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

3 Vijay Elakkya Vijayakumar,¹ Mookambeswaran A.Vijayalakshmi,¹ Sebastien Lacroix-Desmazes,²
4 Krishnan Venkataraman^{1*}

5 ¹Centre for Bio-Separation Technology (CBST), Vellore Institute of Technology (VIT), Vellore,
6 Tamil Nadu, 632014, India ; ²Institut National de la Santé et de la Recherche Médicale, Centre de
7 Recherche des Cordeliers, CNRS, Sorbonne Université, Université de Paris, F-75006 Paris, France

8 *Corresponding Author

9 Dr Krishnan Venkataraman

10 Professor and Director

11 Centre for BioSeparation Technology (CBST)

12 Vellore Institute of Technology (VIT)

13 Vellore-632014

14 bmkrishna1@yahoo.com, krishnan.v@vit.ac.in

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22 **Abstract**

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

38 **Keywords**

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

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46 **Introduction**

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

60 The production of neutralizing alloantibodies against the FVIII protein, known as inhibitors which
61 occurs in 30% of patients with severe haemophilia A and 5% of patients with mild or moderate
62 haemophilia A, is a difficult consequence of therapeutic FVIII infusions (Mancuso and Cannavò
63 2015; Berntorp 2023). The development of FVIII inhibitors has a significant impact on patient quality
64 of life and is associated with increased morbidity and treatment costs. Currently, the primary clinical
65 strategy for reversing inhibitors is to administer substantial amounts of FVIII intravenously regularly
66 for months to years (Dimichele 2012; Franchini and Mannucci 2012). Even though this
67 immunological tolerance induction (ITI) is successful in 60–70% of patients, it has drawbacks,
68 including the requirement for a central catheter for frequent venous access, experiencing anamnesis
69 (inhibitor reappearance) with repeated exposure to FVIII and the extremely high costs and long
70 duration associated with the use of factor products (Sherman et al. 2017; Schep et al. 2018). To date,
71 there is no immunological tolerance prevention strategy clinically available that could prevent
72 haemophilia A patients from developing inhibitors.

73 However, a few methods have recently been tested in preclinical haemophilia A models to establish
74 tolerance to FVIII, avoid or delay the onset of the anti-FVIII immune response, or reduce its
75 amplitude. These strategies include the use of monoclonal antibodies, immunological modulatory
76 drugs, FVIII bypassing agents such as prothrombotic complexes, and viral vector-based gene therapy,
77 among others (Moghimi et al. 2011; Scott et al. 2013; Sack et al. 2014; Sarkar et al. 2014; Kim et al.
78 2015; Mimoun et al. 2023). Oral tolerance, on the other hand, might be a more widely accepted
79 method of inducing prophylactic tolerance and may be easier to evaluate in clinical studies (Wang et
80 al. 2013; Herzog et al. 2017; Tordesillas and Berin 2018). A wide range of antigens, such as food
81 proteins and components of commensal bacteria, are regularly exposed to the gut immune system.
82 Significantly, the gut immune system has developed strictly controlled mechanisms to control
83 unintended or needless inflammatory reactions while still protecting from harmful germs (Hardet and
84 Mingozi 2017; Sricharunrat et al. 2018; Pinheiro-Rosa et al. 2021). However, the expenses of
85 antigen manufacturing and the requirement to shield the antigen against degradation by stomach
86 acidity and proteolytic degradation hamper the translation of this strategy while ensuring effective
87 distribution to the gut-associated immune system. To overcome these hurdles, Henry Daniell and co-
88 workers have developed an oral tolerance protocol to FVIII and FIX (Factor IX) based on the delivery
89 of fusion proteins that are bio-encapsulated in transgenic plant cells (Sherman et al. 2014; Wang et al.
90 2015a; Kwon et al. 2018). However, the amount of FVIII produced by plants may not be compatible
91 with the cost-effective translation to human beings. Thus, there is a need for a cost-effective
92 alternative system for delivering these molecules.

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

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

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

117 In this study, recombinant *B. subtilis* strain was used to express Cholera Toxin B (CTB) subunit-fused
118 potential epitopes of each fragment viz., A₁, A₂, A₃ & C_{1,2} (C₁+ C₂) domains of BDD-FVIII protein;
119 binding of the fusion proteins to ganglioside receptor (GM1) was evaluated. The aim was to develop
120 an alternate cost-effective strategy to produce recombinant FVIII molecules in a prokaryotic system
121 which might be used for the induction of immune tolerance in haemophilic mice.

122 **Materials and methods**

123 **Bacterial strains and Plasmids**

124 In this experimental study, the complete sequence of CTB was obtained from the NCBI database.
125 Sequences of FVIII epitopes from each domain were identified through the Linear Prediction method
126 and more than 2 or more continuous epitopes were fused to obtain a reasonable length of polypeptide
127 which is amenable for expression, essentially cDNA constructs having (CTB (C) followed by
128 individual fragments of FVIII with potential epitopes; viz. F8CA₁, F8CA₂, F8CA₃, F8CC_{1,2}. The
129 cDNA constructs mentioned above were cloned in pUC57 vector and acquired from Genscript. *B.*
130 *subtilis* WB800N (PBS002- MoBiTec, Germany) was used as the host for expression studies.
131 Moreover, *Escherichia coli* DH5 α (Invitrogen Inc.) was used for the construction and amplification of
132 recombinant plasmids. pHT43 shuttle vector (PBS002- MoBiTec, Germany), containing ampicillin
133 and chloramphenicol resistant gene, was used to express the CTB and FVIII fragments with epitopes.

134 **Media composition and culture conditions**

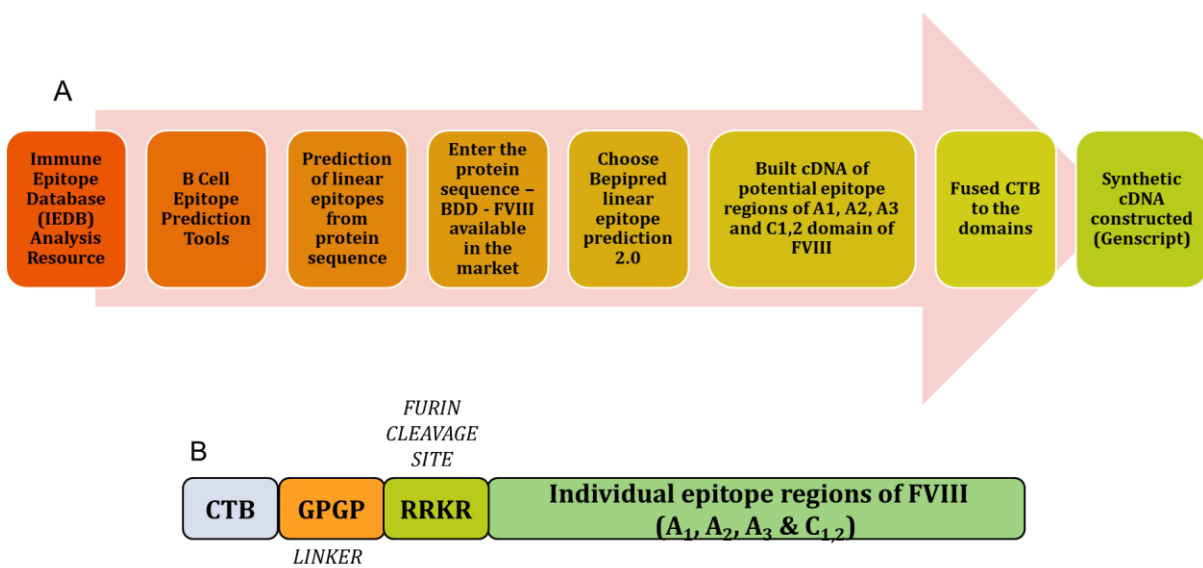
135 Bacterial strains of *E.coli* and *B.subtilis* were grown aerobically at 37°C and 200 rpm in Luria –
136 Bertani (LB) culture medium containing 1% peptone, 0.5% yeast extract and 0.5% sodium chloride
137 and SOC culture medium containing 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl,
138 10mM MgCl₂, 10mM MgSO₄ and 20mM glucose respectively. LB medium was supplemented with
139 ampicillin 100 μ g/mL for *E.coli* and SOC medium with chloramphenicol 10 μ g/mL for *Bacillus*. 20%
140 glycine, competency medium containing 2% LB and 0.5M sorbitol, and washing buffer containing
141 0.5M sorbitol, 50mM Mannitol, and 10% glycerol for *B.subtilis* competent cell preparation. 2X YT
142 agar plates containing 1.6% tryptone, 1% yeast extract, and 0.5% NaCl were used for the
143 transformation of *B.subtilis*.

144 **Plasmid construction and transformation**

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

148 The CTB cDNA and CTB-fused FVIII epitope regions (F8CA₁, F8CA₂, F8CA₃, F8CC_{1,2}) cDNA were
149 codon optimized for expression in *B.subtilis* and synthesized by Genscript in pUC57 cloning vector.

150 F8CA₁, F8CA₂, F8CA₃, F8CC_{1,2} constructs were engineered with a flexible peptide linker, GPGP
 151 (Gly-Pro-Gly-Pro) and a furin cleavage site, RRKR (Arg-Arg-Lys-Arg), between CTB and FVIII
 152 epitope regions as shown in Fig.1b. The coding sequences of CTB and CTB-FVIII epitope regions
 153 were double restriction digested by *Bam*HI and *Xba*I, gel purified (Sigma-GenElute Gel extraction
 154 kit), ligated (T4 DNA *ligase*, NEB) in the *Bam*HI/*Xba*I sites of pHT43 vector and transformed into
 155 DH5 α . The recombinant *B.subtilis* expression plasmids (CTB-pHT43, F8CA₁-pHT43, F8CA₂-pHT43,
 156 F8CA₃-pHT43, and F8CC_{1,2}-pHT43) were propagated in DH5 α and were confirmed by restriction
 157 digestion with *Bam*HI and *Xba*I and sequencing.



158

159 **Fig.1 cDNA construction of CTB fused FVIII epitope regions** (A) FVIII epitope regions (A₁, A₂,
 160 A₃, C_{1,2}) were identified using the Immune Epitope Database Analysis Resource tool by entering the
 161 BDD- FVIII protein sequence and choosing Bepipred linear epitope prediction 2.0 (B cell epitope
 162 prediction) and then fused with cholera toxin B subunit (CTB). (B) CTB-FVIII epitope regions were
 163 engineered with a flexible peptide linker, GPGP, and a furin cleavage site Arg-Arg-Lys-Arg, in
 164 between CTB and FVIII epitope regions

165 Finally, pHT43, CTB-pHT43, and CTB-FVIII epitope regions -pHT43 were transformed into
 166 *B.subtilis* WB800N by electroporation. Briefly, 50 ng of recombinant plasmid was gently mixed with
 167 60 μ L of *B.subtilis* competent cells for 10 min at 4°C. The mixture was then transferred into a pre-

168 cooled electroporation cuvette and subjected to a single electric pulse (22KV/cm, 5ms). A
169 competency medium was added to the electroporated cells which were then cultured for 3h at
170 37°C/300rpm. The positive colonies were selected based on growth on 10µg/mL Chloramphenicol
171 antibiotic agar 2X YT plates.

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

173 To detect expression of CTB and CTB-FVIII predicted epitope proteins, a single transformed colony
174 was inoculated into SOC medium containing chloramphenicol (10µg/mL) and incubated overnight at
175 37°C/180 rpm. Fresh SOC medium was inoculated with the overnight culture to an initial cell density
176 (OD₆₀₀) of 0.1. 1mM IPTG was added to the culture as it reached log-phase (OD₆₀₀ 0.7-0.8) and then
177 incubated for 8h and overnight. The cells were collected by centrifugation at 3000xg for 10min at 4°C
178 and cells were disrupted by sonication. Both the supernatant and pellet were analyzed using 10-15%
179 SDS PAGE. The proteins were then transferred onto a nitrocellulose membrane. Blocking was
180 performed by incubation with 5% skimmed milk in TBS buffer and washed three times with TBST
181 (TBS + Tween20). For immunodetection of the heterologous CTB and CTB fused fragments the
182 membrane was incubated with 1:1000 dilution of polyclonal anti-CTB antibody (Abcam), followed by
183 1:2000 dilution of horseradish peroxidase (HRP) labelled goat anti-rabbit IgG. Binding was detected
184 using DAB (3, 3'-Diaminobenzidine) substrate.

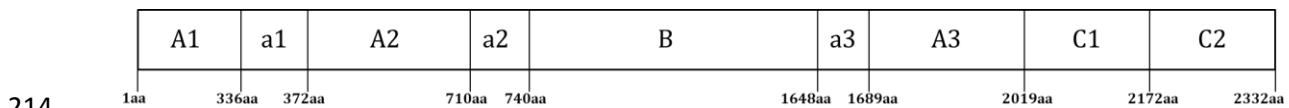
185 **Quantification of CTB and GM1 – Ganglioside binding assay by ELISA**

186 Specific CTB and CTB-fused epitope regions of FVIII were determined by enzyme-linked
187 immunosorbent assay (ELISA). 96 well flat-bottomed plates (NUNC) were coated with expressed
188 *B.subtilis* supernatant and pellet samples in carbonate buffer at 4°C overnight. After blocking with 5%
189 Bovine Serum Albumin for 1h at 37°C the next day, the plates were incubated with 1:2500 dilution of
190 the anti-CTB polyclonal antibody and subsequently incubated with 1:5000 dilution of HRP labelled
191 goat anti-rabbit IgG. The plates were washed with PBST in between every step thrice. Colour was
192 developed using a tetramethyl benzidine (TMB)/H₂O₂ substrate and the reaction was terminated using
193 2M H₂SO₄ and was read at 450nm.

194 To evaluate the binding affinity of the recombinant proteins, a GM1-specific ELISA was performed.
 195 GM1 (50 μ L per well of 3 μ g/mL) diluted with bicarbonate buffer was used to coat 96 well flat-
 196 bottomed plates (NUNC) at 4°C overnight. After blocking with 5% Bovine Serum Albumin for 1h at
 197 37°C the next day, the plates were incubated with the expressed *B.subtilis* supernatant and pellet
 198 samples for 2h at 37°C, and then they were blocked again. The plates were incubated with a 1:2500
 199 dilution of the anti-CTB polyclonal antibody and subsequently incubated with a 1:5000 dilution of
 200 HRP-labelled goat anti-rabbit IgG. The plates were washed with PBST in between every step thrice.
 201 Colour was developed using a TMB/H₂O₂ substrate and the reaction was terminated using 2M H₂SO₄
 202 and was read at 450 nm.

203 **Results**

204 Our goal was to minimize the length of sequence that could be fused with CTB for the successful
 205 *Bacillus subtilis* expression system as FVIII is a lengthy polypeptide with each domain comprising of
 206 A1 (372 aa), A2 (368 aa), A3 (371 aa), C1 (153 aa) and C2 (159 aa). We identified the linear epitopes
 207 of BDD-FVIII by employing the online Bepipred linear epitope prediction tool 2.0 at the Immune
 208 Epitope Database (IEDB) Analysis Resource. The B cell epitope prediction tool was selected for
 209 predicting linear epitopes from the protein sequence provided. Fig.2a represents the linear structure of
 210 FVIII protein and Fig.2b represents the multiple sequence alignment of complete FVIII preproprotein
 211 with BDD-FVIII and predicted immune epitope regions (Bold and colored) taken into consideration
 212 for constructing the recombinant plasmids (F8CA₁, F8CA₂, F8CA₃, F8CC_{1,2}) to be expressed in
 213 *Bacillus subtilis* system.



215 **Fig.2a Complete FVIII protein with domains.**

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216 1 180
217 Complete F8 MQIELSTCFF LCLLRFCFSA TRRYYLGAWE LSWDYMQSDL GELPVDARFP PRVPKSPFPN TSVVYKKTLE VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMSDF LCLTYSYLSH
218 BDD F8 MQIELSTCFF LCLLRFCFSA TRRYYLGAWE LSWDYMQSDL GELPVDARFP PRVPKSPFPN TSVVYKKTLE VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLKNM ASHPVSLHAV GVSYWKASEG AEYDDQTSQR EKEDDKVFPG GSHTYVWQVL KENGPMSDF LCLTYSYLSH
219 F8CA2 .....
220 F8CC1,2 .....
221 F8CA1 ..... DL GELPVDARFP PRVPKSPFPN TSVVYKKTLE VEFTDHLFNI AKPRPPWMGL LGPTIQAEVY DTVVITLK .....
222 F8CA3 .....
223 181 360
224 Complete F8 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TPPEVHSIFL EGHFTFLVRNH RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCEP EPQLRMKNNE
225 BDD F8 VDLVKDLNSG LIGALLVCRE GSLAKEKTQT LHKFILLFAV FDEGKSWHSE TKNSLMQDRD AASARAWPKM HTVNGYVNRS LPGLIGCHRK SVYWHVIGMG TPPEVHSIFL EGHFTFLVRNH RQASLEISPI TFLTAQTLLM DLGQFLLFCH ISSHQHDGME AYVKVDSCEP EPQLRMKNNE
226 F8CA2 .....
227 F8CC1,2 .....
228 F8CA1 .....
229 F8CA3 .....
230 361 540
231 Complete F8 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPTK WHYIAAEEE DWYAPLVA PDRRSYKSQY LNNQPQRIGR KYKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR PNYIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDFG
232 BDD F8 EAEDYDDDLT DSEMDVVRFD DDNSPSFIQI RSVAKKHPTK WHYIAAEEE DWYAPLVA PDRRSYKSQY LNNQPQRIGR KYKVRFMAY TDETFKTREA IQHESGILGP LLYGEVGDTL LIIFKNQASR PNYIYPHGIT DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDFG
233 F8CA2 ..... DVRPLYSRRL PKGVKHLKDF PILPGEIFKY KWTVTVEDFG .....
234 F8CC1,2 .....
235 F8CA1 .....
236 F8CA3 .....
237 541 720
238 Complete F8 PTKSDPRCLT RYSSSFVNME RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVLFSVFD ENRSWYLTEN IQRFLPNPAG VQLEDPEFQA SNIMHSINGY VFDSLQSLVC LHEVAYWYIL SIGAQTDFLS VFFSGYTFKH KMYVEDTLTL PPFSGETVFM SMENPGLWIL GCHNSDFRNR
239 BDD F8 PTKSDPRCLT RYSSSFVNME RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVLFSVFD ENRSWYLTEN IQRFLPNPAG VQLEDPEFQA SNIMHSINGY VFDSLQSLVC LHEVAYWYIL SIGAQTDFLS VFFSGYTFKH KMYVEDTLTL PPFSGETVFM SMENPGLWIL GCHNSDFRNR
240 F8CA2 PTKSDPRCLT RYSSSFVNME RDLASGLIGP LLICYKESVD QRGNQIMSDK RNVLFSVFD ENRSWYLTEN IQRFLPNPAG VQLEDPEFQA SNIMHSINGY VFDSLQSLVC LHEVAYWYIL S .....
241 F8CC1,2 .....
242 F8CA1 .....
243 F8CA3 .....
244 721 900
245 Complete F8 GMTALLKVSS CDKNTGDYWE DSYEDISAYL LSKNNAIEPR SFSQNSRHPS TRQKQFNATT IPENDIEKTD PWFARHTPMP KIQNVSSSDL LMLLRQSPTP HGLSLSDLQE AKYETFSDDP SPGAIDSNNS LSEMTHFRPQ LHHSGDMVFT PESGLQLRLN EKLGTAAATB LKKLDFKVVSS
246 BDD F8 GMTALLKVSS CDKNTGDYWE DSYEDISAYL LSKNNAIEPR SFSQ.....
247 F8CA2 .....
248 F8CC1,2 .....
249 F8CA1 .....
250 F8CA3 .....
251 901 1080
252 Complete F8 TSNNLISTIP SDNLAAGTDN TSSLGPPSMP VHYDSQLDTT LFGKSSPLT ESGGPLSLSE ENNDSKLLS GLMNSQESSW GKNVSSSTESG RLFKGRAGH PALLTKDNAL FKVSISLKT NKTSNNSATN RKTHIDGPSL LIENSPPVWQ NILESDETEK KVTPLIHDRM LMDKNATALR

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253 BDD F8
254 F8CA2
255 F8CC1,2
256 F8CA1
257 F8CA3
258 1081 1260
259 Complete F8 LNHNMSNKTTT SKNMEMVQQK KEGPIPPDAQ NPDMSFFKML FLPESARWIQ RTHGKNSLNS GQGSPSPKQLV SLGPEKSVEG QNFLSEKNKV VVGKGEFTKD VGLKEMVFPF SRNLFLTNDL NLHENNTHNQ EKKIQEIEK KETLIQENVV LPQIHTVTGT KNFMKNLFLF STRQNVESGY
260 BDD F8
261 F8CA2
262 F8CC1,2
263 F8CA1
264 F8CA3
265 1261 1440
266 Complete F8 DGAYAPVLQD FRSLNDSTNR TKKHHTAHFSK KGEENLEGL GNQTKQIVEK YACTTRISPN TSQQNFVTQR SKRALKQFRL PLEETELEKR IIVDDTSTQW SKNMKHLTPS TLTQIDYNEK EKGAITQSPL SDCLTRSHSI PQANRSPLPI AKVSSFFSIR PIYLTRVLFQ DNSSHLPAAS
267 BDD F8
268 F8CA2
269 F8CC1,2
270 F8CA1
271 F8CA3
272 1441 1620
273 Complete F8 YRKKDSGVQE SSHFLQGAKK NNLSLAILTL EMTGDQREVQ SLGTSATNSV TYKKVENTVL PKPDLPKTSG KVELLPKVHI YQKDLFPTET SNGSPGHLDL VEGSLQGTE GAIKWNEANR PGKVPFLRVA TESSAKTFSK LLDPLAWDNH YGTQIPKEEW KSQEKSPKPT AFKKKDTILS
274 BDD F8
275 F8CA2
276 F8CC1,2
277 F8CA1
278 F8CA3
279 1621 1800
280 Complete F8 LNACESNHAI AAINEGQNKP EIEVTWAKQG RTERLCSQNP PVLKRHQREI TRTTLQSDQE EIDYDDTISV EMKKEDFDIY DEENQSPRS FQKKTRHYFI AAVERLWDYG MSSSPHVLNR RAQSGSVPQF KKVVFQEFTE GSFTQPLYRG ELNEHLGLLG PYIRAEVEDN IMVIFRNQAS
281 BDD F8QNP PVLKRHQREI TRTTLQSDQE EIDYDDTISV EMKKEDFDIY DEENQSPRS FQKKTRHYFI AAVERLWDYG MSSSPHVLNR RAQSGSVPQF KKVVFQEFTE GSFTQPLYRG ELNEHLGLLG PYIRAEVEDN IMVIFRNQAS
282 F8CA2
283 F8CC1,2
284 F8CA1
285 F8CA3 KTRHYFI AAVERLWDYG MSSSPHVLNR RAQSGSVPQF KKVVFQEFTE GSFTQPLYRG ELNEHLG
286 1801 1980
287 Complete F8 RPYSFYSSLI SYEEDQRQGA EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD VDLEKDVHSG LIGPLLCHT NTLNPAHGRQ VIVQEFALFF TIFDEKSWY FTEENMERNCR APCNIQMEDP TFKENYRFHA INGYIMDTLP GLVMAQDQRI RWYLLSMGSN ENIHSIHFSG
288 BDD F8 RPYSFYSSLI SYEEDQRQGA EPRKNFVKPN ETKTYFWKVQ HHMAPTKDEF DCKAWAYFSD VDLEKDVHSG LIGPLLCHT NTLNPAHGRQ VIVQEFALFF TIFDEKSWY FTEENMERNCR APCNIQMEDP TFKENYRFHA INGYIMDTLP GLVMAQDQRI RWYLLSMGSN ENIHSIHFSG
289 F8CA2

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290 F8CC1,2 .....
291 F8CA1 .....
292 F8CA3 .....
293 1981 ..... 2160
294 Complete F8 HVFTVRKKEE YKMALYNLYP GVFETVEMLP SKAGIWRVEC LIGEHLHAGM STLFLVYSNK CQTPLGMASG HIRDFQITAS GQYQWAPKL ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
295 BDD F8 HVFTVRKKEE YKMALYNLYP GVFETVEMLP SKAGIWRVEC LIGEHLHAGM STLFLVYSNK CQTPLGMASG HIRDFQITAS GQYQWAPKL ARLHYSGSIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
296 F8CA2 .....
297 F8CC1,2 ..... SIN AWSTKEPFSW IKVDLLAPMI IHGIKTQGAR QKFSSLYISQ FIIMYSLDGK KWQTYRGNST GTLMVFFGNV DSSGIKHNIF
298 F8CA1 .....
299 F8CA3 .....
300 2161 ..... 2340
301 Complete F8 NPPIIARYIR LHPTHYSIRS TLRMELMGCD LNCSMPLGM ESKAISDAQI TASSYFTNMF ATWSPSKARL HLQGRSNAWR PQVNNPKEWL QVDFQKTMKV TGVTTQGVKS LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV KVFQGNQDSF TPVVNSLDPP LLTRYLRIHF QSWVHQIALR
302 BDD F8 NPPIIARYIR LHPTHYSIRS TLRMELMGCD LNCSMPLGM ESKAISDAQI TASSYFTNMF ATWSPSKARL HLQGRSNAWR PQVNNPKEWL QVDFQKTMKV TGVTTQGVKS LLTSMYVKEF LISSSQDGHQ WTLFFQNGKV KVFQGNQDSF TPVVNSLDPP LLTRYLRIHF QSWVHQIALR
303 F8CA2 .....
304 F8CC1,2 NPPIIARYIR LHPTHYSIRS TLRMEL.GCD LNCSMPLGM ESKAISDAQI TASSYFTNMF ATWSPSKARL HLQGRS.....
305 F8CA1 .....
306 F8CA3 .....
307 2341 2352
308 Complete F8 MEVLGCEAQD LY.....
309 BDD F8 MEVLGCEAQD LY.....
310 F8CA2 .....
311 F8CC1,2 .....
312 F8CA1 .....
313 F8CA3 .....
314

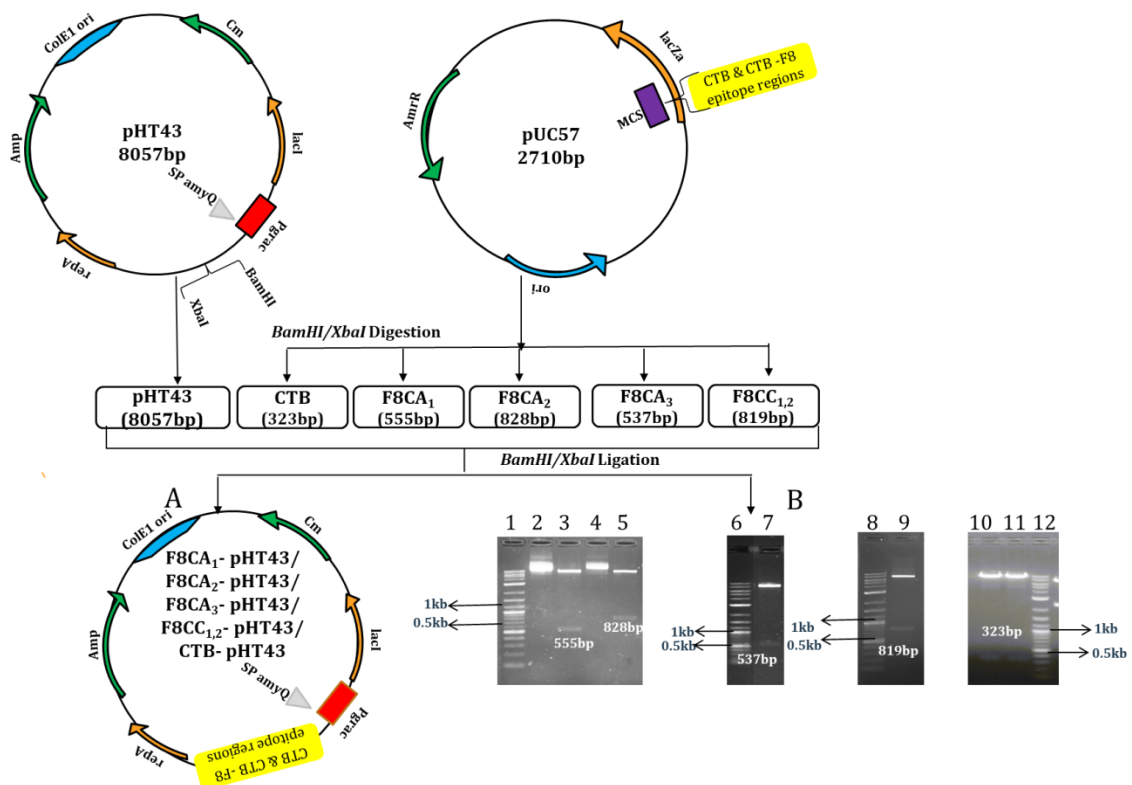
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315 **Fig.2b** Aminoacid sequence of complete FVIII preproprotein, B domain deleted FVIII aligned with the predicted epitope regions. The amino acid sequences that have been
316 differentially colored indicate the stretches with high epitope indexes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

318

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

320 The CTB–FVIII epitope regions were constructed with a flexible peptide linker, (GPGP), and a furin
 321 cleavage site, Arg-Arg-Lys-Arg, between CTB and FVIII epitope regions and were cloned
 322 downstream of the IPTG – inducible pPgrac promoter in pHT43 resulting in CTB-pHT43, F8CA₁-
 323 pHT43, F8CA₂-pHT43, F8CA₃-pHT43 and F8CC_{1,2}-pHT43 (Fig. 3A). The recombinant *B.subtilis*
 324 expression vectors mentioned above were propagated in *E.coli* strain DH5α and isolated using the
 325 Plasmid extraction kit (GenElute Plasmid DNA kit, Sigma). CTB and CTB-fused FVIII epitope
 326 regions cloned in pUC57 vector (codon optimized and synthesized by Genscript) were double
 327 digested with *Bam*HI and *Xba*I, and the products were identified by agarose electrophoresis.
 328 Electrophoresis and sequencing results showed that the CTB and CTB–FVIII epitope regions were
 329 successfully inserted into *B.subtilis* expression vector pHT43 (Fig. 3B). The recombinant plasmids
 330 were then transformed into *B.subtilis* WB800N via electroporation and the positive colonies were
 331 selected on Chloramphenicol resistant 2XYT agar plate.



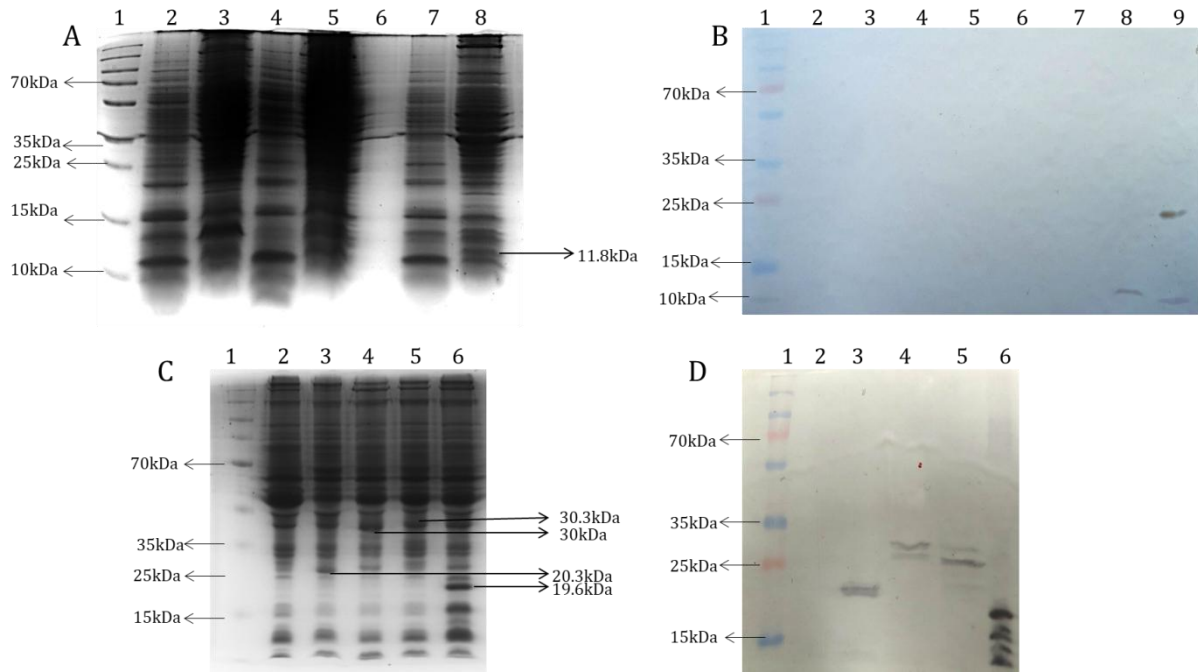
332

333 **Fig.3 Schematic of pHT43-CTB / CTB-FVIII epitope regions (F8CA₁, F8CA₂, F8CA₃, F8CC_{1,2})**

334 (A) CTB and CTB-fused FVIII epitope regions cloned in pUC57 were digested with *Bam*HI and *Xba*I
335 restriction enzymes and inserted into the pHT43 vector, generating 5 constructs of CTB-pHT43,
336 F8CA₁-pHT43, F8CA₂-pHT43, F8CA₃-pHT43 and F8CC_{1,2}-pHT43. (B) Electrophoretogram of
337 ligated plasmids double digested with *Bam*HI and *Xba*I. Lanes 1,6,8,12 - 0.1kb - 10kb DNA ladder,
338 Lane 2,4 – digested pHT43 vector, Lane 3,5,7,9,10-11 – digested F8CA₁-pHT43, F8CA₂-pHT43,
339 F8CA₃-pHT43, F8CC_{1,2}-pHT43 and CTB-pHT43. (pHT 43 vector map – Pgrac : Pgrac promoter
340 consisting of the groE promoter, the lacO operator, ColE1 ori : ColE1 origin replication, Amp :
341 ampicillin resistance, lacI : lacI gene repressor, Cm : chloramphenicol resistance, SPamyQ : amylase
342 Q signal peptide sequence. pUC57 vector map – ori : replication origin oriV_{ColE1}, lacZa : reporter
343 gene, AmpR : ampicillin resistance, MCS : multiple cloning site)

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

346 After induction by IPTG, the expressed fusion proteins in pellet extract from recombinant *B.subtilis*
347 strains were separated by SDS-PAGE under fully denatured and reducing conditions. The SDS-PAGE
348 results showed the expression of the recombinant protein after overnight induction viz. CTB 11.8kDa
349 (Fig. 4A), F8CA₁- 20.3kDa, F8CA₂- 30.3kDa, F8CA₃ – 19.6kDa and F8CC_{1,2} – 30kDa (Fig. 4C). The
350 results also show the expressed protein band in *B.subtilis* were approximately 11.8kDa (Fig. 4B),
351 20.3kDa, 30.3kDa, 19.6kDa and 30kDa in western blotting respectively (Fig. 4D). The western blot
352 analysis was confirmed with anti-CTB polyclonal antibody, thereby confirming the expression of
353 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- rF8CA₁ pellet, Lane 4-rF8CA₂ pellet, Lane 5-rF8CC_{1,2} pellet, Lane 6-

365 rF8CA₃ 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- rF8CA₁ pellet, Lane 4-rF8CA₂

367 pellet, Lane 5-rF8CC_{1,2} pellet, Lane 6- rF8CA₃ pellet

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

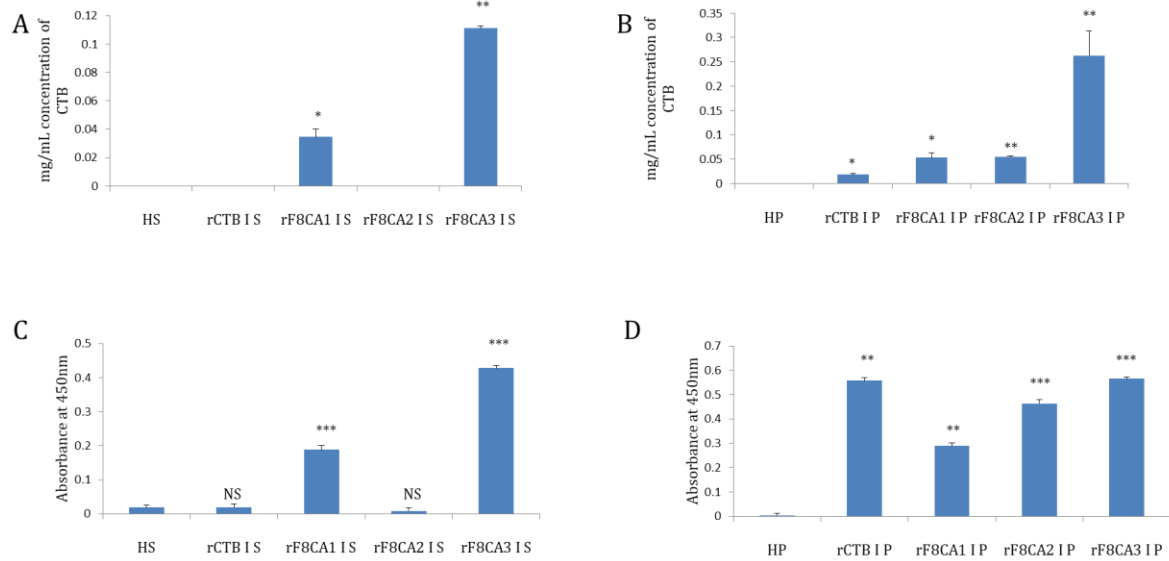
369 Specific CTB and CTB-fused epitope regions of FVIII were determined by enzyme-linked

370 immunosorbent assay (ELISA). The ELISA assay was performed with recombinant strains expressing

371 CTB and CTB-fused FVIII epitope regions (both supernatant and pellet) and quantified using CTB
372 standard plot. Fig.5a and 5b represents the expression pattern of recombinant CTB and CTB-fused
373 proteins. On the whole, rF8CA₃ shows better expression comparatively.

374 **GM1 binding assay by ELISA**

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

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

412 (Sherman et al. 2014). Sriram Krishnamoorthy and colleagues demonstrated in haemophilia A mice
413 that repeated administration of rFVIII-fused with Fc portion IgG1 (rFVIII-Fc) at therapeutically
414 relevant doses significantly reduced antibody responses to rFVIII and also reduced FVIII inhibitors
415 upon subsequent challenge with high doses of rFVIII-Fc (Krishnamoorthy et al. 2016). After
416 appropriate studies for safety and efficacy, the latter strategy may aid in the development of
417 innovative approaches to prevent inhibitor formation in haemophilia A in the future. However, the
418 expenses of generating therapeutic rFVIII-Fc with high yields will be a daunting task, further
419 increasing the costs of the existing treatment, which is already very expensive. Recent work
420 performed in FVIII-deficient mice by Lacroix-Desmazes and colleagues demonstrated that immune
421 tolerance to FVIII may be induced during the intra-utero stage and last long enough to cover the most
422 critical part for FVIII inhibitor development. Immunodominant A2 and C2 domains of FVIII fused to
423 the mouse Fc γ 1 were generated, and co-injected into pregnant FVIII-deficient mice with mouse
424 monoclonal IgG1 as a control group. The offspring were then treated with therapeutic FVIII at 7
425 weeks of age. The anti-FVIII immune response in the offspring was 10 times lower in A2Fc/C2Fc
426 when compared to IgG1 control and proliferation of splenic T cells to FVIII was significantly
427 reduced, suggesting induction of Tregs which was later confirmed by adoptive transfer experiments
428 (Batsuli et al. 2016). Alternatively, Henry Daniell and colleagues demonstrated that prevention of
429 FVIII inhibitor development could be achieved through oral delivery of transplastomic plants
430 expressing recombinant FVIII fused with cholera toxin B subunit. The codelivery of the heavy chain
431 and C2 domain of FVIII was sufficient to suppress inhibitor formation against the entire FVIII
432 molecule proving that efficient induction of FoxP3⁺ and Lap⁺ Treg may provide sufficient suppression
433 so that not all epitopes have to be covered by the orally delivered antigens (Sherman et al. 2014;
434 Kwon et al. 2018). While this approach is highly promising to control the formation of inhibitors, it
435 faces limitations such as general low levels of expression of FVIII by plant cells and might not be
436 compatible with a cost-effective translation of the approach in human beings. With this in mind, we
437 ventured to demonstrate a novel cost-effective strategy to produce epitope regions of FVIII as cholera
438 toxin B subunit fusion proteins in *Bacillus subtilis* which has a strong binding affinity to GM1
439 ganglioside.

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

466 **Conclusion**

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

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

486 **Data availability** Data is available on request from the authors

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490 **Competing Interest** The authors declare no competing interests

491 **Consent for Publication** All authors have consented to publish the paper

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