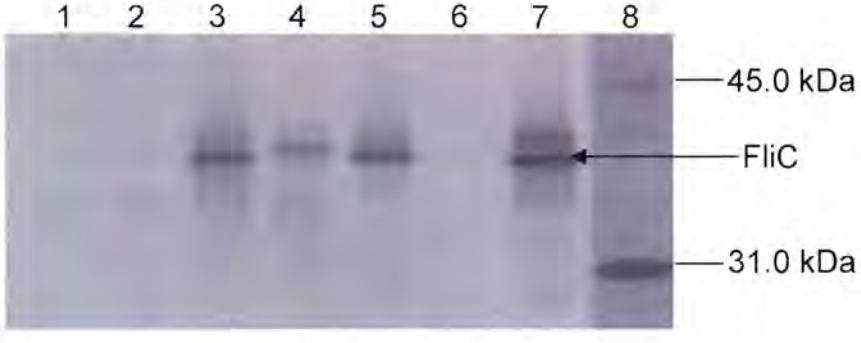


Figure 3.4 (A) 12% SDS-PAGE of cell surface protein fractions of BhFC01 containing different plasmid constructs. Lane 1, pSECNC1; lane 2, pSECNC2; lane 3, pSECNC3; lane 4, pSECNC5; lane 5, pSECNC6, lane 6, BhFC01; lane 7, *B. halodurans* Alk36 and lane 8, low molecular mass ladder. (B) Western blot of (A) confirming NC3 and NC6 are FliC::peptide fusions. No hybridisation was seen for NC1and NC2.

pSECNC3 and pSECNC6 (Fig. 3.4 A) indicating that the chimeric peptide flagellin fusions are over-expressed and polymerised into flagella in these strains. The BhFC01 strain carrying plasmids pSECNC1, pSECNC2, or pSECNC5 did not show expression of the chimeric FliC in the extracellular, intra-cellular or cell wall protein fractions. These results were confirmed with a Western blot using polyclonal rabbit anti-FliC antibodies. Figure 3.4 (B) demonstrated that pSECNC1 and pSECNC2 were not expressed even at low levels. A protein band was seen for pSECNC5 which was only expressed at low levels on the cell surface of BhFC01 (Fig. 3.4 B, lane 4). The band observed was also higher than predicted. BhFC01 carrying pSECNC3 and pSECNC6 (Fig. 3.4 B, lane 3 and 5) and the *B. halodurans* Alk36 positive control (Fig. 3.4 B, lane 7) were all positive for FliC production. The incorporation of polylinkers into the variable region of the *hag* gene of *B. halodurans* Alk36, without interfering with the antigenicity and polymerization of the flagellin protein, is thus feasible.

3.4 DISCUSSION

Through insertions or deletions one can derive useful information on the structure of the FliC protein and in so doing identify regions for cell surface presentation of foreign peptides. Since the domain boundaries for the *S. typhimurium* FliC protein have been well characterised (Namba *et al.*, 1989, Yonekura *et al.*, 2003) sequence alignments were used to determine the extent of sequence homology between *B. halodurans* Alk36, *S. typhimurium*, *B. subtilis* and *E. coli* FliC proteins. This, in combination with secondary structural analysis using the software package 3D-PSSM (Kelley *et al.*, 2000), allowed us to determine the putative variable domain of the *B. halodurans* Alk36 flagellin protein (Fig. 3.2). A putative variable domain was identified between amino acids 127-200 of the *B. halodurans* Alk36 flagellin. This corresponded well to the putative variable domain determined by Sakamoto *et al.* (1992) for *B. halodurans* C-125. Secondary structural analysis showed an area of β-strands and coils between amino acids 129-183 while the rest of the flagellin consisted of coils and α-helices. For this reason the full area from amino acid 129 to 202 was used for the insertion of short polylinkers giving rise to constructs pSECNC2-pSECNC6. Also, the full putative variable region was removed from amino acids 114-202 giving rise to construct pSECNC1. This putative variable domain from *B. halodurans* Alk36 flagellin (73 amino acids) is much smaller than that postulated for

E. coli (187 amino acids) and *S. typhimurium* flagellin (241 amino acids). This is not surprising considering that the total size of the flagellin monomers from *E. coli* (497 amino acids) and *S. typhimurium* (494 amino acids) are much larger than that from *B. halodurans* (272 amino acids) (Kuwajima *et al.*, 1986, Yonekura *et al.*, 2003).

A comprehensive analysis of the domain boundaries was subsequently done by Beatson *et al.* (2006). In addition to the conserved N and C-terminal domains, Beatson *et al.* (2006) also highlighted structural conservation at the N-terminal end of the previously defined variable domain of *S. typhimurium*. These structures were found to be conserved between 205 aligned flagellin sequences. Re-evaluation of *B. halodurans* Alk36, *E. coli* O157, *B. subtilis* 168 and *S. typhimurium* FliC protein revealed that this was in fact the case. These β-hairpins and β-turns are in fact highly conserved between *S. typhimurium* and *B. halodurans* Alk36. These areas have thus not been included as part of the variable domain (Beatson *et al.*, 2006).

With this new information we were able to redefine the variable domain from *B. halodurans* Alk36 FliC protein (between amino acids 159-185). The C-terminal end of the variable domain was characterised using secondary structural analysis done previously (Fig. 3.2). This boundary has been well defined for *S. typhimurium* and is characterised by the start of conserved α-helices. Similarly, *B. halodurans* Alk36 FliC also has α-helices which start at amino acid position 185 as defined by our analysis using 3D-PSSM. This site thus forms the domain boundary between the variable domain and the conserved C-terminal domain.

By correlating the secondary structural analysis with the results from the insertion studies, the refined variable domain seem to hold true as only pSECNC6 had an insertion within this domain and gave rise to functional flagella. However, the β-hairpin region is also amenable to peptide insertions but results in the formation of non-functional flagella (pSECNC3). It is clear that this area is not optimal for flagellar surface display and plays an important role in the functionality of the flagella. Insertions of a peptide close to the newly defined variable domain boundary at the C-terminal end resulted in the loss of flagellar production (pSECNC2). The area at the C-terminal end is more clearly defined and has an exact domain boundary unlike the N-terminal end which potentially has a surface exposed β-hairpin and β-turn.

In all other instances (pSECNC1, pSECNC2 and pSECNC5) no or negligible flagellin production was observed. These results constitute the first direct evidence for the cell surface location of a foreign peptide of at least nine amino acids using two insertion sites at amino acids 153 (pSECNC3) and 180 (pSCENC6) for flagellin display from a Gram-positive bacterium. The identification of insertion sites which do not alter the properties of the FliC protein, including the antigenic properties, indicates the potential use of this over-produced flagellin protein for surface display in *B. halodurans* Alk36. Since the largest inserted peptide was only 9 amino acids, further studies are required in order to determine the size constraints of this expression system. These initial studies also give insight into the secondary structure of the *B. halodurans* Alk36 FliC protein by defining areas which are not important or critical for polymerization into functional flagella.

With the characterization of permissive sites in the *B. halodurans* Alk36 FliC protein for surface display, a selection of peptides and proteins will be inserted within these sites in order to determine the potential of the flagellin display system in bioremediation, vaccine delivery, and biocatalysis. These examples will be outlined in Chapter 4.

Chapter 4.

Cell Surface Display of Heterologous Peptides and Proteins Using *Bacillus halodurans* Alk36 FliC as an Anchoring Motif.

4.1 INTRODUCTION

As described in detail in Chapter 1, microbial cell surface display has potential applications in bioremediation, directed evolution, screening of peptide libraries, oral vaccines, antibody production and whole cell biotransformation (Lee *et al.*, 2003). The focus of this chapter is on the evaluation of the flagellin display system for potential applications in bioremediation, immunology and biocatalysis.

Although microorganisms have cell surfaces with a polyanionic nature which results in non-specific interactions with cations, there may, in some cases, be the need to bind specific cations (Sousa *et al.*, 1996). These include the elimination of cationic inhibitors or the accumulation of precious metals. The display of peptides able to interact with specific cations would thus enhance the bacteria's inherent ability to bind to specific metals ions enabling it to act as a high metal affinity adsorbent. These peptides can then be used for the removal of toxic metals from wastewater. Surface displayed metal binding peptides have been investigated extensively for both Gram-positive and Gram-negative bacteria (Kotbra *et al.*, 1999, Samuelson *et al.*, 2000, Sousa *et al.*, 1996, Xu and Lee, 1999).

Flagella have been used successfully for the display of random peptide libraries using the FLITRX^{TM} system which encompasses the use of the FliC from *E. coli* and a cytoplasmic thioredoxin protein (Lu *et al.*, 1995). Tripp *et al.* (2001) made use of this library to identify peptides with pH or metal ion-sensitive monoclonal antibodies (mAbs) recognition, known as "switch epitopes". They were also able to demonstrate that zinc-sensitive switch epitope peptides recognized by a mAb can be identified using this system. Similarly, Cu_2O- and ZnO-binding peptides were also identified (Thai *et al.*, 2004). Selected sequences identified to have an affinity for ZnO also had

moderate to high affinity for Cu_2O, which suggests that complete discrimination between the two related compounds may be difficult and may require further panning experiments. Flagella have not previously been used for the display of a poly-His peptide, however the display of a metal binding peptide has been shown to enhance ZnO binding in *E. coli* (Thai *et al.*, 2004). This demonstrates the potential use of chimeric flagella for bioremediation.

Flagella have been used extensively for the display of foreign antigenic determinants (Table 1.3). Some of these examples include the use of the *Salmonella* FliC protein to insert and display the CTP3 epitope of the B chain of cholera toxin (Newton *et al.*, 1989). Mice immunized with the live vaccine made antibodies with affinity for the CTP3 epitope and the whole cholera toxin. Since this epitope was able to stimulate an effective immune response Newton *et al.* (1995) created a synthetic oligonucleotide encoding residues 735-752 of the HIV-1 IIIB *env* gene and inserted the oligo into the variable domain of the *fliC* gene of *Salmonella*. This epitope corresponds to the gp41 antigenic region. Mice immunized with the live vaccine had high titres of anti-peptide and anti gp160 antibodies but weak neutralising activity on the HIV-1 IIIB isolates. Sera of mice given two doses of the live vaccine by the oral route had low to no antibody titres. Oligonucleotides corresponding to the specific V3 loop portions of two HIV-1 isolates were also expressed in the flagella of *Salmonella* live vaccine strain (Cattozzo *et al.*, 1997). Intraperitoneally administered recombinant *Salmonella* strains injected into mice resulted in antibodies specific for the heterologous gp 120 of HIV-1 IIIB. Two of the sera were shown to neutralize the HIV-1 MN strain from which the peptide was derived. However, unlike the gp41 epitope, oral administration of the recombinant *Salmonella* strain did evoke specific antibodies against the gp120 (Cattozzo *et al.*, 1997).

The display of larger proteins has been demonstrated for outer membrane proteins (Omps) (Agterberg *et al.*, 1987, O'Callaghan *et al.*, 1990, Francisco *et al.*, 1992, Franscisco *et al.*, 1993), peptidoglycan associated lipoproteins (PAL) (Fuchs *et al.*, 1996), Lpp-OmpA hybrid system (Francisco *et al.*, 1992), autotransporters (Lattemann *et al.*, 2000, Schultheiss *et al.*, 2002), and cell wall bound proteins (Strauss and Gotz, 1996). Flagella have also been exploited for the display of a number of larger proteins even though the general consensus for surface display of

peptides using flagella in Gram-negative bacteria is restricted to 60 aa. These examples include the functional expression of a number of adhesive peptides (Westerlund-Wikström *et al.*, 1997), thioredoxin random peptide library (Lu *et al.*, 1995), green flourescent protein (GFP), and anti-porphyrin antibody sFv (Ezaki *et al.*, 1998). Although Ezaki *et al.* (1998) claimed to have displayed an acid phosphatase, their results do not clearly demonstrate the location of the specific activity. The use of a displayed functional catalytic enzyme with potential applications in biotransformation has as yet not clearly been demonstrated for flagellin cell surface display.

Within this chapter, size constraints of the display sites will be investigated by successfully displaying peptides and proteins of different sizes. As suitable insertion sites within the variable domain of *B. halodurans* Alk36 FliC protein have already been determined (Chapter 3) and small peptides were successfully displayed within these sites, a poly-His peptide (13-14 aa) and a HIV-1 subtype C gp120 V3 loop (29 aa) consensus sequence (Hewer and Meyer, 2003) were inserted into the flagella display system. Both of these examples were small (less than 30 aa). In an attempt to evaluate the ability of the *B. halodurans* Alk36 flagellin display system to successfully display hydrolytic enzymes, a mature thermostable lipase (LipA, 387 aa) from *Geobacillus thermoleovorans* (Genbank, Acession: AF134840) was incorporated into the permissive sites (Cho *et al.*, 2000). This lipase is particularly suited to our display system as it has a temperature optimum of 50°C and a pH optimum of 8. These conditions suit the optimal growth conditions of *B. halodurans* Alk36. It is unlikely that such a large protein will result in functional flagella due to the restriction in channel diameter through which flagellin is transported to the growing tip of the flagella, but may allow for the surface cell wall location of the lipase protein::flagellin sandwich.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, plasmids and growth conditions.

Plasmids pSEC194, pSECNC3, pSECNC6 and *B. halodurans* strain BhFC01 (Δ*hag*) were described previously (Chapter 2 and 3). Plasmid pSECSLipA was kindly provided by Dr E. Berger (Pers. Comm.). This vector contains the *Geobacillus thermoleovorans* lipase gene (Withauer, unpublished) under the control of the *B. halodurans* Alk36 σ^{D} promoter cloned into the pSEC194 vector (section 2.2.3). This enzyme was found to have a temperature optimum of 65°C and a half life of 1 hour at 50°C. All constructs were transformed into *E. coli* DH10B (F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ($^{\varphi}$80d*lac*ZΔM15) Δ*lac*X74 *end*A1 *rec*A1*deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ⁻). All *E. coli* cells were grown at 37°C in Luria-Bertani medium (LB) pH 7. All *B. halodurans* Alk36 strains were grown in LB medium pH 8.5 at 30°C when containing the pSEC194 vector otherwise at 42°C. All *E. coli* and *B. halodurans* Alk36 transformants were selected using 100 µg/ml ampicillin and 10 µg/ml chloramphenicol respectively.

4.2.2 DNA techniques.

All DNA techniques were carried out as described in chapter 2 (section 2.2.2).

4.2.3 Construction of hybrid flagella displaying the poly-His peptide and HIV-1 subtype C gp120 V3 loop peptides.

The synthetic oligonucleotides were derived from the HIV-1 subtype C gp120 V3 loop (Hewer and Meyer, 2003) (Table 4.1). Oligonucleotides were annealed according to the method described by IDT (Integrated DNA Technologies, www.idtdna.com). Oligonucleotides were diluted in STE buffer (10 mM Tris pH8, 50 mM NaCl, 1 mM EDTA) to a final concentration of 20 µM. A working stock was made (5 µM) and equal amounts of complementary oligonucleotides were mixed together. Samples were boiled for 5 minutes and allowed to cool in a waterbath. The resulting product was restricted with the appropriate restriction enzymes and ligated to pSECNC6

Chapter 3). This gave rise to pSECNHivC6. The annealed oligonucleotide was ligated directly into pSECNC3 at the *Sma*I site to give pSECNHivC3.

Table 4.1. HIV-1 III clade C gp120 V3 loop and poly-His oligonucleotides used for annealing and subsequent insertion into pSECNC3 and pSECNC6. Amino acid sequence in bold is the sequence of the inserted heterologous peptide. Restriction digest sites are in red.

Oligo name	Oligo sequence	Peptide sequence (Construct Name)	Restriction enzyme sites
HIVCF	5'- GGG CGT CGA CAC GCG TCC AAA TAA TAA TAC GCG TAA ATC AAT TCG TAT TGG ACC AGG ACA AAC GTT TTA TGC AAC GGG AGA TTG GAT CCG CGG-3'	QLPVV**TRPNNNTRKSIRIGPG QTFYATGD**WIRGGS (pSECNHivC3)	*Sal*I/*Bam*HI
HIVCR	5'-CCG CGG ATC CAA TCT CCC GTT GCA TAA AAC GTT TGT CCT GGT CCA ATA CGA ATT GAT TTA CGC GTA TTA TTA TTT GGA CGC GTG TCG ACG CCC-3'	VD**TRPNNNTRKSIRIGPGQT FYATGD**WIQ (pSECNHivC6)	
HisF	5'- TCG AGA CAT CAT CAT CAT CAT CAC-3'	QLPDSR**HHHHHH**GS (pSECNHisC3)	*Xho*I/*Bam*HI
HisR	5'- GAT CCG TGA TGA TGA TGA TGA TGT-3'		
HisF2	5'- TCG AGA CAT CAT CAT CAT CAT CAC A-3'	VDSR**HHHHHH**RIQ (pSECNHisC6)	*Xho*I/*Bam*HI
HisR2	5'- GAT CCT GTG ATG ATG ATG ATG ATG T-3'		

Similarly a set of complementary oligonucleotides were designed for the insertion of a poly-His peptide into pSECNC3 and pSECNC6 at the *Xho*I/*Bam*HI insertion sites within the flagellin protein (Table 4.1). Unlike the HIV peptide the oligonucleotides for the poly-His peptide, once annealed, contained the necessary restriction enzyme site overhangs for direct ligation into pSECNC3 and pSECNC6 digested with

*Bam*HI/*Xho*I. Annealed oligonucleotides HisF/HisR and HisF2/HisR2 were ligated into pSECNC3 and pSECNC6 to give pSECNHisC3 and pSECNHisC6 respectively. Ligations were carried out according to the FastLink ligation kit and transformed into *E. coli* DH10B cells by electroporation. All constructs were confirmed to be correct by polymerase chain reaction (PCR) and sequencing analysis. PCR amplification was carried out as recommended by the supplier (BioLine). Primers used were FliNterRev with NC5F (Table 4.2) and HisF or HisF2 with FliNterRev (Table 4.2) for pSECNHivC6, pSECNHisC3 and pSECNHisC6 respectively. Constructs were transformed into *B. halodurans* BhFC01 (Δ*hag*).

4.2.4 Construction of hybrid flagella displaying the *Geobacillus thermoleovorans* LipA protein.

The vectors pSECNC3 and pSECNC6 was digested with *Sma*I and *Bam*HI. The mature *lipA* gene (Genbank, Accession: AF134840) from *G. thermoleovorans* lacking the signal sequence (29 aa) was PCR amplified using primers as described in Table 4.2. The PCR product (1161 bp) was digested with the appropriate restriction enzymes and ligated to pSECNC3 and pSECNC6 to give pSECNLipC3 and pSECNLipC6 respectively.

Table 4.2. List of primers and their corresponding nucleotide sequences. Restriction enzyme sites are underlined.

Primer Name	Primer sequence	Restriction enzyme sites
FliNterRev	5'CTCCTCGAGCGACCTTCTGAAACAGC3'	*Pst*I
NC5F	5'CACGTCGACTCGAGCCCGGGATCCTTTAATACG CAAAAATTACTC 3'	*Sal*I
NMLipC6F	5'GTACCCGGGAGCAGCTTCACGCGCCAA C3'	*Sma*I
NMLipC6R	5'GTAGGATCCAAGGCCCGAAGCTCGCCAG3'	*Bam*HI
FliDNR2	5'CGAGGATCCAAGACCGGCAGAGTTAATGTC3'	*Bam*HI
UpFor	5'GCGGATCCGTGTGGTGACATTTGAC3'	*Bam*HI

4.2.5 Transformation and integration of the chimeric *hag* gene in the chromosome of *B. halodurans* BhFC01.

Integration of pSECHisC6 was carried out according to Chapter 2 giving rise to *B. halodurans* strain BhFHC02 (section 2.2.5). After the double cross-over event colonies unable to grow on chloramphenicol plates were confirmed to contain the integrated chimeric *hag* gene by PCR analysis using two chromosomal primers UpFor and FliDNR2 (Table 2.1).

4.2.6 Protein extraction and analysis.

Two separate protein extractions were carried out using an ultra-centrifugation protocol for whole flagella and the NaOH extraction method for flagellin subunit isolation (Chapter 2, section 2.2.6). Protein extracts from the different cell fractions were isolated as described in Chapter 2. All cultures were grown overnight and reached stationary phase (OD_{540} 3-5).

In order to obtain whole flagella for metal binding assays bacterial cells were grown in 50 ml LB pH 8.5 to early log phase. Cells were harvested at 8000 X *g* for 10 minutes and resuspended in 10 ml phosphate buffer saline (PBS) (pH 7.5). The cell suspension was subjected to 3 times five second pulses using an Ultra-Turrax (Janke & Kunkel, IKA-WERK) homogeniser. Cells were again centrifuged at 8000 X *g* for 10 minutes to remove cell debris and the supernatant then centrifuged at 40 000 X *g* for 1 hour to pellet the whole flagella. Flagella were then resuspended in 50 µl PBS buffer. Protein concentrations were determined according to the Bradford method (1976). All proteins were run on a 10% SDS-PAGE as described by Laemmli (1970).

4.2.7 Western blot analysis.

Western blot analysis was carried out according to Gallagher *et al.* (1997). Polyclonal rabbit anti-flagellin antibodies were generated using *B. halodurans* Alk36 flagellin protein. Anti-6His antibodies were obtained from AEC Ammersham. MEIV3b 4 antibodies were kindly supplied Dr Meyer (University of Johannesberg) (Hewer and Meyer, 2003).

4.2.8 Detection and isolation of FliC::LipA fusion protein.

Lipase activity of transformed BhFC01 containing pSECNLipC3 and pSECNLipC6 was detected using Tributyrin plates (tryptone 1 g, yeast extract 0.5 g, sodium chloride 1 g, agar 1.5 g, tributyrin [Merck] 1 ml, make up 100ml water) pH 8.5 (Mourey and Kilbertus, 1975). Cell wall and intracellular protein fractions of positive transformants were isolated using the methods described above but the cell surface proteins were included as part of the cell wall. This allowed for the isolation of non-denature proteins. All protein fractions were resuspended in 25 mM Tris-HCl buffer (pH 7.5). For SDS-PAGE and zymogram analysis protein samples were prepared by the addition of loading dye and incubated for 30 minutes at 37°C but were not heat denatured. After electrophoresis, gels were incubated overnight in 25 mM Tris buffer with 2.5% Triton X-100. PAGE were then submerged in 25 mM Tris buffer (pH 7.5) and incubated for 30 minutes at room temperature. Gels were stained for lipase activity using 0.1% α-naphthyl acetate and 0.2% Fast Red TR salt in 25 mM Tris buffer (Takahashi *et al.*, 1998).

In order to determine lipase activity on the cell surface of recombinant *B. halodurans* BhFC01 a whole ce l lipase assay was performed. Cells (7.5 ml) were harvested in triplicate after 8, 24, 48 and 72 hours (6000 X *g* for 10 minutes). Cells were washed once in 25 mM Tris (pH 7.5). The cell pellet was resuspended in a final volume of 5 ml 25 mM Tris (pH 7.5). Lipase assays were performed immediately.

4.2.9 Lipase assays.

Lipase assays were carried out at 50°C. The reaction buffer was made just before use (0.01% *p*-nitrophenolpalmitate, 0.45% Na-deoxycholate, 0.11% Arabic acid in 0.1 M phosphate buffer pH 8). Reaction mixtures consisted of 25 µl diluted sample and 600 µl of the reaction buffer preheated to 50°C. Change in OD_{410} was monitored and change in OD was recorded every minute. Lipase activity was determined using *p*-nitrophenylpalmitate (*p*NPP) as substrate (Vorderwülbecke *et al.*, 1992). One unit of lipase activity was defined as the amount of enzyme that liberated 1 mmol *p*-nitrophenol from *p*NPP (*p*-nitrophenylpalmitate) per min (Vorderwülbecke *et al.*,

1992). The extinction co-efficient of *p*-nitrophenol at 410 nm (pH 8.0) is 15 (1 x $nmol^{-1}$ x cm^{-1} =ml x μmol^{-1} x cm^{-1})

4.2.10 Metal Binding Analysis of Chimeric Flagellin Proteins.

For whole and denatured chimeric flagella, (cell surface protein fraction) metal binding quantification of the displayed poly-His peptide was assayed directly using Ni-NTA alkaline phosphatase (AP) conjugate. Methods were followed according to manufacturer (QIAGEN). Cell surface proteins (2 µg) were immobilised in each well of a Maxisorp Nunc-Immuno plate. After incubation of the Ni-NTA AP conjugate (QIAGEN) 200 µl of substrate solution (50 µl *p*-nitrophenyl phosphate sodium salt (250 mg) in reagent grade water, 3 ml Diethanolamine (1 M) and 15 µl $MgCl_2$ (0.05 mM), pH 9.8) was added. The reaction was incubated at 37°C for 1 hour and stopped with 50 µl NaOH (3 M). Colour development was read at OD_{405}.

Denatured hybrid flagella were also isolated using the MagneHis Protein Purification system provided by Promega (cat # TM060). The method was carried out as described by the kit technical manual with the following modifications. 5 ml of an over night culture was pelleted at 8000 X *g* for 10 minutes and resuspended in 5 ml phosphate buffer (pH 7.5). The sample was then centrifuged at 8000 X *g* to remove all non-soluble cell debris. The supernatant containing the flagella fraction was precipitated using an equal volume of 5% TCA solution for 30 minutes at room temperature. The crude protein extract was centrifuged at 18000 X *g* for 30 minutes to collect the soluble proteins. The pellet was resuspended in 200 µl MagneHis cell lysis reagent and incubated at room temperature for 10 minutes to ensure complete resuspension of the protein pellet. Protein isolation and purification was continued as described in the technical manual and finally eluted in 100 µl elution buffer.

4.3 RESULTS

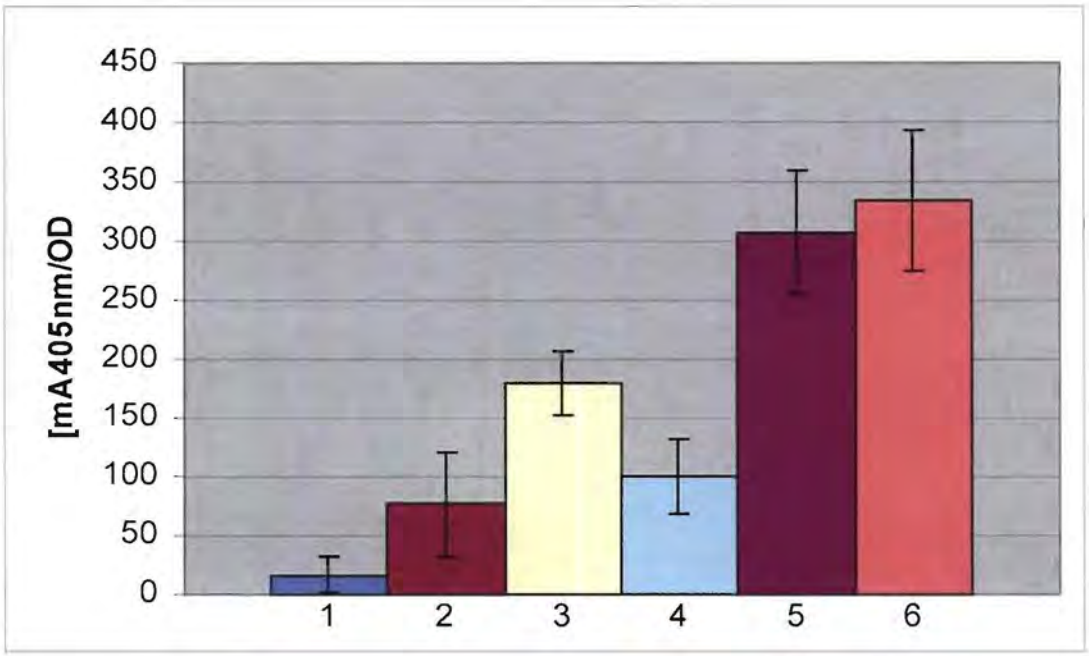
4.3.1 Expression and functionality of the chimeric flagellin poly-His peptide.

Whole cell binding assays were initially done as described by Samuelson *et al.* (2000). However, excessive wash steps resulted in the shearing of the flagella from the *B. halodurans* Alk36 cells resulting in false negative results. For this reason two separate protein extractions were done using an ultracentrifugation protocol for whole flagella and the NaOH extraction method for flagellin subunit isolation.

The NaOH method will allow for analysis of individual chimeric flagellin subunits as a metal binding substrate. It was demonstrated using both extraction methods that the whole and flagellin subunits carrying the poly-His peptide were able to bind more Ni^{2+} than did the wild type *B. halodurans* Alk36 flagella (Fig. 4.1). The site at which the poly-His peptide is displayed also plays a significant role in metal binding abilities with the NC6 site being more accessible than the NC3 site to the Ni-NTA conjugate (Fig. 4.1). The results showed the same trend for both whole flagella and flagellin subunits. Vector pSECNHisC6 was successfully integrated into the chromosome of *B. halodurans* BhFC01 to create a new strain BhFHC02. This new strain carrying a single copy of the chimeric flagellin gene was able to produce hybrid flagellin, and bind equal amounts of Ni-NTA conjugate.

The Magne-His protein purification system selectively purifies proteins carrying an exposed poly-His peptide. By using this purification system we were able to demonstrate the accessibility of the poly-His peptide since hybrid flagella were purified to a high level using this system (Fig. 4.2).

A



B

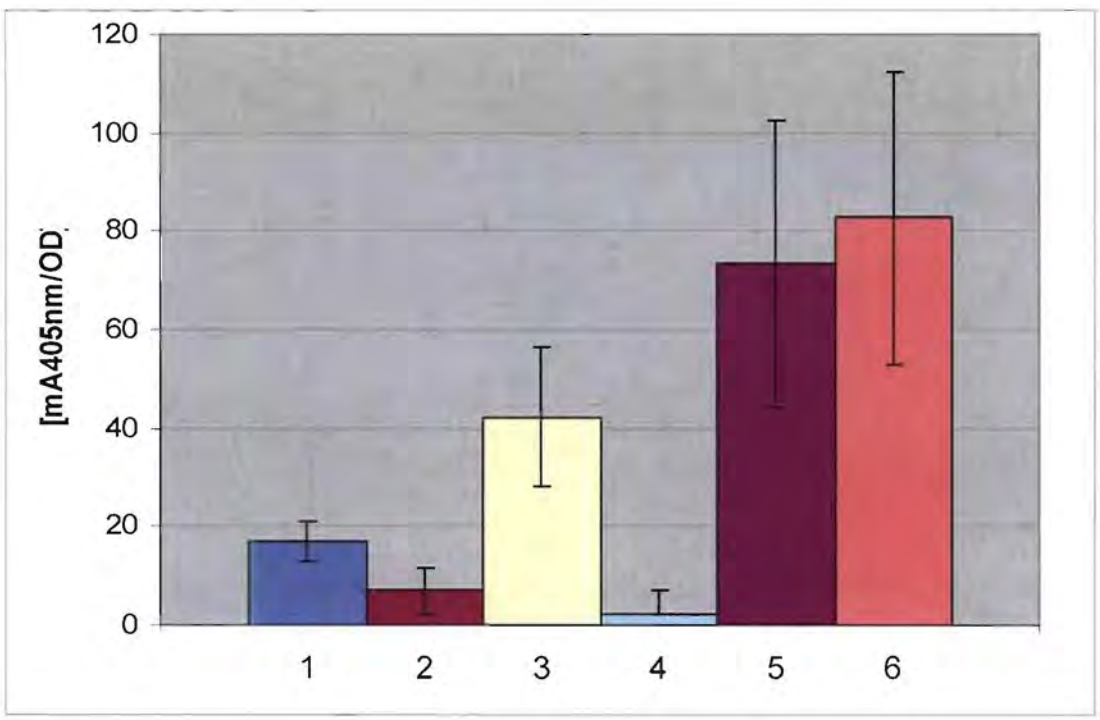


Figure 4.1 Histogram representations of results from Ni^{2+}- binding assay. Results demonstrate binding of (A) poly-His::flagellin monomers and (B) whole poly-His::flagella, to Ni-NTA conjugate. Bar 1, *B halodurans* Alk36; bar 2, BhFC01(pSECNC3); bar 3, BhFC01(pSECNHisC3); bar 4, BhFC01 (pSECNC6); bar 5, BhFC01 (pSECNHisC6); and bar 6, BhFHC02 (Integrated pSECNHisC6).

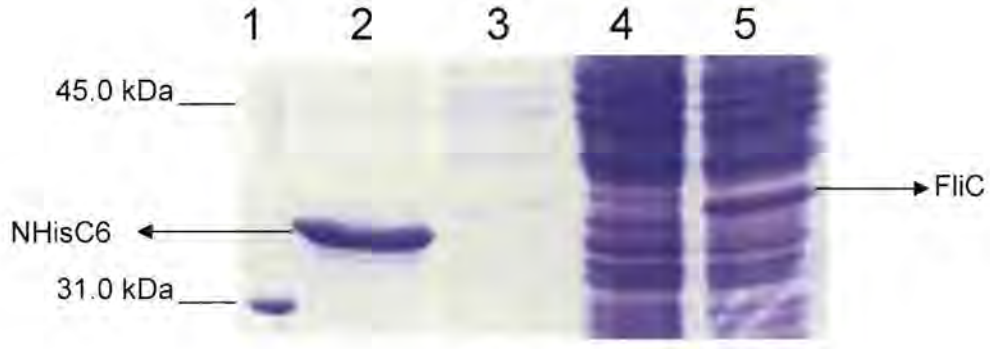


Figure 4.2 SDS-PAGE showing purified hybrid flagella using the Magne-His protein purification system of the surface exposed poly-His peptide at the NC6 insertion site of FliC from *B. halodurans* Alk36. Lane 1, low molecular mass ladder; lane 2, purified hybrid flagella from BhFC01 (pSECNHisC6); lane 3, purified flagella from *B. halodurans* Alk36; lanes 4 and 5, are the unbound protein fraction after purification from both the BhFC01 (pSECNHisC6) and *B. halodurans* Alk36. Note the unbound FliC protein for the *B. halodurans* Alk36 (lane 5).

4.3.2 Surface display of the gp-120 V3 loop region of HIV-1 subtype C.

Construction of pSECNHivC6 was successful and confirmed to be correct by sequence analysis and PCR. Sequence analysis also confirmed that the inserted oligonucleotides were in the correct reading frame. The total size of the peptide inserted into pSECNC3 and pSECNC6 was predicted to be 35 aa and 29 aa respectively. However, sequence analysis revealed an extra 18 bp of unknown origin at the 5' end of the inserted gp120 V3 epitope for pSECNHivC3 (Fig. 4.3). Insertion of the extra nucleotides did not affect the reading frame of the sequence but ultimately resulted in the insertion of a 40 aa peptide.

Transformation of pSECNHivC3 and pSECNHivC6 into *B. halodurans* BhFC01 was confirmed by PCR analysis. A single clone of each construct was grown to stationary phase and protein fractions harvested. OD_{540} of *B. halodurans* BhFC01 carrying pSECNHivC6 and pSECNHivC3 were both 1.87. Cell surface fractions of the pSECNHivC6 and pSECNHivC3 were compared to the *B. halodurans* BhFC01 carrying the pSECNC6 construct on a 10 % SDS-PAGE (Fig. 4.4).

5'- **CAG CTG CCG GAC TCG AGC CCG GGC GTC GAC** ACG CGT CCA

Q L P V S S P G V D T R P

AAT AAT AAT ACG CGT AAA TCA ATT CGT ATT GGA CCA GGA CAA ACG

N N N T R K S I R I G P G Q T

TTT TAT GCA ACG GGA GAT **TGG ATC CGC GGG GGA TCC**- 3'

F Y A T G D **W I R G G S**

Figure 4.3. The nucleotide and amino acid sequence of the inserted HIV-1 subtype C gp 120 V3 loop consensus epitope within the flagellin in pSECNC3 vector. Red indicates the gp120 peptide sequence and black bold is the *hag* gene. The blue sequence is 6 extra amino acids inserted during the cloning process for pSECNHivC3.

Size differences of the respective clones can be clearly seen. pSECNHivC3 did not show any production or display of the chimeric flagellin protein (Fig 4.4).

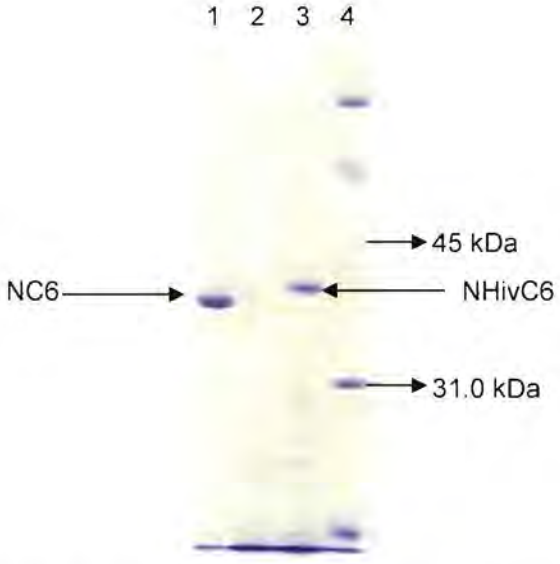


Figure 4.4. A 10 % SDS-PAGE showing the successful expression and display of the chimeric flagellin::Hiv gp120 V3 loop epitope. Lane 1, pSECNC6 (control); lane 2, pSECNHivC3; lane 3, pSECNHivC6; and lane 4, low molecular mass ladder.

Western blot analysis using the anti-flagellin antibodies confirmed that the FliC::MEIV3b fusion when inserted into pSECNC3 was not displayed sufficiently. Insertion of the gp 120 peptide within the pSECNC6 vector showed successful display of the chimeric flagellin (Fig 4.5). Antibodies generated against the Multiple Epitope Immunogen of the HIV subtype C gp 120 V3 loop MEIV3b (Hewer and Meyer, 2003) bound successfully to the exposed HIV-1 C gp120 epitope on the flagella at concentrations of 500 ng of protein for pSECNHivC6 (Fig 4.6). There was no antibody interaction between MEIV3b 4 antibodies and the pSECNHivC3 cell surface protein fraction.

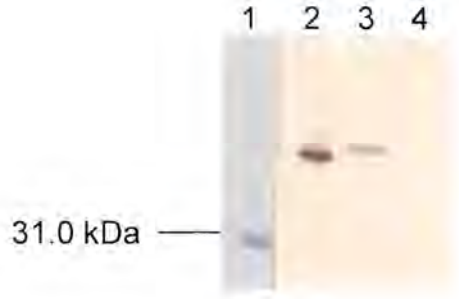


Figure 4.5. Western blot analysis using anti-flagellin antibodies of BhFC01 containing different constructs.. Lane 1, low molecular mass ladder; lane 2, pSECNC6; lane 3, pSECNHivC6; and lane 4,pSECNHivC3.

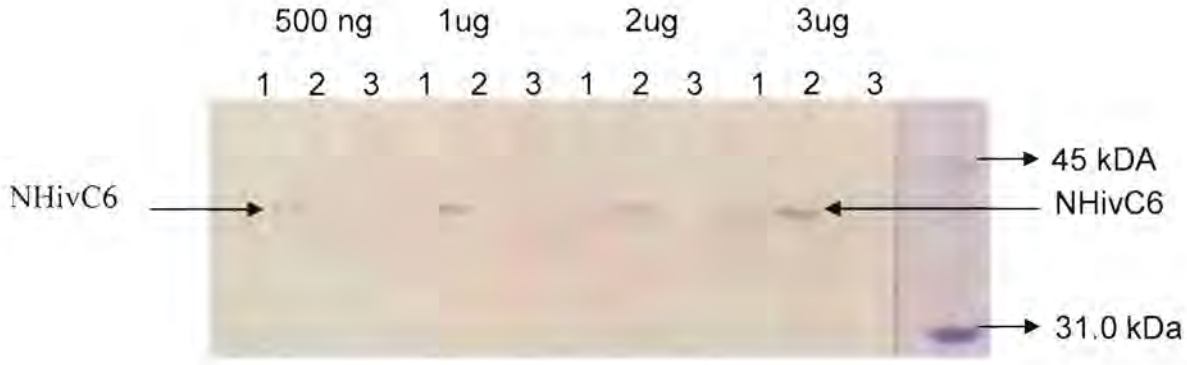


Figure 4.6. Western blot analysis using MEIV3b 4 antibodies of BhFC01 carrying flagellin::gp120 V3 loop peptide fusion constructs. Lane 1, pSECNC6 (negative control); lane 2, pSECNHivC6; and lane 3 the pSECNHivC3.

4.3.3 Surface display of a thermostable lipase using the *B. halodurans* Alk36 flagellin display system.

Construction of pSECNLipC3 and pSECNLipC6 was confirmed to be correct by PCR and sequence analysis. The total size of the LipA protein inserted into pSECNC3 and pSECNC6 including the multiple cloning site was 397 aa. Both constructs were successfully transformed into *B. halodurans* BhFC01 and colonies were transferred to tributyrin plates to confirm the expression of the FliC::LipA fusion (Fig. 4.7). Clearing zones are indicative of either lipase or esterase activity.

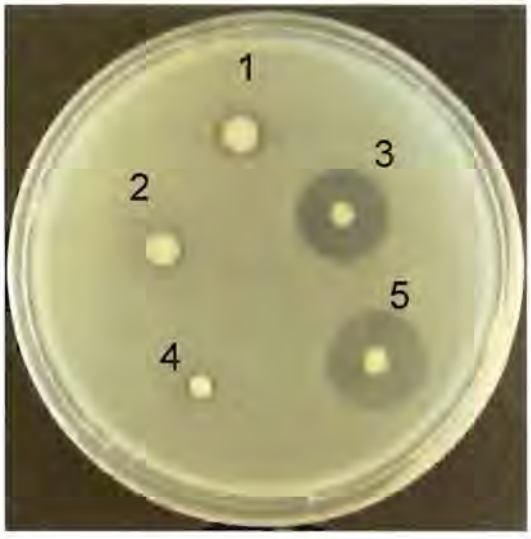


Figure 4.7. Lipase activity of recombinant BhFC01 harboring pSECNLipC3 and pSECNLipC6 on an agar plate containing tributyrin. Clearing zones are indicative of lipase activity. Colony 1, pSEC194; colony 2, pSECNC3; colony 3, pSECNLipC3; colony 4, pSECNC6; and colony 5, pSECNLipC6.

A non-denaturing protein purification method was used to isolate the chimeric lipase fusions from the various cellular compartments from recombinant BhFC01 in stationary phase so as not to destroy the lipase activity. These protein fractions were separated on an SDS-PAGE and activity was detected using a Fast Red TR salt. A large 76 kDa protein corresponding to the chimeric flagellin was observed in the cell wall and intracellular protein fractions (Fig. 4.8).

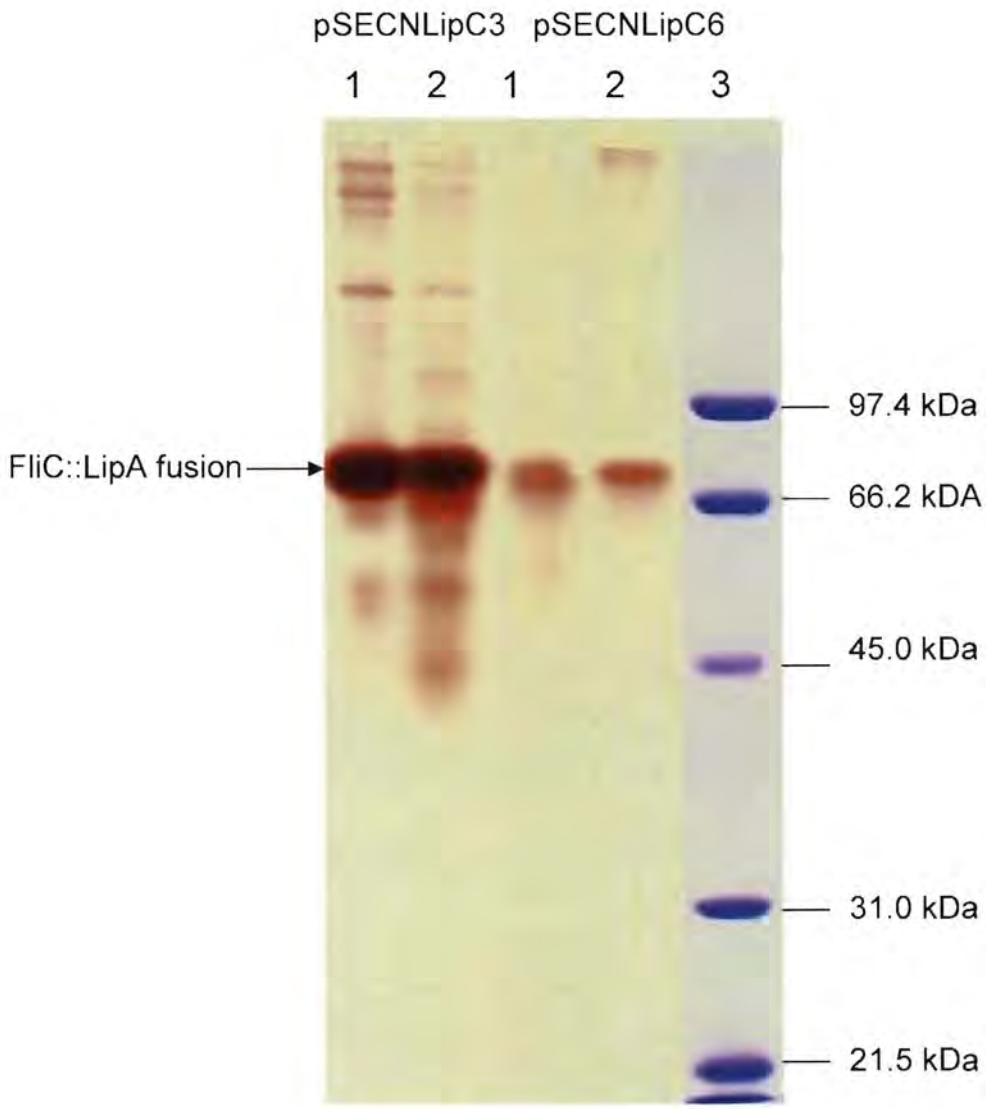


Figure 4.8. Zymogram of lipase activity from cell wall and intracellular protein fractions of BhFC01 harboring pSECNLipC3 and pSECNLipC6 grown for 24 hours. Lane 1, cell wall protein fraction; lane 2, intracellular protein fraction; lane 3, BioRad low molecular mass marker.

The time course of growth versus lipase activity of whole BhFC01 cells harboring pSECNLipC3 and pSECNLipC6 are shown in Fig. 4.9. Whole cell lipase activity increased up to 24 hours and 48 hours for the pSECNlipC3 and pSecNlipC6 in BhFC01 respectively. Maximum whole cell activity levels reached 5.57 and 2.78 U/mg protein for pSECNLipC3 and pSECNLipC6 respectively. The lack of lipase activity for whole cells containing vector pSECSLipA demonstrated that the activity was associated with the surface exposed FliC::LipA fusion since the mature (lacking the signal sequence) LipA is not fused to the flagella but is under the control of the σ^D promoter. The absence of the signal sequence results in the lipase being located intracellularly. Extracellular lipase activity is high in proportion to the whole cell lipase activity. There is also an increase in extracellular lipase activity for pSECNLipC3, pSECNlipC6 and pSECSLipA over time with the latter being associated with cell lysis.

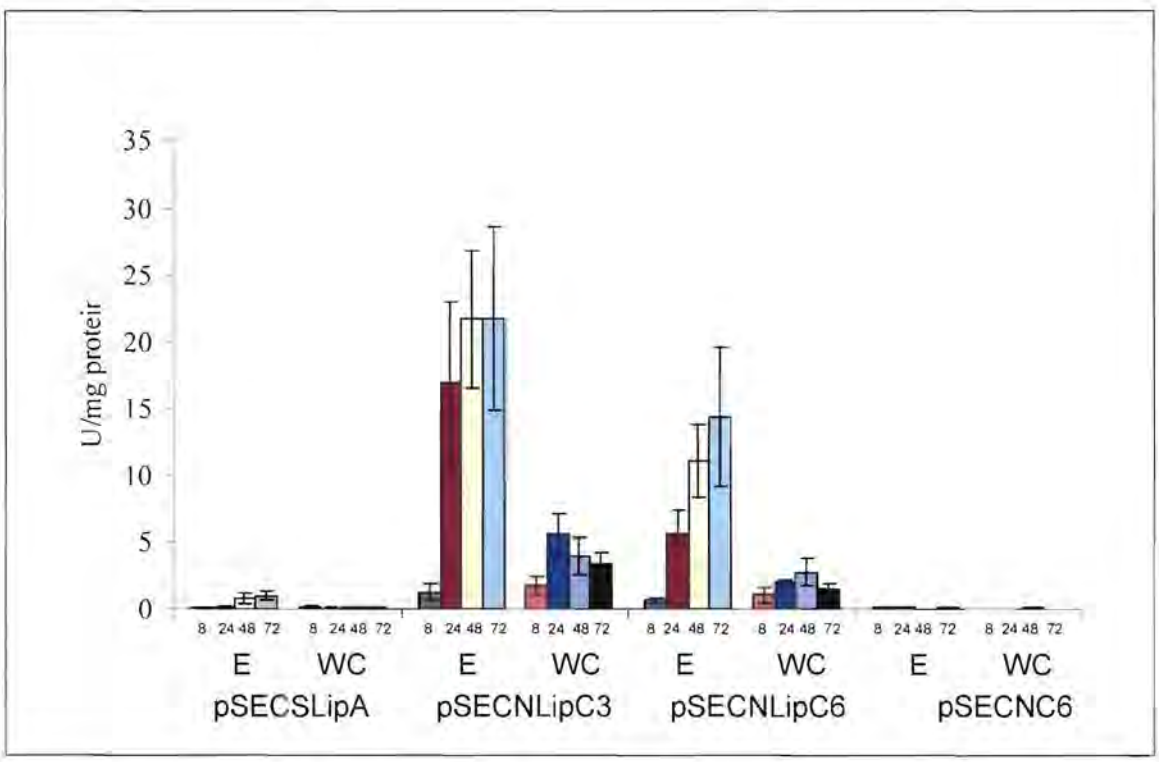


Figure 4.9. Histogram representation of lipase assays of extracellular (E) and whole cells (WC) over a period of 8 to 72 hours for *B. halodurans* BhFC01 carrying expression vectors pSECSLipA, pSECNlipC3, pSECNLipC6 and pSECNC6 (negative control).

4.4 DISCUSSION

B. halodurans Alk36 possesses the unique ability among *Bacillus* sp. to over produce flagella for extended culture times (Chapter 2). This, together with the fact that Gram-positive bacteria have a much thicker cell wall making them more rigid, has made this organism a prime target for the development of flagella surface display technologies. In order to demonstrate the robustness and versatility of this system we evaluated the expression of polypeptides and proteins as flagellin fusions. We also evaluated the biological functionality of these polypeptide fusions.

The most common application for bacterial flagellin surface display to date has been the development of live vaccine delivery systems for mucosal immunizations. Flagella are potent immunogens and numerous reports show that immunization with purified hybrid flagella or whole *Salmonella* cells carrying hybrid flagella induces cellular and humoral immune responses in laboratory animals against the chimeric flagellin epitope (Cattozzo *et al.*, 1997 and Westerlund-Wikström, 2000). We have successfully demonstrated the potential of this application by inserting a clade C gp120 synthetic epitope of 29 amino acids in the variable region of the *B. halodurans* Alk36 flagellin protein and shown the production of the flagellin::peptide fusions on the bacterial cell surface as determined by SDS-PAGE analysis. This was only true for the pSECNC6 vector whereas the pSECNC3 vector did not result in the surface display of the chimeric flagellin. A combination of peptide size (40 aa) and location (just outside the putative variable domain) could have contributed to the failure of pSECNHivC3 chimeric flagellin gene product to polymerize into functional flagella.

Although successful translocation of the hybrid flagellin to the cell surface was demonstrated (pSECNHivC6), this does not however, confirm the correct folding and display of the inserted peptide. For this reason Western blot analysis using the specific polyclonal antibodies to the HIV peptides was done and this resulted in cross reactivity with the hybrid flagella. The detection levels were as low as 500 ng of hybrid flagella indicating cross-reactivity between the antigenic epitope and its corresponding antibody. As an effective immunogen this epitope is still in an experimental stage and viral inhibition studies involving animal models would need to be carried out in order to determine the efficacy of this specific epitope as a potential

vaccine. Possibilities also exist for the insertion of other antigenic epitopes or even a range of different epitopes displayed on the same flagella. This has already been demonstrated to a certain extent in Gram-negative bacteria by expressing two foreign adhesive peptides on the same flagellin filament (Tanskanen *et al.*, 2000, Majander *et al.*, 2005a). This resulted in the display of several different effector molecules simultaneously. The combination of the flagella to act as an effective adjuvant (McSorley *et al.*, 2002) and the potential for large scale chimeric flagellin over-production and harvesting make this a potentially attractive candidate as a vaccine delivery system.

The traditional method of performing an enzymatic reaction involves the use of purified, free or immobilized enzyme. As enzyme cost is a concern, enzyme immobilization was developed as a strategy for more efficient enzyme use. However, the immobilization step might lead to problems with enzyme activity and stability. An elegant solution to this problem would be to express the active enzyme on the bacterial surface, thus creating a cost-effective system without the need for enzyme purification and eliminating the mass transport problems associated with intracellular expression (Wernérus and Ståhl, 2004). A number of attempts have been recorded for the use of *B. subtilis* in a whole cell biocatalyst. These have primarily focussed on the use of the major cell wall binding proteins such as peptidoglycan hydrolases as anchoring motifs. These include the cell wall binding domain of the major autolysin CwlB (Tsuchiya *et al.*, 1999, Kobayashi *et al.*, 2000, Kobayashi *et al.*, 2002). This was used as an anchoring motif to display both bacterial and fungal lipases on the bacterial cell surface. One of the major problems encountered was instability of the displayed proteins and after 12 hours of cell growth the amount of enzyme recorded was found to decline significantly despite using a protease deficient host strain (Kobayashi *et al.*, 2000).

Kim *et al.* (2005) used a molecular chaperone PrsA as an anchoring motif to display *Clostridium cellum* cellulose bound to the cell membrane of *B. subtilis*. Cellulase activity could however only be detected bound to *B. subtilis* protoplasts. Nguyen and Schumann (2006) expressed α-amylase on the surface of *B. subtilis* cells by fusing it to the C-terminal region of *S. aureus* fibronectin binding protein B containing the known sorting motif LPXTG. The *srtA* gene coding for sortase A of *Listeria*

monocytogenes was expressed in the cells. Amylase expression was detected in log phase cultures after induction by IPTG. Ezaki *et al.* (1998) claimed some cell surface expression of the green fluorescent protein (GFP) and bacterial alkaline phosphatase (BAP) as flagellin fusions in *E. coli* as shown by PAGE analysis and Western blot. No quantitative measurements of BAP were shown and no images of GFP by fluorescent microscopy were included in their report. Most of the expression was reported to be in the intracellular fraction. It was also not clear how stable their constructs were found to be. The above examples also have the added disadvantage of having to be induced by IPTG.

In evaluating our system we chose a thermostable lipase enzyme cloned from a *Geobacillus* sp. as a model enzyme. When cloned and expressed in our system the flagellin::LipA fusion was detected in all the protein fractions isolated using zymographic and lipase assay analysis. However, it did not produce functional flagella on the cell surface for both pSECNLipC3 and pSECNLipC6 as determined by SDS-PAGE analysis of a cell surface isolated protein fraction. This was probably due to the amino acid composition of the inserted protein interfering with the polymerization of chimeric flagellin into flagella. Zymogram analysis indicated that most of the enzyme activity was found associated with the cell wall as well as intracellularly. Plate and whole cell bio-assays showed the presence of significant lipase activity associated with the cell wall/surface and extracellular medium indicating transport of the flagellin::LipA fusion to the cell wall and exposure of the active site to the extracellular environment. The extracellular activity is most likely due to cell lysis as shown by the increase in lipase activity of strain BhFC01 carrying the pSECSLipA. This construct lacks a signal sequence to direct the heterologous lipase to the extracellular environment. This holds true for both insertion sites within the flagellin.

The fact that a high concentration of enzyme activity could be measured for up to 72 hours indicates the positive effect of the Gram-positive flagellin display system for heterologous protein display. This system was found to function effectively when the constitutive flagellin promoter system was used thereby negating the need for an inducible expression cassette. Although cell lysis was shown to occur this still needs to be determined using numerous intracellular enzyme controls.

Future work will involve the expression of an extremophile enzyme in our system and evaluation of expression at various elevated growth temperatures as *B. halodurans* Alk36 is capable of growth over a range of temperatures up to 60°C. Although *B. halodurans* Alk36 is capable of growing up to 60°C, all lipase assays (LipA) were done at 50°C since LipA has a greater stability and half life at this temperature (Cho *et al.*, 2000). It would also be of interest to utilise the flagellin secretory pathway for the production of various biologically active proteins. This could be done by removing the cap protein (FliD) as has been demonstrated by Majander *et al.* (2005b) for *E. coli*.

The increasing accumulation of heavy metal contaminants in our environment due to industrial and agricultural pollution is a growing concern for public health. Most remediation is of groundwater and soil, and most sites are contaminated with a combination of heavy metals and organic compounds (Watanabe and Baker, 2000). Bacterial sequestration of toxic metals has previously been investigated using only non-engineered bacteria (Pollmann *et al.*, 2006), but recombinant DNA technology offers the possibility to improve the metal-binding capacity of the bacteria. For some applications where bioremediation is in use, it might be beneficial to have the biologically active moiety displayed at the outer surface of the microbe. This could include the expression of tailor-made highly specific ligands for the affinity capture of certain pollutants such as heavy metals. Recent efforts have focussed on the development of ligands with increased affinity and selectivity for the target metals using phage display technology (Naik *et al.*, 2004, Kehoe and Kay, 2005). In some cases these ligands have also been expressed on bacterial surfaces to create bacteria able to bind precious metals such as gold and platinum thereby creating an opportunity for bio-mining (Brown, 1997) and biomimetics (Sarikaya *et al.*, 2003). Histidine-rich metal-binding peptides have been previously shown to function as effective bioadsorbants. Both *E. coli* and *Staphylococci* have been shown to harbour increased Ni- and Cd-binding capacity when these polyhistidyl peptides were surface displayed (Samuelson *et al.*, 2000, Xu *et al.*, 1999). We used a poly-histidyl peptide as our model system. We were able to show cell surface display of the chimeric flagellin fusions and functionality by demonstrating binding of the chimeric flagellin monomers as well as whole flagella to metal ions. The successful use of whole

flagella potentially allows for the extrapolation of the results to whole cells. When the construct containing the chimeric flagellin poly-His peptide fusion was integrated using a double cross-over event, there was no noticeable decrease in the amount of chimeric flagellin produced, or metal binding, indicating that the high levels of flagellin production is not dependent on the gene being present on a multi-copy vector. This is a significant advantage as such recombinant strains could be used to sequester metals without the need for antibiotic selection to maintain the vector in the cells.

This is the first report of the successful display of a range of heterologous polypeptides and proteins as cell surface fusions using chimeric flagellin from a Gram-positive bacterium.

Chapter 5

Concluding Remarks and Future Prospects

Cell surface display of heterologous peptides and proteins has applications in many fields such as bioremediation, immunology and biotransformation, and has been discussed extensively in Chapter 1. This interest, together with the discovery of a highly expressed 34 kDa flagellin protein on the cell surface of *B. halodurans* Alk36, led us to the evaluation of this protein as a display system. In order to implement a display system using *B. halodurans* Alk36 flagellin, we had to develop genetic tools to create selected mutations in the endogenous *B. halodurans* Alk36 *hag* gene. This included construction of a shuttle/integration vector for targeted gene inactivation, optimisation of *B. halodurans*Alk36 transformation systems, and methods to integrate manipulated genes back into the chromosome. This integration mechanism also results in the removal of any plasmid DNA from *B. halodurans* Alk36 chromosome. The constructed vector (pSEC194) is both an expression vector and a shuttle vector allowing us to perform all genetic manipulations in *E.coli* before introducing the construct into *B. halodurans* Alk36. The vector pSEC194 carrying the *hag* gene has a σ^D promoter which is not active in *E. coli*, thus preventing gene expression and allowing for a highly stable cloning vector system. Once developed, we utilised the *B. halodurans*i Alk36 display system to display a poly-His tag, Hiv-1 clade C gp120 V3 loop peptide and a thermostable lipase (LipA) from *G. thermoleovorans*.

Alignments of the *B. halodurans* Alk36 flagellin protein revealed 99.9% identity to the *B. halodurans* C-125 *hag* gene at the nucleotide level. Sakamoto *et al.* (1992) postulated a variable domain for *B. halodurans* C-125 FliC to be between amino acids 137-200 following alignment of the flagellin sequence to that of *B. subtilis*. However, the *B. subtilis* domain was speculative and not well characterised. We therefore characterized a permissible insertion site within the flagellin protein for heterologous peptide display by inserting peptides in a number of different sites, and also used the multiple flagellin sequence alignment (Beatson *et al.*, 2006) in order to more precisely determine the variable domain for the *B. halodurans* Alk36 FliC protein (159-185 aa). In this manner, we identified only a single insertion site (NC6) which did not interfere

with the polymerization of flagellin into functional flagella. Furthermore, motility was restored to *B. halodurans* strain BhFC01 (Δ *hag*) when peptide fragments were inserted into NC6.

The *B. halodurans* Alk36 display system offers a range of applications and certain advantages over the previously reported *E. coli* and *Salmonella* flagellin display systems. After cultivation high concentrations of chimeric peptide fusions can easily be isolated by either mechanical or chemical treatments. Neither intracellular nor secreted proteins are included in the cell surface fraction thereby enabling easy purification. Chimeric flagellin fusions make up approximately 70% of the proteins isolated in this cell fraction as visualised by the isolated cell surface fractions run on SDS-PAGE gels (Chapter 2). Cell growth is not impaired by the production of chimeric flagellin fusions. The expression system is very versatile as custom designed peptides, ranging from epitopes to metal binding peptides can be displayed (this study). Functional metal binding peptides and proteins have been displayed on the cell surface of *E. coli* for environmental and biosensor applications (Lu *et al.*, 1995, Tripp *et al.*, 2001, and Thai *et al.*, 2004). Future studies will include peptide display for improved metal binding capabilities. This, however, requires improvement of efficiencies of transformation of *B. halodurans* Alk36. This could potentially be achieved by targeted gene inactivation of the methylation restriction pathway to enhance transformation efficiencies. Meinhardt (pers. comm.) was able to improve electroporation transformation efficiencies in *Bacillus licheniformis* by deleting the methylation restriction system.

The flagellin display system can also be used to display proteins such as enzymes on the cell surface. In this study we were able to successfully display a lipase (LipA) on the cell surface of *B. halodurans* Alk36. This flagellin::lipase fusion is stable and continues to accumulate for up to 48 hours. This is in contrast to the current *B. subtilis* lipase display system (Kobayashi *et al.*, 2000) were the lipase fusion is degraded after 12 hours. These displayed enzymes can then be mutated or rearranged and selected for novel substrate specificities or improved activity (directed evolution).

The abundance of flagellin monomers on the cell surface increases the potential to display large amounts of chimeric fusions. This was apparent when the cell surface

fractions of *B. subtilis* and *B. halodurans* Alk36 were compared (Crampton *et al.*, 2007). However, a comparison and quantification of chimeric flagellin production by *B. halodurans* Alk36 with *E. coli* and *Salmonella* systems is extremely difficult to perform due to the lack of information on growth conditions, different methodologies for flagellin harvesting and lack of quantitative data for the latter bacteria. Ezaki *et al.* (1998) reported that as the size of the displayed peptide increases so the relative quantity of hybrid flagella decreases. This was observed in this study as the hybrid flagellin containing the lipase fusions did not form flagella and were only detectable using zymographic studies.

To date, few flagellin display systems are commercially utilised. An exception is the FLITRX that is used as a peptide display system for evaluating peptide interactions with various targets (Lu *et al.*, 1995). In this study commercially viable heterologous antigens were displayed successfully demonstrating the potential of the flagellin display system to be utilized as a vaccine delivery vehicle for mucosal immunizations. The co-expression of antigens and mucosal adhesion proteins may also enhance the immune response. Future work in flagellin display will include overcoming the limitations of display technology and finding direct applications that will lead to commercial success. One aspect which could be pursued is the development of vaccines for domestic animals since it would be easier to obtain Food and Drug Administration (FDA) approval.

Further aspects of flagellin display that will be investigated include the development of a dual display system using both the FliC and FliD, as has been done for *E. coli* (Majander *et al.*, 2005a). A dual display system has the advantage of directing the flagellin displayed antigens to the epithelial surface for orally administered vaccines and in so doing, elicit a stronger immune response. A second prospect is to evaluate the type III secretory system for the secretion of heterologous peptides. Although this has been sshown to have potential in *E. coli* (Majander *et al.*, 2005b), *B. halodurans* Alk36 has many extracellular proteases and these will need to be inactivated using the integration system developed in this study. In this manner, many *B. subtilis* strains have been developed for industry with increased secretory capabilities as a result of being protease deficient (Wu *et al.*, 1991).

The underlying mechanism of *B. halodurans* Alk36 FliC expression and its regulation is also of interest as it seems to be very different from that observed in *B. subtilis*. Transcriptomic and proteomic studies of *B. halodurans* Alk36 could reveal sporulation control mechanisms. The onset of sporulation in *B. subtilis* is associated with the suppression of flagellin synthesis. By extrapolating the sporulation control mechanisms from *B. halodurans* Alk36 to *B. subtilis*, one could engineer a strain of *B. subtilis* which is capable of over-expressing FliC. *B. subtilis* has the advantage of being a GRAS (generally regarded as safe) organism and fermentation processes are well documented. Many protease deficient strains are also readily available (Wu *et al.*, 1991).

In conclusion, the present study developed the genetic tools for the manipulation of the Gram-positive bacterium *B. halodurans* Alk36. Using these tools we were able to create suitable host strains for the evaluation of FliC as a display system for heterologous peptides and proteins. To this end we were able to identify suitable display sites within the FliC protein. These sites were then successfully exploited for the display of metal binding, antigenic, and a bioactive protein, ranging from 13 to 397 amino acids.

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