# Inactivation of genes encoding extracellular proteases in *Bacillus halodurans* BhFC01 and the impact on its modified flagellin type III secretion pathway towards improving peptide expression

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**Abstract**

The flagellin type III secretion pathway of *Bacillus halodurans* BhFC01 (Δ*hag*) was modified by inactivation of the gene encoding FliD protein. This prevents polymerization of the flagellin monomers as encoded by the *hag* gene into functional flagella. Using this host background, an in-frame chimeric flagellin fusion polypeptide on a multi-copy vector was expressed and heterologous peptides were shown to be secreted. Heterologous peptides were however only observed in the growth medium during log phase growth. Degradation of the fusion peptides was observed during stationary phase indicating extra-cellular proteases were problematic. A number of key protease genes were subsequently identified and inactivated which successfully enhanced the stability and concentration of secreted heterologous peptides during stationary phase growth. This is the first demonstration in a Gram-positive system of a modified flagellar type III secretion apparatus showing successful secretion of un-polymerised heterologous peptide fusion monomers.

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## Introduction

Gram-negative bacteria use specialized secretion systems to secrete selected proteins to the outside of the cell. One such system is the type III secretion system which mediates, among others, the export of virulence proteins (9). The exported substrates of type III secretion systems do not contain cleavable Sec-dependant signal sequences as is present in the type I secretion system (1). The flagellar type III secretion apparatus is, however, a specific variant which efficiently secretes the flagellin subunit protein FliC. This occurs in both Gram-negative as well as Gram-positive bacteria. The bacterial flagellum is a super-molecular structure consisting of a basal body, a hook and a filament. Most of the flagellar components are translocated across the cytoplasmic membrane by the flagellar type III protein export apparatus in the vicinity of the flagellar base, diffuse down the narrow channel through the nascent structure and self assemble at its distal end with the help of a cap structure. Flagellar proteins synthesized in the cytoplasm are targeted to the export apparatus with the help of flagellum-specific chaperones and pushed into the channel by an ATPase. They are then polymerized into flagellin filaments exterior to the cell. It was also shown that mutant strains of *E. coli* with a non-functional FliD (cap structure) fail to assemble flagellum leading to diffusion of FliC monomers into the culture medium (13).

Stahl and La Vallie (17) suggested the use of the *B subtilis* flagellum specific export apparatus for exporting heterologous proteins from host cells. Their approach involved a genetically engineered *B subtilis* strain whereby the flagellin gene *hag*, (named *fliC* in *Escherchia coli)* was inactivated (12). Fusion constructs were then generated whereby the heterologous gene was attached via its 5' terminus to the 3' terminus of the full length flagellin gene. An alternative construct was proposed utilizing a shorter portion of the flagellin gene which was truncated at its 3' terminus. By using this system they were able to detect the expected fusion

proteins by Western blot. The FliD protein which directs polymerization of the FliC monomers was however still present. The development of a modified flagellar type III secretion apparatus to secrete foreign polypeptides into the growth medium of *E. coli* was first reported by Majander et al. (13). They created a suitable host strain by inactivating the *fliC* and *fliD* genes on the *E. coli* chromosome. They then successfully complemented the Δ*fliC* gene with a number of *fliC* constructs containing various heterologous polypeptides from pathogenic organisms cloned as in-frame fusions. These were found to be secreted into the medium with varying degrees of efficiency. Majander et al. (13) concluded that N-terminal FliC fragments lacking the C terminus needed the *fliC* 5' untranslated promoter region for efficient secretion of heterologous peptides via the flagellar type III secretion apparatus in *E. coli*. Végh et al. (20) identified a flagellum-specific secretion signal in *Salmonella* flagellin, localizing it to a specific N-terminal fragment of FliC, which when fused to an 8 kDa human protein domain was sufficient to mediate secretion of this protein module through the flagellum specific export pathway of a flagellum deficient *S typhimurium* strain.

A flagellin surface display expression system was developed in the alkaliphilic *Bacillus halodurans* Alk36 strain by inactivation of the *hag* gene encoding FliC and subsequent complementation on a multi-copy vector of in-frame chimeric flagellin fusions (3). As this strain was found to produce FliC at a significant concentration a study was initiated in order to evaluate the ability of this strain to secrete heterologous peptides as in-frame flagellin fusions using a modified flagellar type III secretion apparatus. In this publication we describe the development of an efficient heterologous peptide expression system utilizing the flagellar type III secretion apparatus of *B halodurans* BhFC01 (3). The *fliD* gene was inactivated and the system further optimized through the identification and inactivation of a number of key protease genes which successfully enhanced the stability of secreted heterologous peptides.

As a model system to evaluate heterologous secretion the HIV-1 subtype C gp120 epitope was used as described by Hewer and Meyer (7). This is the first demonstration in a Gram-positive system of a modified flagellar type III secretion apparatus showing successful secretion of un-polymerised heterologous fusion peptides into the growth medium.

## MATERIALS AND METHODS

**Bacterial strains, culture medium and culture conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *B halodurans* BhFC01 and associated mutant strains were grown in Luria Bertani (LB) medium at pH 8.5 at 30, 42 or 52°C as specified. *E. coli* DH10B was grown at 37°C in LB medium at pH 7. All *E. coli* and *B. halodurans* transformants were selected using 100 μg/ml ampicillin and 10 μg/ml chloramphenicol respectively.

**DNA techniques.** Plasmid DNA was isolated using a Plasmid Midi Kit (Qiagen). Restriction enzymes were used as specified by the manufacturer (Fermentas and Roche Diagnostics). All mini-preps were done using Perfectprep Plasmid Mini Kit (Eppendorf). All DNA manipulations were done in *E. coli*, which was transformed using electroporation (5). Transformation into *B. halodurans* BhFC01 and associated mutant strains was carried out according to the protoplast method of Kudo et al. (10) with modifications as described by Crampton et al. (3). *Thermus aquaticus* DNA polymerase was used for polymerase chain reaction (PCR) as recommended by the supplier (BIOLINE) and all primers used are shown in Table 2.

TABLE. 1 Bacterial strains and plasmids used in this work

| Strain or plasmid | Genotype, genetic markers and relevant characteristics | Reference |
|---|---|---|
| *B halodurans* BhFC01 | Δ*hag* | Crampton et al. (3) |
| *B halodurans* BhFC04 | Δ*hag*, Δ*wprA* | this study |
| *B halodurans* BhFD01 | Δ*hag*, Δ*wprA*, Δ*fliD* | this study |
| *B halodurans* BhFD02 | Δ*hag*, Δ*wprA*, Δ*fliD*, Δ*alp* | this study |
| *B halodurans* BhFD03 | Δ*hag*, Δ*wprA*, Δ*fliD*, Δ*alp*, Δ*apr* | this study |
| *B halodurans* BhFD04 | Δ*hag*, Δ*wprA*, Δ*fliD*, Δ*alp*, Δ*apr*, Δ*vpr* | this study |
| *B halodurans* BhFD05 | Δ*hag*, Δ*wprA*, Δ*fliD*, Δ*alp*, Δ*apr*, Δ*vpr*, Δ*asp* | this study |
| *E. coli* DH10B | (F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ($^{\varphi}$80d*lac*ZΔM15)Δ*lac*X74*end*A1 *rec*A1 *deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*Lλ$^-$ | Invitrogen |
| pSEC194 | Cm$^R$, Ap$^R$, thermosensitive integration vector | Crampton et al. (3) |
| pSECNC6 | pSEC194 containing FliC-NC6 fusion protein | Crampton et al. (3) |
| pSECNHIVC6 | pSECNC6 containing HIV-1 gp120 epitope as a fusion protein | Crampton et al. (3) |

**Inactivation of the *B. halodurans* BhFC01 major cell wall protease gene (*wprA*).** Due to the high sequence homology between the genomes of *B halodurans* Alk36 and *B halodurans* C-125 primers were designed according to the sequenced genome of *B halodurans* C-125 which is published in the DNA Data Bank of Japan (http://www.gib.genes.nig.ac.jp). The cell wall protease gene (*wprA*) is located at position 2202614 to 2205772 on the *B halodurans* C-125 genome. A defective *wprA* gene was created by designing primers in order to amplify two fragments of the *wprA* gene by PCR amplification. These fragments contained part of the N-terminal (primers wprA1F/wprA1R) and C-terminal (primers wprA2F/wprA2R) regions of

the *wprA* gene. The vector pSEC194 was digested with *Sac*I/*Xba*I, ligated to both fragments and transformed into *E. coli* DH10B to create the plasmid pSECwprA containing the defective *wprA* gene. The defective *wprA* gene was integrated into the *B halodurans* BhFC01 chromosome. The method used for integration was a combination of two different methods by Biswas et al.. (2) and Poncet et al. (15). Putative single crossover (sco) colonies were screened with primers M13F and WprAChrR. One of the N-terminal sco clones was used to force a double crossover (dco) event. PCR amplification with primers WprAChrF and WprAChrR proved that the dco event did occur. This strain was named *B halodurans* BhFC04 (Δ*hag*, Δ*wprA*).

**Inactivation of the *fliD* gene on chromosome of *B halodurans* BhFC04:** The *fliD* gene is located at position 3717115 to 3718713 on the *B halodurans* C-125 genome. A defective *fliD* gene was constructed by designing primers in order to amplify two fragments of the *fliD* gene by PCR amplification. These were 1.5 kb and 0.989 kb respectively and contained part of the N-terminal (primers $\sigma^D$Kpn/MC120805) and C-terminal (primers FliDCF2/FliDCR2) regions of the *fliD* gene. The vector pSEC194 was digested with *Kpn*I /*Hind*II, ligated to both fragments and transformed into *E. coli* DH10B to create the plasmid pSECFliD containing the defective *fliD* gene. This plasmid was then transformed into *B halodurans* BhFC04 and integrated via a dco event as described in previous section. This strain was named *B halodurans* BhFD01 (Δ*hag*, Δ*wprA* Δ*fliD*).

1 TABLE 2. List of primers and their corresponding nucleotide sequences

2

| Primer name | Nucleotide sequence[a] | Restriction site |
|---|---|---|
| $\sigma^D$Kpn | 5’CTCGGTACCCTCGCGTTACGCTCTTTCTGT3’ | *Kpn*I |
| MC120805 | 5’CGAGGATCCCGTATTTAAAGAGGAACGTAA3’ | *Bam*HI |
| FliDCF2 | 5’CGAGGATCCCGAGCAGTGATTACAGATTG3’ | *Bam*HI |
| FliDCR2 | 5’CGACCCGGGCAGAGAGCTCATTATGCTTCTC3’ | *Sma*I |
| M13F | 5’TGACCGGCAGCAAAATG3’ | |
| WprA1-F | 5’GCGAGCTCTGCAGCGTACTACAACCA3’ | *Sac*I |
| WprA1-R | 5’GCGGATCCAGCTGATAACGCTACGTA3’ | *Bam*HI |
| WprA2-F | 5’GCGGATCCTAGCGGACCTGTAGATGT3’ | *Bam*HI |
| WprA2-R | 5’GGTCTAGATGCCTTGTCCTTCGCTGTA3’ | *Xba*I |
| WprAChrR | 5’CGACACCCTCTTTACGTATAGTC3’ | |
| WprAChrF | 5’ - CAGCTCGATACGGAGCGG - 3’ | |
| alp1F | 5’CTTGGTACCGCGTGGGAATGTTGCA3’ | *Kpn*I |
| alp1R | 5’CTTGGATCCTGCACTTCTACCGCTGAG3’ | *Bam*HI |
| alp2F | 5’CTTGGATCCGGCTTCACCTCATGTGA3’ | *Bam*HI |
| alp2R | 5’CTCCCGGGTGGTTGTCACAGCAGCGG3’ | *Sma*I |
| apr1F | 5’GCAGGTACCGTTGGTGTTCAAGATGTTTACG3’ | *Kpn*I |
| apr1R | 5’CAGGATCCAGGCGTTGCTTGAGACGTACCA3 | *Bam*HI |
| apr2F | 5’GCAGGATCCGGACAGGAAGCGAACCTCAAG3’ | *Bam*HI |
| apr2R | 5’GCACCCGGGCAAGTCCTAGAGTACAATAAC3’ | *Sma*I |
| vpr1F | 5' CGTGTTACCGATGTGTAGTGCCTTATC3’ | *Kpn*I |
| vpr1Rev2 | 5' CTTGGATCCTTCATACGTCTCGCCATCGAG3' | *Bam*HI |

| | | |
|---|---|---|
| vpr2For | 5’CGTGGATCCCGAAGGTACGATCATCGTA3’ | *Bam*HI |
| vpr2Rev2 | 5'GTCCCGGGAAGCACGAGTGGATTCATGGTATA3' | *Sma*I |
| asp1F | 5’GTAGGTACCCTCGATGCGAAAGTTCTCGATG3’ | *Kpn*I |
| asp1R | 5’GCAGGATCCGTACCAGCCACGTGAGTTCCG3’ | *Bam*HI |
| asp2F | 5’GCAGGATCCGCTAGATACTCTGGTGTTATGG3’ | *Bam*HI |
| asp2R | 5’GATCCCGGGCCTCCTATCATACCCAAATGAG | *Sma*I |
| FliCup | 5’CGAGGTACCAGGAGTTTGTCCTTCTG 3 | *Kpn*I |
| VCF6 | 5’CACGTCGACTCGAGCCCGGGATGGATCCAGAAT | *Sal*I,*Xho*I,*Sma*I |
| | GCACAATCAGCTATTGAC3’ | *Bam*HI |
| VNR6 | 5’GACGTCGACAGTGTGGTCAGTAATATCCTC3’ | *Sal*I |
| FliDNR3 | 5’CGAGTCAACAAGACCGGCAGAGTTAATGTC3’ | *Hind*II |

[a] Restriction enzyme sites underlined

**Construction of extra-cellular protease deficient strains.**

All the defective protease gene constructs used for inactivation of the respective proteases were constructed as two fragments containing part of the N- and C-terminal regions of the gene of interest. The two fragments were digested with *Kpn*I/*Bam*HI and *Bam*HI/*Sma*I respectively and ligated in a three way ligation to the thermo-sensitive vector pSEC194 digested with *Kpn*I/*Hind*II and transformed into *E. coli* DH10B to obtain a plasmid construct containing a defective copy of the protease gene. These constructs were then transformed into the relevant *B halodurans* strains and integrated into the chromosome via a dco event as described in the previous section. PCR analysis was used to confirm the event. All primers used are listed in Table 2.

In order to create the protease defective construct *B halodurans* BhFD02 the *alp* protease gene located at position 740001 to 741119 on the *B halodurans* C-125 genome was

inactivated. PCR amplification of the N-terminal (primers alp1F/alp1R) and C-terminal (primers alp2F/alp2R) regions of the *alp* gene gave rise to the defective *alp* gene fragments which ligated to vector pSEC194 created the plasmid pSECalp which was integrated into the *B halodurans* BhFD01 chromosome to create BhFD02.

Construction of *B halodurans* BhFD03 led to inactivation of the prepro-alkaline protease (*apr*) gene located at position 751087 to 753465 on the *B halodurans* C125 genome. PCR amplification of the N-terminal (primers apr1F/apr1R) and C-terminal (primers apr2F/apr2R) regions gave rise to the defective *apr* gene fragments which ligated to vector pSEC194 created the plasmid pSECapr which was integrated into the *B halodurans* BhFD02 chromosome to create BhFD03.

Inactivation of the *vpr* gene located at position 905382 to 902983 on the *B halodurans* C125 genome created *B halodurans* BhFD04. PCR amplification of the N-terminal (primers vpr1F/vpr1R) and C-terminal (primers vpr2F/vpr2R) regions gave rise to the defective *vpr* gene fragments which ligated to vector pSEC194 created the plasmid pSECvpr which was integrated into the *B halodurans* BhFD03 chromosome to create BhFD04.

Inactivation of the extracellular alkaline serine protease gene designated *asp* located at position 927497 to 928582 on the *B halodurans* C125 genome created *B halodurans* BhFD05. PCR amplification of the N-terminal (primers asp1F/asp1R) and C-terminal (primers asp2F/asp2R) regions gave rise to the defective *asp* gene fragments which ligated to vector pSEC194 created the plasmid pSECasp which was integrated into the *B halodurans* BhFD04 chromosome to create BhFD05.

**Evaluation of type III secretion efficiency among the different *B halodurans* mutant strains using an antigenic peptide derived from the HIV gp 120 V3 loop as a model construct.**

The pSECNHIVC6 construct containing the HIV subtype C gp 120 V3 loop antigenic peptide of 27 amino acids (7) was fused as an in-frame sandwich fusion to the flagellin protein gene and used as a model construct in order to determine secretion efficiency among the different protease deficient mutant strains.

**Precipitation of secreted proteins and preparation of cell lysates.**

Cultures were grown at 30ºC in 30 ml LB (pH 8.5 or pH 7.6). The cells were precipitated and the supernatant collected. An equal volume of 10% TCA (trichloroacetic acid) was added to the supernatant and incubated on ice for 30 minutes. The precipitate was pelleted at 15 000 X *g* for 10 minutes. The dry pellet was resuspended in 500 µl Tris buffer (pH 9.0). Samples (20 µl/lane) were evaluated on 10% SDS-PAGE gels

**Purification of FliC-NC6 monomers.**

*B. halodurans* (pSECNC6) was grown to an $OD_{600}$ of 3. Flagellin (FliC-NC6) fusion monomers were purified from crude culture supernatants on an ÄKTA FPLC system using a Toyopearl 650M strong anion exchange resin. The FliC-NC6 monomers were eluted off the column using an increasing NaCl gradient from 0-0.5 M in 20 mM sodium phosphate buffer, pH 7.4. The FliC-NC6 containing fractions were collected and protein precipitation was performed with 10% TCA as described above.

**One-dimensional SDS-PAGE and protease zymography.**

Protein electrophoresis was performed in a vertical gel electrophoresis system (10 cm x 10 cm x 0.1 cm; CBS Scientific) employing the Tris-glycine system (11). Protein samples (~20 μg) were loaded per well. The molecular mass marker used is the prestained PageRuler #SM1811 (Fermentas, St Leon-Rot, Germany). Following electrophoresis, the gels were stained using colloidal Coomassie Blue G-250 gel stain (8).

For protease zymograms the different protease deficient strains were grown into stationary phase (24 hours) in LB pH 8.5 at 37$^{o}$C. Cells were precipitated and the supernatants (30 ml) were concentrated via ultrafiltration (10 kDa cut-off) to 0.5 ml. The concentrated samples were analysed for protease activity on zymograms containing either gelatin (1 mg/ml) or purified FliC-NC6 monomers (100 μg/ml) as substrate in the resolving gel. Following electrophoresis, gels were washed 2 x 30 min in 1% Triton X-100 to remove the SDS and incubated in 0.1 M glycine, pH 8.5 for 3.5 hours at 37$^{o}$C. The gels were subsequently stained for at least 1 hour in amido black solution (0.1% amido black in 30% methanol/10% acetic acid). The gels were destained in 30% methanol/10% acetic acid until the background was sufficiently reduced to indicate white clearing bands of proteolytic activity on a blue-black background.

**Directed proteomics: bioinformatics-based identification of extra-cellular proteases.**

The gel region corresponding to proteolytic activity was excised from the zymogram and sent to the Fingerprints Tryptic peptides were separated on a high resolution nano-liquid chromatography system (Dionex Ultimate 3000) and peptide sequence information obtained using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific). Sequence information

was then searched using a local Mascot (Matrix Science) search engine against the NCBInr database for identification.

**Control for cell lysis**

The possibility of cell lysis was monitored by detecting the release of isocitrate dehydrogenase (ICDH), a NADP-linked cytoplasmic marker enzyme, into the supernatant fluid as described by Coxon et al. (4).

## RESULTS

**Secretion of heterologous peptides by the modified type III secretion pathway of *B halodurans.***

Successful inactivation of the *fliD* gene was achieved and verified by PCR analysis giving rise to *B halodurans* strain BhFD01. This strain also contained an inactivated *wprA* cell wall protease gene. In an attempt to improve the stability of the secreted heterologous peptides two other protease genes were inactivated on the chromosome, namely *alp* and *apr*. This gave rise to strains BhFD02 and BhFD03. These strains were evaluated for the expression of the HIV antigenic peptide in both log and stationary phase (Fig. 1).

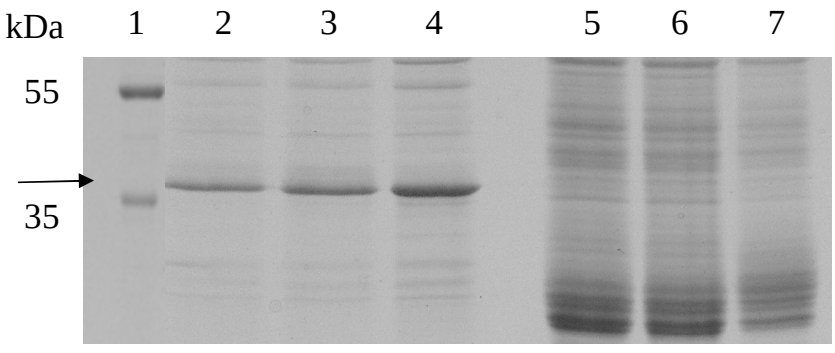


FIG. 1. HIV fusion peptide being secreted in log ($OD_{600}$ 2) and stationary phase ($OD_{600}$ 4) from different *B halodurans* genetic backgrounds. Lanes 2-4 contains log phase and lanes 5-7

stationary phase cultures. Lanes 1, molecular mass marker (Fermentas); 2, BhFD01; 3, BhFD02; 4, BhFD03; lane 5, BhFD01; lane 6, BhFD02 and lane 7, BhFD03. The arrow indicates the HIV antigenic fusion peptide.

As strain BhFD03 showed no improvement with regard to stability of secreted peptides the *vpr* protease gene was inactivated giving rise to strain BhFD04. The proteolytic activity in the supernatants of stationary phase BhFD03 and BhFD04 strains was monitored by evaluating extra-cellular samples from both strains on a gelatine zymogram (Fig. 2).

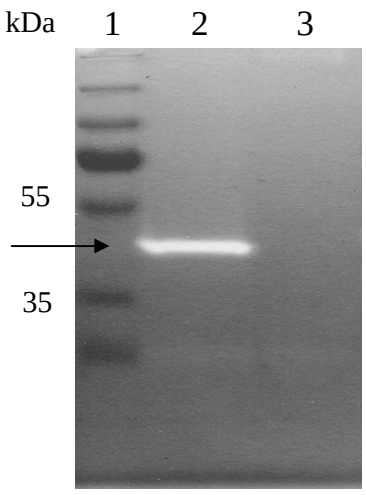


FIG. 2. Zymographic analysis of the protease profile of strains BhFD03 and BhFD04**.** Extra-cellular samples from stationary phase cultures $OD_{600}$ of 4. were tested for proteolytic activity on a SDS-PAGE gel containing gelatine as substrate. Lanes 1, molecular mass marker (Fermentas); 2, BhFD03 and 3, BhFD04. The arrow indicates the zone of proteolytic activity corresponding to the active protease present in strain BhFD03.

As can be seen by the zymogram (Fig. 2) a zone of proteolytic activity of approximately 45 kDa disappeared with the inactivation of the *vpr* gene in strain BhFD04. This was smaller

than the predicted molecular weight of 88 kDa as deduced from the amino acid protein sequence. This, however, corresponds to the findings of Sloma et al.. (1991) who observed a similar major difference between the expected and actual size of the purified Vpr protein from *Bacillus subtilis.* They attributed the size differences to the presence of a prosequence as well as possible C-terminal processing or proteolysis.

In order to minimize the effect of proteolytic activity prevalent at the higher pH range a strategy was followed whereby the different *B. halodurans* strains were grown at a lower pH which is sub-optimal for alkaline protease activity. The different mutant strains were then compared with regard to their ability to secrete the HIV antigenic fusion peptide during stationary phase when grown at pH 7.6 (Fig. 3).

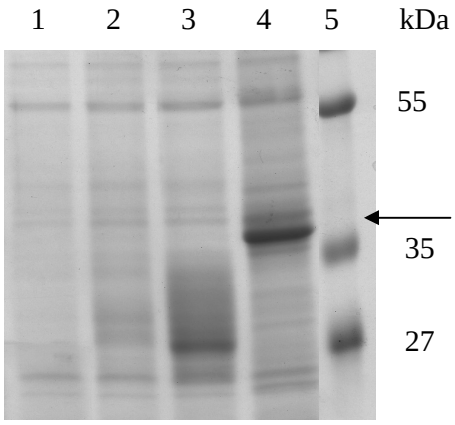


FIG. 3. Comparison of different genetic backgrounds secreting HIV antigenic fusion peptide at pH 7.6 during stationary phase at $OD_{600}$ 4**.** Lanes 1, BhFD01; 2, BhFD02; 3, BhFD03, 4, BhFD04 and lane 5, molecular mass marker (Fermentas). The arrow indicates the HIV antigenic fusion peptide.

It can clearly be seen that the stability of the HIV antigenic fusion peptide in the supernatant was greatly enhanced by the BhFD04 strain when grown into stationary phase at pH 7.6. At pH 8.5 this peptide was, however, completely degraded (data not shown). This indicated the presence of at least one other protease targeting the heterologous fusion peptides secreted by *B. halodurans.*

**Identification of key proteases affecting the stability of secreted heterologous peptides.**

In order to identify the protease(s) involved in the degradation of the flagellin-fusion peptides secreted by BhFD04, a flagellin protein zymogram was developed by incorporating purified FliC-NC6 fusion monomers as substrate. A zone indicating enzyme activity was identified at approximately 22 kDa (Fig. 4). The purified FliC migrated in the resolving gel to 36 kDa (size of FliC monomers) making it impossible to determine whether any proteases were present above 36 kDa. This was overcome by repeating the zymography experiment with an agarose overlay (containing FliC-NC6 as substrate in polymerised agarose) From this overlay, no other zones of proteolytic activity were observed above 36 kDa (results not shown). The gel region between 18 kDa and 25 kDa was excised from a preparative gel and all proteins were identified in that region using 1-D nano LC-MS/MS. The most likely protease candidate was selected based on database search scores, molecular weights similar to the protease activity region around 22 kDa as seen on the zymogram and presence of homologues on the *B halodurans* C-125 genome database. The identified protease was an extra-cellular alkaline serine protease, designated *asp.* The gene was located at position 927497 to 928582 on the *B halodurans* C-125 genome database. This protease was subsequently inactivated on the *B halodurans* genome giving rise to strain BhFD05.

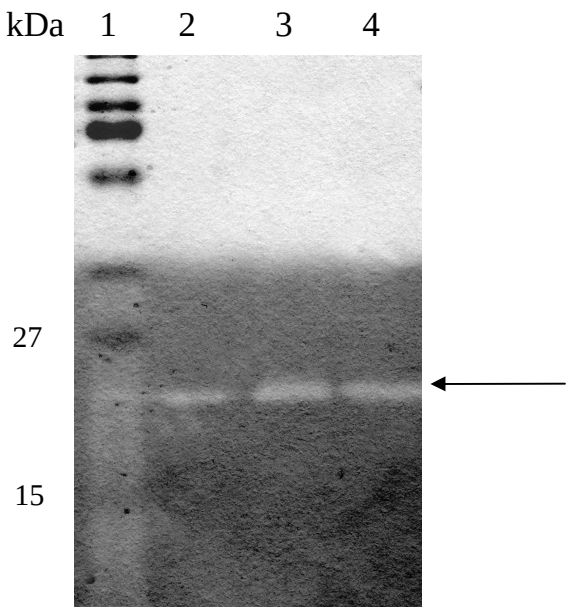


FIG. 4. Protease zymogram containing FliC-NC6 monomers as substrate. Extra-cellular samples from stationary phase cultures were tested for proteolytic activity. Lanes 1, molecular mass marker (Fermentas); 2, BhFC01; 3, BhFD03: and 4, BhFD04. The arrow indicates the zone of proteolytic activity.

The different strains were then evaluated for their ability to secrete the HIV antigenic peptide flagellin fusions in stationary phase at pH 8.5. The supernatants were run on a SDS-PAGE gel and compared for peptide expression (Fig. 5).

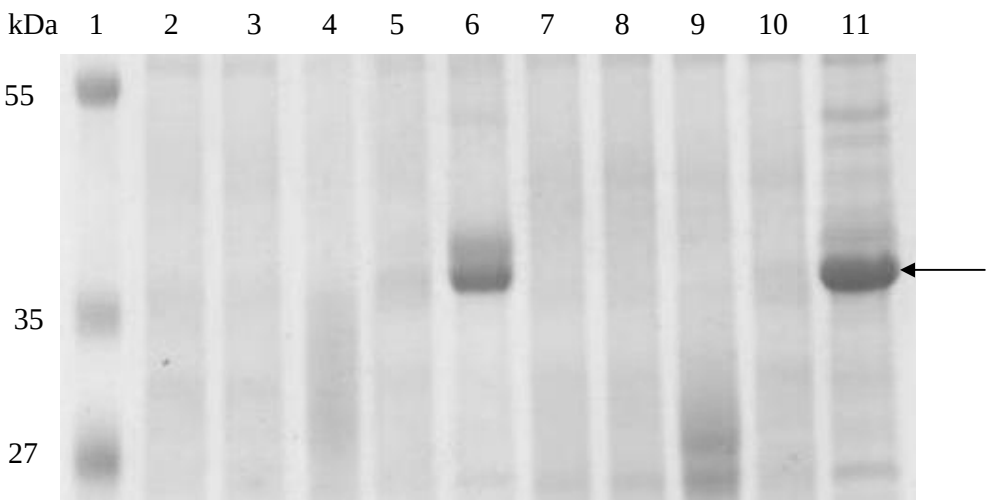

FIG. 5. Comparison of different genetic backgrounds secreting HIV antigenic fusion peptide at pH 8.5 during stationary phase. Samples were taken at different $OD_{600}$: lanes 2-6 ($OD_{600}$ 2), lanes, 7-11 ($OD_{600}$ 5). . Lane 1, molecular mass marker (Fermentas); Lanes 2 and 7, strain BhFD01; lanes 3 and 8, BhFD02; lanes 4 and 9, BhFD03; lanes 5 and 10, BhFD04 and lanes 6 and 11, BhFD05. The arrow indicates the HIV antigenic fusion peptide.

As can be seen from Fig. 5 the inactivation of the *asp* protease was found to significantly improve the stability of the secreted HIV antigenic fusion peptide in the culture medium when grown to stationary phase at pH 8.5.

## DISCUSSION

The flagellum-specific type III export system of *B. halodurans* Alk36 was modified for the secretion of heterologous flagellin fusion peptides by inactivation of the *fliD* gene. However, degradation of the fusion peptides occurred during stationary phase growth due to proteolytic activity. An important limiting factor for the use of *B subtilis* and related strains for the production of heterologous proteins is the expression of several extra-cellular proteases into the growth medium which degrade the secreted heterologous proteins (19). The synthesis of extra-cellular proteases in *Bacillus* is tightly regulated and induction usually occurs following the transition from the exponential to the stationary phase of growth except for *wprA* which is expressed constitutively in exponentially growing cells and up-regulated during stationary phase (18). A strategy to circumvent the proteolytic degradation of recombinant proteins expressed by *B subtilis* focussed on the generation of *B subtilis* strains defective in 6 proteases (21), 7 proteases (23) and 8 proteases (22). A similar strategy was followed for stabilizing the secreted recombinant fusion peptides produced by *B. halodurans* Alk36. As *B. halodurans* is

alkaliphilic it does not harbour metallo-proteases and therefore homologues to the key alkaline protease genes as inactivated on the *B subtilis* genome resulting in the creation of *B subtilis* WB800 were identified. However, a combined inactivation of the *apr* (homologue of *epr*), *alp* (homologue of *aprE*) and *wprA* genes did not significantly improve stability of recombinant peptides in the medium. In creating strain *B. halodurans* BhFD04 with the inactivation of the *vpr* protease, increased stability of the fusion peptide was achieved during stationary phase growth at pH 7.6. However, this pH is not optimum for growth of *B. halodurans* and it became apparent at a higher pH that proteolytic activity was still problematic. Through a combination of zymography and bioinformatics the *asp* protease was identified which showed specific activity against flagellin-fusion monomers. The *asp* gene (another homologue of the *B subtilis aprE* gene) was subsequently inactivated giving rise to strain *B. halodurans* BhFD05. This strain showed significantly improved stability of recombinant fusion peptides in the supernatant at alkaline pH and during stationary phase growth. It has been reported that engineered strains of *B subtilis* deficient in extra-cellular proteases show increased susceptibility to cellular lysis (19). When peptide expression studies were carried out over a twenty-four hour time period with *B. halodurans* BhFD05 no decrease in optical density was measured and the intra-cellular enzyme marker isocitrate dehydrogenase (ICDH) could not be detected in the supernatant. Harwood et al. (6) reported that ICDH is relatively refractile to the activity of *B. subtilis* proteases and was used successfully as reliable indicator of cellular lysis.

The flagellar type III secretion system developed in *E. coli* by Majander et al. (13) was shown to secrete heterologous polypeptides in amounts and purity that are sufficient for biotechnological applications. Our system shows a similar profile in that the expressed HIV antigenic fusion peptide constitutes approximately 50% of total secreted protein, as

determined by fluorescence densitometry analysis using Sypro® Ruby protein staining. A comparative study would, however, need to be carried out in order to compare expression efficiencies. The *B. halodurans* BhFC01 strain has been successfully engineered for type III secretion of flagellin fusion peptides resulting in strain BhFD05. Future work will now focus on evaluating the expression of a range of peptides in order to validate the system. As reported by Majander et al. (13) there is a significant difference in the efficiencies of polypeptides secreted by *E. coli.* Some polypeptides were expressed at high concentrations intra-cellularly, but were not secreted while others were secreted at concentrations which ranged from 1 to 15 mg/L. In addition, heterologous promoters can be evaluated in the *B. halodurans* system and flagellum-specific export signals identified as described in *Salmonella* (20) and in *E. coli* (13). An interesting aspect of both the *Salmonella* and the *B subtilis* type III secretion systems (20, 17) is that their host strains are described as flagellin deficient but apparently still possess a functional gene encoding the flagellar capping protein FliD, which should in fact substantially hinder secretion (14). When the construct pSECNHIVC6 is expressed in either *B. halodurans* BhFC01 or BhFC04 (both of which contain a functional FliD) surface display is achieved as documented by Crampton et al. (3) as the fusion peptide monomers are polymerised into flagella and remain attached to the cell wall.

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