The use of *Bacillus subtilis* as a cost-effective expression system for production of Cholera Toxin B fused factor VIII epitope regions applicable for inducing oral immune tolerance

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Abstract

Coagulation factor replacement therapy for the X-linked bleeding disorder Haemophilia, characterized by a deficiency of coagulation protein factor VIII (FVIII), is severely complicated by antibody (inhibitors) formation. The development of FVIII inhibitors drastically alters the quality of life of the patients and is associated with a tremendous increase in morbidity as well as treatment costs. The ultimate goal of inhibitor control is antibody elimination. Immune tolerance induction (ITI) is the only clinically established approach for developing antigen-specific tolerance to FVIII. This work aims to establish a novel cost-effective strategy to produce FVIII molecules in fusion with cholera toxin B (CTB) subunit at the N terminus using the *Bacillus subtilis* expression system for oral tolerance, as the current clinical immune tolerance protocols are expensive. Regions of B-Domain Deleted (BDD)-FVIII that have potential epitopes were identified by employing Bepipred linear epitope prediction; 2 or more epitopes in each domain were combined and cDNA encoding these regions were fused with CTB and cloned in the *Bacillus subtilis* expression vector pHT43 and expression analysis was carried out. The expressed CTB-fused FVIII epitope domains showed strong binding affinity towards the CTB-receptor GM1 ganglioside. To conclude, *Bacillus subtilis* expressing FVIII molecules might be a promising candidate for exploring for the induction of oral immune tolerance.

Keywords

Haemophilia A; Factor VIII; Epitopes; *Bacillus subtilis*; Ganglioside receptor(GM1); Cholera Toxin B; Oral Tolerance

Introduction

Haemophilia A is an X-linked bleeding disorder characterized by a deficiency of coagulation protein factor VIII (FVIII). Individuals with severe haemophilia A are in danger of sudden and potentially fatal bleeding. To prevent and cure bleeding episodes, the current standard of care and the cornerstone of haemophilia management is intravenous (IV) infusions of plasma-derived or recombinant factor VIII concentrate (Batsuli et al. 2016; Volkers et al. 2019). FVIII is made as a single polypeptide with a signal peptide having 19 amino acids plus a 2332 amino acid. The six different domains that make up the structure of FVIII are arranged in the following order: A1-a1-A2-a2-B-a3-A3-C1-C2. FVIII undergoes glycosylation and at least two intracellular cleavages of the B domain before being released as a heterodimer. As a result, circulating FVIII is made up of two non-covalently linked chains: a heavy chain with A1-A2-B domains and a light chain with A3-C1-C2 domains (Chavin 1984; Lenting et al. 1998; Shen et al. 2008; Kosloski et al. 2009). The B domain is dispensable for FVIII to have its full pro-coagulant activity; as a result, the most recent recombinant FVIII molecules created for therapeutic use are B domain-deleted FVIII (BDD- FVIII) molecules.

The production of neutralizing alloantibodies against the FVIII protein, known as inhibitors which occurs in 30% of patients with severe haemophilia A and 5% of patients with mild or moderate haemophilia A, is a difficult consequence of therapeutic FVIII infusions (Mancuso and Cannavò 2015; Berntorp 2023). The development of FVIII inhibitors has a significant impact on patient quality of life and is associated with increased morbidity and treatment costs. Currently, the primary clinical strategy for reversing inhibitors is to administer substantial amounts of FVIII intravenously regularly for months to years (Dimichele 2012; Franchini and Mannucci 2012). Even though this immunological tolerance induction (ITI) is successful in 60–70% of patients, it has drawbacks, including the requirement for a central catheter for frequent venous access, experiencing anamnesis (inhibitor reappearance) with repeated exposure to FVIII and the extremely high costs and long duration associated with the use of factor products (Sherman et al. 2017; Schep et al. 2018). To date, there is no immunological tolerance prevention strategy clinically available that could prevent haemophilia A patients from developing inhibitors.

However, a few methods have recently been tested in preclinical haemophilia A models to establish tolerance to FVIII, avoid or delay the onset of the anti-FVIII immune response, or reduce its amplitude. These strategies include the use of monoclonal antibodies, immunological modulatory drugs, FVIII bypassing agents such as prothrombotic complexes, and viral vector-based gene therapy, among others (Moghimi et al. 2011; Scott et al. 2013; Sack et al. 2014; Sarkar et al. 2014; Kim et al. 2015; Mimoun et al. 2023). Oral tolerance, on the other hand, might be a more widely accepted method of inducing prophylactic tolerance and may be easier to evaluate in clinical studies (Wang et al. 2013; Herzog et al. 2017; Tordesillas and Berin 2018). A wide range of antigens, such as food proteins and components of commensal bacteria, are regularly exposed to the gut immune system. Significantly, the gut immune system has developed strictly controlled mechanisms to control unintended or needless inflammatory reactions while still protecting from harmful germs (Hardet and Mingozzi 2017; Sricharunrat et al. 2018; Pinheiro-Rosa et al. 2021). However, the expenses of antigen manufacturing and the requirement to shield the antigen against degradation by stomach acidity and proteolytic degradation hamper the translation of this strategy while ensuring effective distribution to the gut-associated immune system. To overcome these hurdles, Henry Daniell and co-workers have developed an oral tolerance protocol to FVIII and FIX (Factor IX) based on the delivery of fusion proteins that are bio-encapsulated in transgenic plant cells (Sherman et al. 2014; Wang et al. 2015a; Kwon et al. 2018). However, the amount of FVIII produced by plants may not be compatible with the cost-effective translation to human beings. Thus, there is a need for a cost-effective alternative system for delivering these molecules.

Cholera toxin B subunit (CTB) is a non-toxic pentameric part of cholera toxin that binds to monosialotetrahexosylganglioside (GM1 – ganglioside) receptor. The GM1 ganglioside receptor, a glycolipid is expressed in most mammalian cells, including epithelial cells and antigen – presenting cells (APCs)(Sánchez and Holmgren 2008; Chunfeng et al. 2013). The GM1-ganglioside receptor helps absorbing and presenting the toxin to the immune system. Fusion of CTB to therapeutic proteins facilitates their effective oral delivery for induction of oral tolerance or delivers functional proteins to

sera or as an adjuvant to accelerate protective immunity(Ruhlman et al. 2007; Stratmann 2015; Xiao et al. 2016)

Bacillus subtilis is a unique probiotic and an excellent bio-control bacterium that is non-pathogenic and is classified as "Generally recognized as safe" (GRAS). It is a popular vehicle for heterologous antigen production and protective immunization (Mou et al. 2016; Li et al. 2019). Recombinant live vector vaccines using *Bacillus subtilis* strains have been used to deliver antigens orally in the form of spores or vegetative cells. In addition to its simplicity in growing, it can produce heterologous proteins effectively in comparison to other delivery vehicles. In reality, it is a common host choice in the synthesis of industrial enzymes because it grows quickly in a basic nutritional media, secretes significant amounts of heterologous proteins into the culture medium, and is highly cost-effective (Wickramasuriya et al. 2021; Shafaati et al. 2022). Although *Escherichia coli* is commonly utilized to produce heterologous proteins, coliform lipopolysaccharide (LPS) contamination is always a concern. *Bacillus* species, unlike *E. coli*, are gram-positive bacteria and so do not contain LPS (Amuguni and Tzipori 2012; Jiang et al. 2019; Neef et al. 2021) *B. subtilis* has unusual resistance capabilities and can live in harsh environments such as freezing temperatures, desiccation, and chemical exposure. This property, in addition to making *B. subtilis* strains easy to transport and store, makes them an attractive vehicle for the transfer of heterologous antigens to harsh settings such as the gastrointestinal tract (Hu et al. 2011; Zhang et al. 2020).

In this study, recombinant *B. subtilis* strain was used to express Cholera Toxin B (CTB) subunit-fused potential epitopes of each fragment viz., A_1, A_2, A_3 & $C_{1,2}$ (C_1+ C_2) domains of BDD-FVIII protein; binding of the fusion proteins to ganglioside receptor (GM1) was evaluated. The aim was to develop an alternate cost-effective strategy to produce recombinant FVIII molecules in a prokaryotic system which might be used for the induction of immune tolerance in haemophillic mice.

Materials and methods

Bacterial strains and Plasmids

In this experimental study, the complete sequence of CTB was obtained from the NCBI database. Sequences of FVIII epitopes from each domain were identified through the Linear Prediction method and more than 2 or more continuous epitopes were fused to obtain a reasonable length of polypeptide which is amenable for expression, essentially cDNA constructs having (**C**TB (C) followed by individual fragments of FVIII with potential epitopes; viz. $F8CA_1$, $F8CA_2$, $F8CA_3$, $F8CC_{1,2}$. The cDNA constructs mentioned above were cloned in pUC57 vector and acquired from Genscript. *B. subtilis WB800N* (PBS002- MoBiTec, Germany) was used as the host for expression studies. Moreover, *Escherichia coli DH5α* (Invitrogen Inc.) was used for the construction and amplification of recombinant plasmids. pHT43 shuttle vector (PBS002- MoBiTec, Germany), containing ampicillin and chloramphenicol resistant gene, was used to express the CTB and FVIII fragments with epitopes.

Media composition and culture conditions

Bacterial strains of *E.coli* and *B.subtilis* were grown aerobically at 37°C and 200 rpm in Luria – Bertani (LB) culture medium containing 1% peptone, 0.5% yeast extract and 0.5% sodium chloride and SOC culture medium containing 2% tryptone, 0.5% yeast extract, 10nM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$ and 20mM glucose respectively. LB medium was supplemented with ampicillin 100µg/mL for *E.coli* and SOC medium with chloramphenicol 10µg/mL for *Bacillus*. 20% glycine, competency medium containing 2% LB and 0.5M sorbitol, and washing buffer containing 0.5M sorbitol, 50mM Mannitol, and 10% glycerol for *B.subtilis* competent cell preparation. 2X YT agar plates containing 1.6% tryptone, 1% yeast extract, and 0.5% NaCl were used for the transformation of *B.subtilis*.

Plasmid construction and transformation

FVIII epitope regions were identified using BDD-FVIII amino acid sequence by employing Bepipred linear epitope prediction tool 2.0 at the Immune Epitope Database (IEDB) Analysis Resource (*https://tools.iedb.org/bcell*). Fig.1a gives the flowchart for constructing the FVIII epitope regions.

The CTB cDNA and CTB-fused FVIII epitope regions ($F8CA_1$, $F8CA_2$, $F8CA_3$, $F8CC_{1,2}$) cDNA were codon optimized for expression in *B.subtilis* and synthesized by Genscript in pUC57 cloning vector.

F8CA$_1$, F8CA$_2$, F8CA$_3$, F8CC$_{1,2}$ constructs were engineered with a flexible peptide linker, GPGP (Gly-Pro-Gly-Pro) and a furin cleavage site, RRKR (Arg-Arg-Lys-Arg), between CTB and FVIII epitope regions as shown in Fig.1b. The coding sequences of CTB and CTB-FVIII epitope regions were double restriction digested by *BamHI* and *XbaI*, gel purified (Sigma-GenElute Gel extraction kit), ligated (T4 DNA *ligase*, NEB) in the *BamHI/XbaI* sites of pHT43 vector and transformed into DH5α. The recombinant *B.subtilis* expression plasmids (CTB-pHT43, F8CA$_1$-pHT43, F8CA$_2$-pHT43, F8CA$_3$-pHT43, and F8CC$_{1,2}$-pHT43) were propagated in *DH5α* and were confirmed by restriction digestion with *BamHI* and *XbaI* and sequencing.

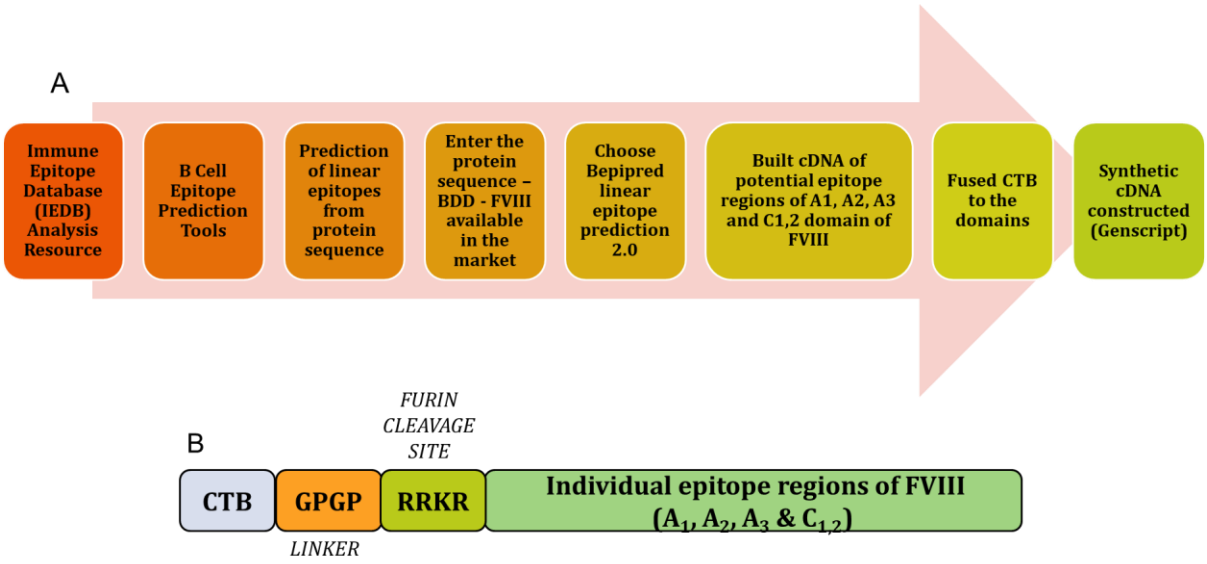

Fig.1 cDNA construction of CTB fused FVIII epitope regions (A) FVIII epitope regions (A$_1$, A$_2$, A$_3$, C$_{1,2}$) were identified using the Immune Epitope Database Analysis Resource tool by entering the BDD- FVIII protein sequence and choosing Bepipred linear epitope prediction 2.0 (B cell epitope prediction) and then fused with cholera toxin B subunit (CTB). **(B)** CTB-FVIII epitope regions were engineered with a flexible peptide linker, GPGP, and a furin cleavage site Arg-Arg-Lys-Arg, in between CTB and FVIII epitope regions

Finally, pHT43, CTB-pHT43, and CTB-FVIII epitope regions -pHT43 were transformed into *B.subtilis WB800N* by electroporation. Briefly, 50 ng of recombinant plasmid was gently mixed with 60 µL of *B.subtilis* competent cells for 10 min at 4°C. The mixture was then transferred into a pre-

cooled electroporation cuvette and subjected to a single electric pulse (22KV/cm, 5ms). A competency medium was added to the electroporated cells which were then cultured for 3h at 37°C/300rpm. The positive colonies were selected based on growth on 10µg/mL Chloramphenicol antibiotic agar 2X YT plates.

Expression of CTB and CTB-FVIII epitope regions by the recombinant *B.subtilis*

To detect expression of CTB and CTB-FVIII predicted epitope proteins, a single transformed colony was inoculated into SOC medium containing chloramphenicol (10µg/mL) and incubated overnight at 37°C/180 rpm. Fresh SOC medium was inoculated with the overnight culture to an initial cell density (OD_{600}) of 0.1. 1mM IPTG was added to the culture as it reached log-phase (OD_{600} 0.7-0.8) and then incubated for 8h and overnight. The cells were collected by centrifugation at 3000xg for 10min at 4°C and cells were disrupted by sonication. Both the supernatant and pellet were analyzed using 10-15% SDS PAGE. The proteins were then transferred onto a nitrocellulose membrane. Blocking was performed by incubation with 5% skimmed milk in TBS buffer and washed three times with TBST (TBS + Tween20). For immunodetection of the heterologous CTB and CTB fused fragments the membrane was incubated with 1:1000 dilution of polyclonal anti-CTB antibody (Abcam), followed by 1:2000 dilution of horseradish peroxidase (HRP) labelled goat anti-rabbit IgG. Binding was detected using DAB (3, 3'-Diaminobenzidine) substrate.

Quantification of CTB and GM1 – Ganglioside binding assay by ELISA

Specific CTB and CTB-fused epitope regions of FVIII were determined by enzyme-linked immunosorbent assay (ELISA). 96 well flat-bottomed plates (NUNC) were coated with expressed *B.subtilis* supernatant and pellet samples in carbonate buffer at 4°C overnight. After blocking with 5% Bovine Serum Albumin for 1h at 37°C the next day, the plates were incubated with 1:2500 dilution of the anti-CTB polyclonal antibody and subsequently incubated with 1:5000 dilution of HRP labelled goat anti-rabbit IgG. The plates were washed with PBST in between every step thrice. Colour was developed using a tetramethyl benzidine (TMB)/H_2O_2 substrate and the reaction was terminated using 2M H_2SO_4 and was read at 450nm.

To evaluate the binding affinity of the recombinant proteins, a GM1-specific ELISA was performed. GM1 (50 µL per well of 3 µg/mL) diluted with bicarbonate buffer was used to coat 96 well flat-bottomed plates (NUNC) at 4°C overnight. After blocking with 5% Bovine Serum Albumin for 1h at 37°C the next day, the plates were incubated with the expressed *B.subtilis* supernatant and pellet samples for 2h at 37°C, and then they were blocked again. The plates were incubated with a 1:2500 dilution of the anti-CTB polyclonal antibody and subsequently incubated with a 1:5000 dilution of HRP-labelled goat anti-rabbit IgG. The plates were washed with PBST in between every step thrice. Colour was developed using a TMB/H_2O_2 substrate and the reaction was terminated using 2M H_2SO_4 and was read at 450 nm.

Results

Our goal was to minimize the length of sequence that could be fused with CTB for the successful *Bacillus subtilis* expression system as FVIII is a lengthy polypeptide with each domain comprising of A1 (372 aa), A2 (368 aa), A3 (371 aa), C1 (153 aa) and C2 (159 aa). We identified the linear epitopes of BDD-FVIII by employing the online Bepipred linear epitope prediction tool 2.0 at the Immune Epitope Database (IEDB) Analysis Resource. The B cell epitope prediction tool was selected for predicting linear epitopes from the protein sequence provided. Fig.2a represents the linear structure of FVIII protein and Fig.2b represents the multiple sequence alignment of complete FVIII preproprotein with BDD-FVIII and predicted immune epitope regions (Bold and colored) taken into consideration for constructing the recombinant plasmids (F8CA$_1$, F8CA$_2$, F8CA$_3$, F8CC$_{1,2}$) to be expressed in *Bacillus subtilis* system.

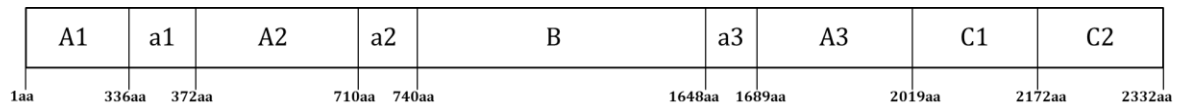

Fig.2a Complete FVIII protein with domains.

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            1                                                                                                                                                                                            180
Complete F8 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL GELPVDARFP PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMASDP LCLTYSYLSH
     BDD F8 MQIELSTCFF LCLLRFCFSA TRRYYLGAVE LSWDYMQSDL GELPVDARFP PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMASDP LCLTYSYLSH
      F8CA2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
   F8CC1,2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA1 .......... .......... .......... ........DL GELPVDARFP PRVPKSFPFN TSVVYKKTLF VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLK.. .......... .......... .......... .......... .......... .......... ..........
      F8CA3 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
            181                                                                                                                                                                                          360
Complete F8 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCPE EPQLRMKNNE
     BDD F8 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TTPEVHSIFL EGHTFLVRNH RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCPE EPQLRMKNNE
      F8CA2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
   F8CC1,2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA3 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
            361                                                                                                                                                                                          540
Complete F8 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPKT WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR PYNIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVED.G
     BDD F8 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPKT WVHYIAAEEE DWDYAPLVLA PDDRSYKSQY LNNGPQRIGR KYKKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR PYNIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDFG
      F8CA2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDFG
   F8CC1,2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA3 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
            541                                                                                                                                                                                          720
Complete F8 PTKSDPRCLT RYYSSFVNME RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVILFSVFD ENRSWYLTEN IQRFLPNPAG VQLEDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL SIGAQTDFLS VFFSGYTFKH KMVYEDTLTL FPFSGETVFM SMENPGLWIL GCHNSDFRNR
     BDD F8 PTKSDPRCLT RYYSSFVNME RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVILFSVFD ENRSWYLTEN IQRFLPNPAG VQLEDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL SIGAQTDFLS VFFSGYTFKH KMVYEDTLTL FPFSGETVFM SMENPGLWIL GCHNSDFRNR
      F8CA2 PTKSDPRCLT RYYSSFVNME RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVILFSVFD ENRSWYLTEN IQRFLPNPAG VQLEDPEFQA SNIMHSINGY VFDSLQLSVC LHEVAYWYIL S......... .......... .......... .......... .......... ..........
   F8CC1,2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA3 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
            721                                                                                                                                                                                          900
Complete F8 GMTALLKVSS CDKNTGDYYE DSYEDISAYL LSKNNAIEPR SFSQNSRHPS TRQKQFNATT IPENDIEKTD PWFAHRTPMP KIQNVSSSDL LMLLRQSPTP HGLSLSDLQE AKYETFSDDP SPGAIDSNNS LSEMTHFRPQ LHHSGDMVFT PESGLQLRLN EKLGTTAATE LKKLDFKVSS
     BDD F8 GMTALLKVSS CDKNTGDYYE DSYEDISAYL LSKNNAIEPR SFSQ...... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
   F8CC1,2 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA1 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
      F8CA3 .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
            901                                                                                                                                                                                         1080
Complete F8 TSNNLISTIP SDNLAAGTDN TSSLGPPSMP VHYDSQLDTT LFGKKSSPLT ESGGPLSLSE ENNDSKLLES GLMNSQESSW GKNVSSTESG RLFKGKRAHG PALLTKDNAL FKVSISLLKT NKTSNNSATN RKTHIDGPSL LIENSPSVWQ NILESDTEFK KVTPLIHDRM LMDKNATALR
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BDD F8  ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CC1,2 ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
        1081                                                                                                        1260
Complete F8  LNHMSNKTTS SKNMEMVQQK KEGPIPPDAQ NPDMSFFKML FLPESARWIQ RTHGKNSLNS GQGPSPKQLV SLGPEKSVEG QNFLSEKNKV VVGKGEFTKD VGLKEMVFPS SRNLFLTNLD NLHENNTHNQ EKKIQEEIEK KETLIQENVV LPQIHTVTGT KNFMKNLFLL STRQNVEGSY
BDD F8  ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CC1,2 ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
        1261                                                                                                        1440
Complete F8  DGAYAPVLQD FRSLNDSTNR TKKHTAHFSK KGEEENLEGL GNQTKQIVEK YACTTRISPN TSQQNFVTQR SKRALKQFRL PLEETELEKR IIVDDTSTQW SKNMKHLTPS TLTQIDYNEK EKGAITQSPL SDCLTRSHSI PQANRSPLPI AKVSSFPSIR PIYLTRVLFQ DNSSHLPAAS
BDD F8  ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CC1,2 ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
        1441                                                                                                        1620
Complete F8  YRKKDSGVQE SSHFLQGAKK NNLSLAILTL EMTGDQREVG SLGTSATNSV TYKKVENTVL PKPDLPKTSG KVELLPKVHI YQKDLFPTET SNGSPGHLDL VEGSLLQGTE GAIKWNEANR PGKVPFLRVA TESSAKTPSK LLDPLAWDNH YGTQIPKEEW KSQEKSPEKT AFKKKDTILS
BDD F8  ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CC1,2 ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
        1621                                                                                                        1800
Complete F8  LNACESNHAI AAINEGQNKP EIEVTWAKQG RTERLCSQNP PVLKRHQREI TRTTLQSDQE EIDYDDTISV EMKKEDFDIY DEDENQSPRS FQKKTRHYFI AAVERLWDYG MSSSPHVLRN RAQSGSVPQF KKVVFQEFTD GSFTQPLYRG ELNEHLGLLG PYIRAEVEDN IMVTFRNQAS
BDD F8  ..... ..... ..... .....QNP PVLKRHQREI TRTTLQSDQE EIDYDDTISV EMKKEDFDIY DEDENQSPRS FQKKTRHYFI AAVERLWDYG MSSSPHVLRN RAQSGSVPQF KKVVFQEFTD GSFTQPLYRG ELNEHLGLLG PYIRAEVEDN IMVTFRNQAS
F8CA2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CC1,2 ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
F8CA3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ...KTRHYFI AAVERLWDYG MSSSPHVLRN RAQSGSVPQF KKVVFQEFTD GSFTQPLYRG ELNEHLG... ..... .....
        1801                                                                                                        1980
Complete F8  RPYSFYSSLI SYEEDQRQGA EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD VDLEKDVHSG LIGPLLVCHT NTLNPAHGRQ VTVQEFALFF TIFDETKSWY FTENMERNCR APCNIQMEDP TFKENYRFHA INGYIMDTLP GLVMAQDQRI RWYLLSMGSN ENIHSIHFSG
BDD F8  RPYSFYSSLI SYEEDQRQGA EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD VDLEKDVHSG LIGPLLVCHT NTLNPAHGRQ VTVQEFALFF TIFDETKSWY FTENMERNCR APCNIQMEDP TFKENYRFHA INGYIMDTLP GLVMAQDQRI RWYLLSMGSN ENIHSIHFSG
F8CA2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
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  F8CC1,2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
    F8CA1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
    F8CA3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
           1981                                                                                                                                                                                        2160
Complete F8  HVFTVRKKEE YKMALYNLYP GVFETVEMLP SKAGIWRVEC LIGEHLHAGM STLFLVYSNK CQTPLGMASG HIRDFQITAS GQYGQWAPKL ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
   BDD F8  HVFTVRKKEE YKMALYNLYP GVFETVEMLP SKAGIWRVEC LIGEHLHAGM STLFLVYSNK CQTPLGMASG HIRDFQITAS GQYGQWAPKL ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
    F8CA2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
  F8CC1,2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......SIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
    F8CA1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
    F8CA3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
           2161                                                                                                                                                                                        2340
Complete F8  NPPIIARYIR LHPTHYSIRS TLRMELMGCD LNSCSMPLGM ESKAISDAQI TASSYFTNMF ATWSPSKARL HLQGRSNAWR PQVNNPKEWL QVDFQKTMKV TGVTTQGVKS LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV KVFQGNQDSF TPVVNSLDPP LLTRYLRIHP QSWVHQIALR
   BDD F8  NPPIIARYIR LHPTHYSIRS TLRMELMGCD LNSCSMPLGM ESKAISDAQI TASSYFTNMF ATWSPSKARL HLQGRSNAWR PQVNNPKEWL QVDFQKTMKV TGVTTQGVKS LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV KVFQGNQDSF TPVVNSLDPP LLTRYLRIHP QSWVHQIALR
    F8CA2  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
  F8CC1,2  NPPIIARYIR LHPTHYSIRS TLRMEL.GCD LNSCSMPLGM ESKAISDAQI TASSYFTNMF ATWSPSKARL HLQGRS.... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
    F8CA1  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
    F8CA3  .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
           2341       2352
Complete F8  MEVLGCEAQD LY........
   BDD F8  MEVLGCEAQD LY........
    F8CA2  .......... ..........
  F8CC1,2  .......... ..........
    F8CA1  .......... ..........
    F8CA3  .......... ..........
```

Fig.2b Aminoacid sequence of complete FVIII preproprotein, B domain deleted FVIII aligned with the predicted epitope regions. The amino acid sequences that have been differentially colored indicate the stretches with high epitope indexes as predicted by the Linear Bepipred prediction method. Each domain is colored differently and each highlighted region has 2 or more epitopes fused (Epitope index more than 1)

Construction of recombinant plasmids for expression in *B.subtilis*

The CTB–FVIII epitope regions were constructed with a flexible peptide linker, (GPGP), and a furin cleavage site, Arg-Arg-Lys-Arg, between CTB and FVIII epitope regions and were cloned downstream of the IPTG – inducible pPgrac promoter in pHT43 resulting in CTB-pHT43, $F8CA_1$-pHT43, $F8CA_2$-pHT43, $F8CA_3$-pHT43 and $F8CC_{1,2}$-pHT43 (Fig. 3A). The recombinant *B.subtilis* expression vectors mentioned above were propagated in *E.coli* strain DH5α and isolated using the Plasmid extraction kit (GenElute Plasmid DNA kit, Sigma). CTB and CTB-fused FVIII epitope regions cloned in pUC57 vector (codon optimized and synthesized by Genscript) were double digested with *BamHI* and *XbaI*, and the products were identified by agarose electrophoresis. Electrophoresis and sequencing results showed that the CTB and CTB–FVIII epitope regions were successfully inserted into *B.subtilis* expression vector pHT43 (Fig. 3B). The recombinant plasmids were then transformed into *B.subtilis* WB800N via electroporation and the positive colonies were selected on Chloramphenicol resistant 2XYT agar plate.

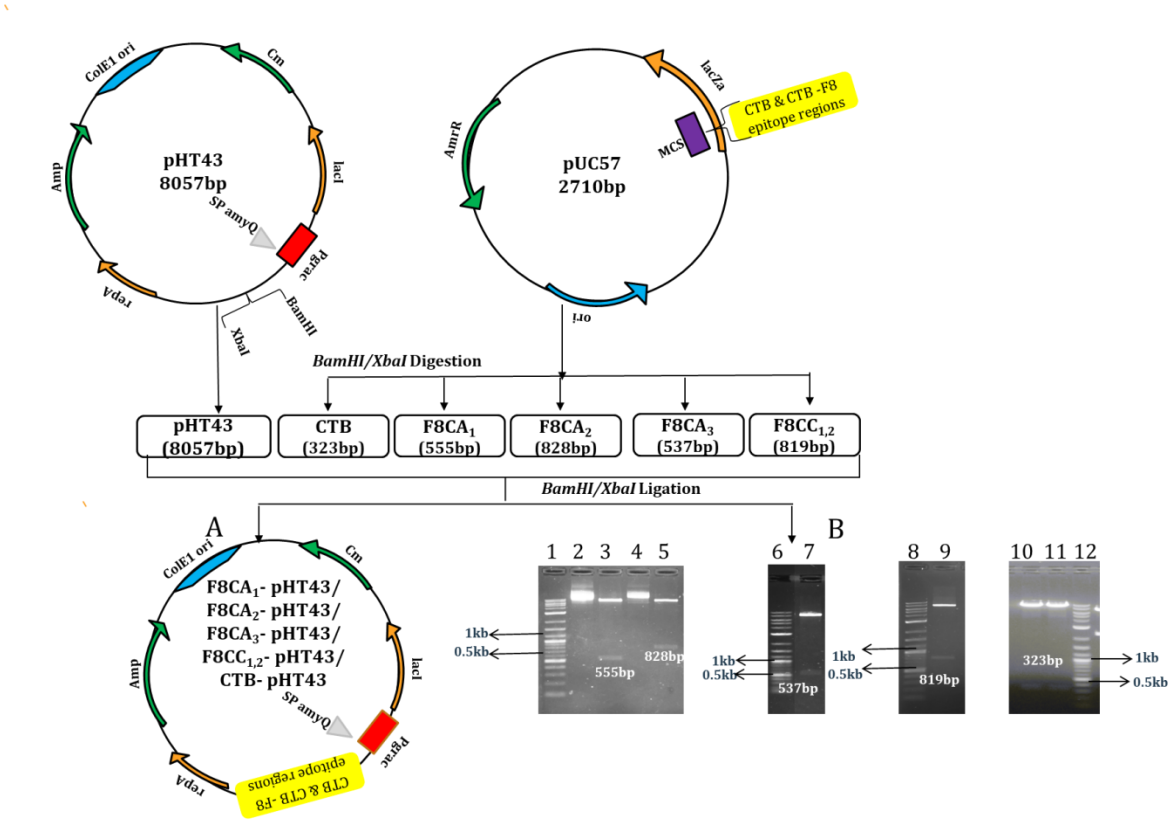
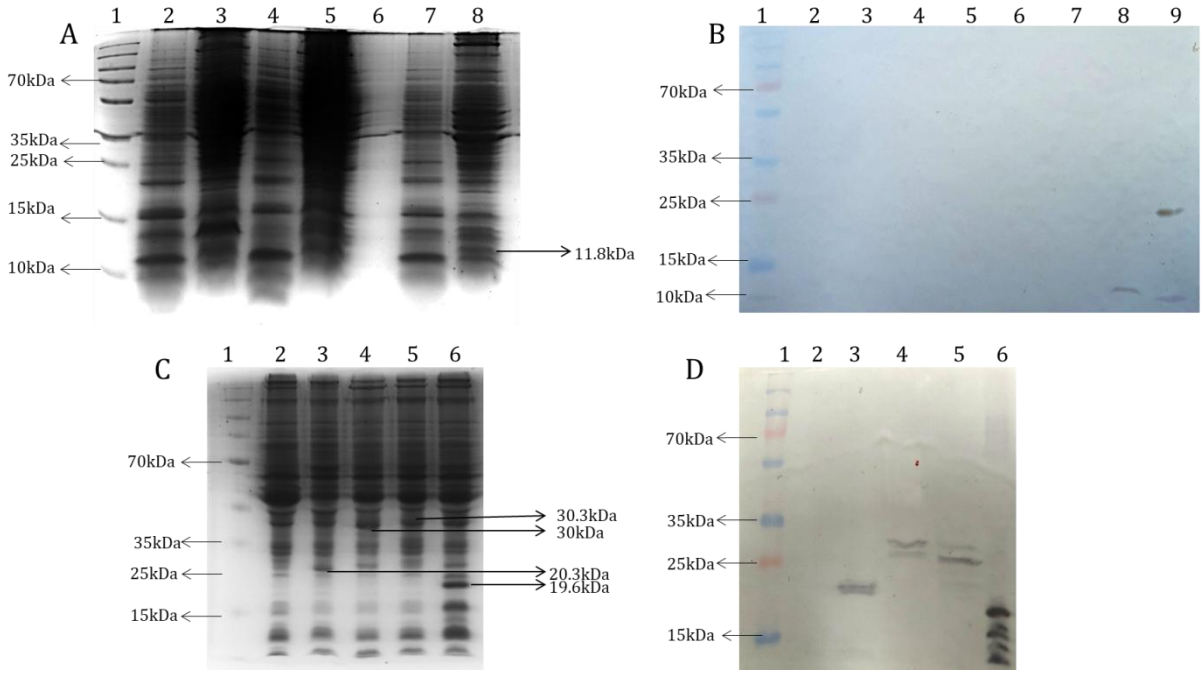


Fig.3 Schematic of pHT43-CTB / CTB–FVIII epitope regions ($F8CA_1$, $F8CA_2$, $F8CA_3$, $F8CC_{1,2}$) **(A)** CTB and CTB-fused FVIII epitope regions cloned in pUC57 were digested with *BamHI* and *XbaI* restriction enzymes and inserted into the pHT43 vector, generating 5 constructs of CTB-pHT43, $F8CA_1$-pHT43, $F8CA_2$-pHT43, $F8CA_3$-pHT43 and $F8CC_{1,2}$-pHT43. **(B)** Electrophoretogram of ligated plasmids double digested with *BamHI* and *XbaI*. Lanes 1,6,8,12 - 0.1kb - 10kb DNA ladder, Lane 2,4 – digested pHT43 vector, Lane 3,5,7,9,10-11 – digested $F8CA_1$-pHT43, $F8CA_2$-pHT43, $F8CA_3$-pHT43, $F8CC_{1,2}$-pHT43 and CTB-pHT43. (pHT 43 vector map – Pgrac : Pgrac promoter consisting of the groE promoter, the lacO operator, ColE1 ori : ColE1 origin replication, Amp : ampicillin resistance, lacI : lacI gene repressor, Cm : chloramphenicol resistance, SPamyQ : amylase Q signal peptide sequence. pUC57 vector map – ori : replication origin $oriV_{ColE1}$, lacZa : reporter gene, AmpR : ampicillin resistance, MCS : multiple cloning site)

Induced expression of CTB or CTB-fused epitope regions of FVIII domains in *B.subtilis WB800N*

After induction by IPTG, the expressed fusion proteins in pellet extract from recombinant *B.subtilis* strains were separated by SDS-PAGE under fully denatured and reducing conditions. The SDS-PAGE results showed the expression of the recombinant protein after overnight induction viz. CTB 11.8kDa (Fig. 4A), $F8CA_1$- 20.3kDa, $F8CA_2$- 30.3kDa, $F8CA_3$ – 19.6kDA and $F8CC_{1,2}$ – 30kDa (Fig. 4C). The results also show the expressed protein band in *B.subtilis* were approximately 11.8kDA (Fig. 4B), 20.3kDa, 30.3kDa, 19.6kDa and 30kDa in western blotting respectively (Fig. 4D). The western blot analysis was confirmed with anti-CTB polyclonal antibody, thereby confirming the expression of CTB and CTB-fused FVIII epitope regions.



354

355 **Fig.4 SDS-PAGE and western blot analysis of the recombinant CTB and CTB-FVIII epitope**
356 **regions in *Bacillus subtilis* WB800N (A)** SDS-PAGE analysis of rCTB in *WB800N* , Lane 1- 10kDa
357 to 250kDa Protein ladder, Lane 2- *WB800N* supernatant, Lane 3- *WB800N* pellet, Lane 4- rCTB
358 uninduced supernatant, Lane 5-rCTB uninduced pellet, Lane 6-Empty, Lane 7- rCTB induced
359 supernatant, Lane 8-rCTB induced pellet **(B)** Western Blot analysis of rCTB in *WB800N* , Lane 1-
360 10kDa to 250kDa Protein ladder, Lane 2- *WB800N* supernatant, Lane 3- *WB800N* pellet, Lane 4-
361 rCTB uninduced supernatant, Lane 5-rCTB uninduced pellet, Lane 6-Empty, Lane 7- rCTB induced
362 supernatant, Lane 8-rCTB induced pellet, Lane 9 – CTB standard 25ng. **(C)** SDS-PAGE analysis of
363 rCTB fused FVIII epitope regions in *WB800N* Lane 1- 10kDa to 250kDa Protein ladder, Lane 2-
364 *WB800N* pellet, Lane 3- rF8CA$_1$ pellet, Lane 4-rF8CA$_2$ pellet, Lane 5-rF8CC$_{1,2}$ pellet, Lane 6-
365 rF8CA$_3$ pellet **(D)** Western Blot analysis of rCTB fused FVIII epitope regions in *WB800N* Lane 1-
366 10kDa to 250kDa Protein ladder, Lane 2- *WB800N* pellet, Lane 3- rF8CA$_1$ pellet, Lane 4-rF8CA$_2$
367 pellet, Lane 5-rF8CC$_{1,2}$ pellet, Lane 6- rF8CA$_3$ pellet

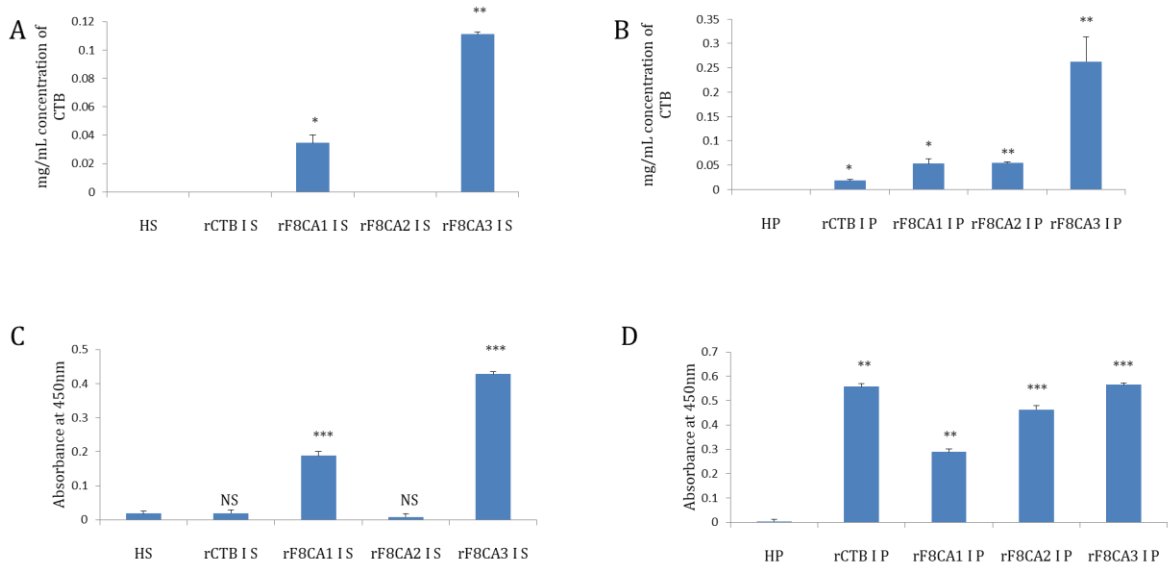
368 **Quantification of CTB and CTB-fused epitope regions of FVIII domains in *B.subtilis WB800N***

369 Specific CTB and CTB-fused epitope regions of FVIII were determined by enzyme-linked
370 immunosorbent assay (ELISA). The ELISA assay was performed with recombinant strains expressing

CTB and CTB-fused FVIII epitope regions (both supernatant and pellet) and quantified using CTB standard plot. Fig.5a and 5b represents the expression pattern of recombinant CTB and CTB-fused proteins. On the whole, rF8CA_3 shows better expression comparatively.

GM1 binding assay by ELISA

The plasma membrane receptor GM1-Ganglioside is the specific receptor for CTB *in vivo* and for appropriate receptor binding, a pentameric structure is required. Therefore, a GM1 binding assay using ELISA has been performed to ensure that the CTB and CTB-fused FVIII epitope regions are in the correct configuration *in vivo*. This ELISA assay was performed using 3µg/mL GM1 in bicarbonate buffer with 0.5mg of expressed CTB and CTB fused FVIII epitopes recombinant strains. The positive control used here was standard CTB (0.1µg) whereas the negative control is BSA (3µg) as the coating protein. As shown in Fig.5c and 5d, the absorbance of recombinant strains (both supernatant and pellet) were significantly higher than that of Wildtype (*WB800N*). The results confirmed that the interest proteins expressed by *B.subtilis* were able to fold successfully in the form of a pentamer with native conformation and the sites required for binding of the CTB pentamer to GM1 are conserved. Also rCTB and CTB fused F8A1, F8A2, F8A3 shows better affinity directly proportional to the expression of the recombinant proteins than F8C1,2 (not detectable) as evidenced by poor expression levels. There was an inconsistent binding of CTB-$C_{1,2}$ domains and the reasons for the same are not clear in our experiments. FVIII C1 and C2 domains are very hydrophobic in nature and may be poorly soluble. C2 domains are known to bind to membrane phospholipid Phosphatidyl-Serine. Both C1 and C2 domains are involved in endocytosis of FVIII after binding to membrane phospholipids. In addition, poor accessibility of GM1 for CTB binding could also be one of the probable reasons, we are unable to see the consistent binding. Hence, the data is not shown. It should also be noted that rCTB had the binding affinity to GM1 and not to BSA (Data not shown).



394

395 **Fig.5 Expression and pentamer formation of *B.subtilis* derived CTB and CTB-fused FVIII**
396 **epitope regions. (A)** Quantification of CTB and CTB fused FVIII epitope regions of supernatant in
397 *B.subtilis* strain expressing recombinant proteins. **(B)** Quantification of CTB and CTB fused FVIII
398 epitope regions of pellet in *B.subtilis* strain expressing recombinant proteins respectively**. (C)** The
399 GM1-dependent ELISA was performed for supernatant by coating the well of the microtiter plate with
400 GM1 or BSA to make sure that CTB fused FVIII epitope regions are in the correct configuration and
401 able to form pentameric structure *in vivo*. **(D)** The GM1-dependent ELISA was performed for pellet
402 by coating the well of the microtiter plate with GM1 or BSA to make sure that CTB fused FVIII
403 epitope regions are in the correct configuration and able to form pentameric structure *in vivo*. H:
404 *WB800N*; S: Supernatant; P: Pellet; I: Induced; r: Recombinant. Results were presented as average ±
405 SD. Asterisk symbol represents P value (* <0.05, **<0.01; ***<0.001) compared to the Host

406 **Discussion**

407 FVIII is a highly immunogenic molecule that, when administered at low antigen levels to
408 experimental animals and haemophilia A patients, can elicit strong antibody responses. The majority
409 of inhibitors bind to A2, A3, or C2 domains (Wang et al. 2015b). Animal studies indicate that
410 toleration to specific molecular components, such as the combination of the A2 and C2 domains; can
411 prevent the production of inhibitors against FVIII, whereas a single domain may not be enough

(Sherman et al. 2014). Sriram Krishnamoorthy and colleagues demonstrated in haemophilia A mice that repeated administration of rFVIII-fused with Fc portion IgG1 (rFVIIIFc) at therapeutically relevant doses significantly reduced antibody responses to rFVIII and also reduced FVIII inhibitors upon subsequent challenge with high doses of rFVIIIFc (Krishnamoorthy et al. 2016). After appropriate studies for safety and efficacy, the latter strategy may aid in the development of innovative approaches to prevent inhibitor formation in haemophilia A in the future. However, the expenses of generating therapeutic rFVIIIFc with high yields will be a daunting task, further increasing the costs of the existing treatment, which is already very expensive. Recent work performed in FVIII-deficient mice by Lacroix-Desmazes and colleagues demonstrated that immune tolerance to FVIII may be induced during the intra-utero stage and last long enough to cover the most critical part for FVIII inhibitor development. Immunodominant A2 and C2 domains of FVIII fused to the mouse Fcγ1 were generated, and co-injected into pregnant FVIII-deficient mice with mouse monoclonal IgG1 as a control group. The offspring were then treated with therapeutic FVIII at 7 weeks of age. The anti-FVIII immune response in the offspring was 10 times lower in A2Fc/C2Fc when compared to IgG1 control and proliferation of splenic T cells to FVIII was significantly reduced, suggesting induction of Tregs which was later confirmed by adoptive transfer experiments (Batsuli et al. 2016). Alternatively, Henry Daniell and colleagues demonstrated that prevention of FVIII inhibitor development could be achieved through oral delivery of transplastomic plants expressing recombinant FVIII fused with cholera toxin B subunit. The codelivery of the heavy chain and C2 domain of FVIII was sufficient to suppress inhibitor formation against the entire FVIII molecule proving that efficient induction of $FoxP3^+$ and Lap^+ Treg may provide sufficient suppression so that not all epitopes have to be covered by the orally delivered antigens (Sherman et al. 2014; Kwon et al. 2018). While this approach is highly promising to control the formation of inhibitors, it faces limitations such as general low levels of expression of FVIII by plant cells and might not be compatible with a cost-effective translation of the approach in human beings. With this in mind, we ventured to demonstrate a novel cost-effective strategy to produce epitope regions of FVIII as cholera toxin B subunit fusion proteins in *Bacillus subtilis* which has a strong binding affinity to GM1 ganglioside.

This study successfully used the *Bacillus* expression system to express the CTB-fused FVIII epitope regions using an engineered host strain *WB800N* that has higher competency to uptake foreign DNA molecules. These predicted epitope regions were also part of the T cell epitopes involved in the immune response of forming inhibitors predominantly to intravenous FVIII in conventional haemophilic E17 mice (Steinitz et al. 2012). N-terminal CTB-fused FVIII epitope regions were successfully cloned in a pHT43 vector containing Pgrac strong promoter and signal peptide amy-Q gene. This expression system has desirable features such as unbiased codon usage and prevents the formation of inclusion bodies with no endotoxin, making it a good platform for the production of our recombinant proteins (Amuguni and Tzipori 2012; Souza et al. 2021). With this in mind, the *B.subtilis* expression system was chosen to express the epitope regions of the highly complex molecule FVIII. Cholera Toxin has a potent mucosal adjuvant activity and can also function as a carrier molecule with many potential applications in cell biology. When conjugated with various antigens, the non-toxic component of the cholera toxin B (CTB) subunit is known for its adjuvant properties. The adjuvant effect of CTB is dependent on the binding of the pentameric form to GM1 gangliosides present on intestinal cellular surfaces. It has been shown to induce oral tolerance against co-administered foreign antigens in some autoimmune and allergic diseases and thus serves as an effective mucosal carrier molecule for autoantigens (Sun et al. 1994, 2010; Baldauf et al. 2015; Stratmann 2015). The expression vector constructed in this study includes the recognition sequence between CTB and FVIII epitope regions for the ubiquitous protease furin. Currently, the cleavage site motif is Arg-Arg-Lys-Arg. Insertion of this cleavage site between CTB and epitope regions of FVIII allows for effective cleavage and release of FVIII upon feeding the fusion proteins, as they will cross the stomach and reach the digestive track where, by virtue of binding to GM1 through CTB moiety, will be endocytosed by epithelial cells. The linker GPGP was also added to direct the protein folding. As shown in the GM1-ELISA binding assay, the *B.subtilis* strains producing different CTB-fused FVIII epitope regions demonstrated a strong affinity for GM1 ganglioside but not for BSA suggesting that these CTB fused FVIII epitopes are highly specific.

Conclusion

The main goal of the study was to develop a cost-effective strategy to produce FVIII fragments that could be easily scalable to obtain high yields in a relatively short time. *Bacillus* expression system overcomes major limitations of protein production by eliminating expensive media composition, elaborate purification strategies, cold storage, transportation, and importantly inclusion bodies and endotoxin production which is a major disadvantage of using the *E.coli* expression system. Oral delivery of *Bacillus subtilis* recombinant strain proteins are emerging as an effective approach due to its probiotic nature lacking pathogenicity and has shown to be effective as a recombinant vehicle system. This study opens the door for exploring the *Bacillus subtilis* expression system for producing recombinant proteins which might be useful in oral immune tolerance studies.

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Author Contributions VEV conceived the ideas, carried out literature surveys, executed experiments, and wrote, read, and edited the manuscript. MAV conceived ideas, read, wrote and edited the manuscript. SLD conceived ideas, read, wrote and edited the manuscript. KV conceived and provided the idea for the article, carried out literature survey, and wrote, read, edited, and communicated the manuscript to the journal.

Data availability Data is available on request from the authors

Statements and Declarations

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Competing Interest The authors declare no competing interests

Consent for Publication All authors have consented to publish the paper

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