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Protease-resistant single-domain antibodies inhibit *Campylobacter jejuni* motility

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Camelid heavy-chain antibody variable domains (V_HHs) are emerging as potential antimicrobial reagents. We have engineered a previously isolated V_HH (FlagV1M), which binds *Campylobacter jejuni* flagella, for greater thermal and proteolytic stability. Mutants of FlagV1M were obtained from an error-prone polymerase chain reaction library that was panned in the presence of gastrointestinal (GI) proteases. Additional FlagV1M mutants were obtained through disulfide-bond engineering. Each approach produced V_HHs with enhanced thermal stability and protease resistance. When the beneficial mutations from both approaches were combined, a hyperstabilized V_HH was created with superior stability. The hyperstabilized V_HH bound *C. jejuni* flagella with wild-type affinity and was capable of potently inhibiting *C. jejuni* motility in assays performed after sequential digestion with three major GI proteases, demonstrating the remarkable stability imparted to the V_HH by combining our engineering approaches.

Keywords: *C. jejuni*/motility/protease resistance/protein stability/V_HH

Introduction

Campylobacter jejuni is a non-spore forming gram-negative bacterium and one of the most common food-borne pathogens in humans (Man, 2011). Infected individuals suffer from symptoms ranging from acute diarrhea to severe neurological disorders such as Guillain–Barré syndrome, reactive arthritis and inflammatory bowel disease (Altekruse *et al.*, 1999; Hughes *et al.*, 1999; Willison and O'Hanlon, 1999; Humphrey *et al.*, 2007; Man, 2011). Strategies to control or eliminate *Campylobacter* prevalence in poultry, particularly in chickens, are expected to improve poultry product safety and reduce the incidence of *Campylobacter*-induced gastroenteritis in humans. We recently illustrated a reduction-at-source approach to address the problem by orally administering *C. jejuni*-specific V_HH pentabodies to chickens, reducing their *C. jejuni* bacterial load by two to three orders of magnitude (Riazi *et al.*, 2013). While our approach was successful, we believe that the isolation or engineering of more robust heavy-chain antibody variable domains (V_HHs) with greater gastrointestinal (GI) tract stability would eventually lead to a more efficacious strategy to control *C. jejuni* infections.

Stability is a key determinant of therapeutic antibody efficacy and strategies to increase antibody stability without compromising specificity and target binding affinity are desirable. For oral immunotherapeutics to succeed in the GI tract, they must resist proteolytic digestion. Monoclonal antibodies and larger antibody fragments (i.e. Fabs, scFvs) have shown limited success in the GI tract (Reilly *et al.*, 1997; Singh *et al.*, 2008; Hussack *et al.*, 2011). Single-domain antibodies, particularly V_HHs from *Camelidae*, have the potential for oral immunotherapeutic development due to their high intrinsic stability and their amenability to screening, selection and engineering. Even so, orally delivered V_HHs still require considerable stabilization as demonstrated by the significant level of V_HH degradation in the stomach of pigs (Harmsen *et al.*, 2006). A number of options exist to increase thermal, chemical and proteolytic stability of antibody fragments. These approaches include error-prone polymerase chain reaction (PCR) (Leung *et al.*, 1989; Daugherty *et al.*, 2000), DNA shuffling/random mutagenesis (Harmsen *et al.*, 2006), site-directed mutagenesis (Wörn and Plückthun, 1998), phage library screening under selection pressures such as heat, acidic pH and proteases (Jespers *et al.*, 2004; Famm *et al.*, 2008; Dudgeon *et al.*, 2012). Specific mutations introducing disulfide bonds have also been used to increase antibody stability (Young *et al.*, 1995; Hagihara *et al.*, 2007; Saerens *et al.*, 2008; Hussack *et al.*, 2011, 2012; Kim *et al.*, 2012, 2013).

Here, our aim was to improve the biophysical properties of a *C. jejuni* flagella-binding V_HH (Riazi *et al.*, 2013) through two different approaches: by panning an error-prone PCR library in the presence of the major GI tract proteases and by disulfide-bond engineering. On their own, both mutations produced variants that were more stable compared with the parent V_HH. When the beneficial mutations were combined, a hyperstabilized V_HH with superior biophysical properties was produced; it retained function in terms of binding *C. jejuni* flagella and potently reduced *C. jejuni* motility *in vitro*.

Materials and methods

Construction of the mutant V1 library by error-prone PCR

The vector pMED1 was first modified in order to remove tag sequences which were prone to protease digestion (data not shown). The new vector is named pMED6. For error-prone PCR, 10 ng of DNA coding for V_HH 'FlagV1M' (hereafter referred to as 'V1'), a V_HH specific to *C. jejuni* flagella (Riazi *et al.*, 2013), was used as the initial template and amplified in a 50-μl reaction using a random mutagenesis PCR kit (GeneMorph II Random Mutagenesis kit, Stratagene, Mississauga, ON, Canada) and

primers MJ7BACK and MJFOR 11 (Table I) for 30 cycles (95°C, 30 s; 55°C, 30 s; 72°C, 60 s) followed by a 10 min extension at 72°C. The PCR products (~500 bp in length) were purified with a QIAquick PCR purification kit (Qiagen, Mississauga, ON, Canada), digested with SfiI for 6 h at 50°C (New England BioLabs, Pickering, ON, Canada) and re-purified using the same kit. Two hundred micrograms of pMED6 vector were also digested overnight at 50°C followed by PstI/XhoI digestion for 2 h. The digested vector was purified with a QIAquick PCR purification kit (Qiagen) and the DNA was eluted in sterile distilled H_2O (Arbabi-Ghahroudi *et al.*, 2009*a*). Forty-five micrograms of digested V_HH fragments were ligated with 150 μg of SfiI-digested pMED6 phagemid vector (3 : 1 molar ratio, respectively) (Arbabi-Ghahroudi *et al.*, 2009*a*) using a LigaFast Rapid DNA ligation system and its protocol (Promega, Madison, WI, USA). The ligated material was transformed into commercial electrocompetent *Escherichia coli* TG1 cells (Stratagene) as described previously (Arbabi-Ghahroudi *et al.*, 2009*a*) and a library size of $\sim 2 \times 10^9$ transformants was obtained. The V_HH fragments from 30 colonies were PCR-amplified and sequenced to analyze the complexity of the library. The library was grown for 3–4 h at 37°C, 250 rpm in 2xYT/ampicillin supplemented with glucose (2% w/v). The bacterial cells were pelleted, resuspended in the same medium, and stored as glycerol stocks at −80°C as described previously (Arbabi-Ghahroudi *et al.*, 2009*a*).

Protease panning of the V1 error-prone PCR library

Panning experiments were essentially performed as described previously (Arbabi-Ghahroudi *et al.*, 1997, 2009*a*), except that rescued and amplified phage from the initial library and each round of panning were pre-treated with chicken GI tract fluid (Loc Carrillo *et al.*, 2005) as well as pepsin, chymotrypsin and trypsin proteases. Three phage aliquots (125 μl each; 1×10^{12} phage particles) were prepared in 1 mM Tris–HCl buffer, pH 7.8. To the first phage aliquot, 12.5-μl GI tract chicken protease extract (diluted 1 in 10 in phosphate buffered saline, PBS) was added and incubated for 2 h at 37°C. The second phage aliquot was incubated with an equimolar mixture of chymotrypsin/trypsin (Roche, Laval, QC, Canada) (2.5 μM of each protease in Round 1, 7.5 μM in Round 2 and 10 μM in Rounds 3 and 4; final concentration) in 1 mM HCl plus 20 mM $CaCl_2$ and incubated for 15 min in Round 1, 45 min in Round 2 and 60 min in Rounds 3 and 4. To the third phage aliquot, different concentrations of pepsin (Roche) were prepared in PBS and added along with 1/10 volume of 100 mM HCl, pH 2.0, to the 125 μl phage aliquot (2.5 μM in Round 1, 7.5 μM in Round 2 and 10 μM in Rounds 3 and 4; final concentrations). The protease reactions were stopped either by adding 12.5 μl of protease inhibitor cocktail (Roche) for chicken protease and trypsin/chymotrypsin or by adding 0.5 volume of 1 M Tris–HCl, pH 7.5, for pepsin. The protease-treated phage aliquots were mixed and used for panning. A total of four rounds of panning were performed against *C. jejuni* flagella as described previously (Arbabi-Ghahroudi *et al.*, 2009*a*). Briefly, wells of a 96-well Maxisorp™ plate (Nunc, Rochester, NY) were coated with 15 μg of flagella or PBS (as a blank) overnight at 4°C. The wells were rinsed with PBS and blocked with PBS/1% (w/v) casein for 2 h at 37°C. Mixed protease-treated phage particles (100 μl) were added to the blocked wells and incubated for 2 h at 37°C. The wells were washed six times with PBST (PBS with 0.1% (v/v) Tween-20) and six times with PBS. The bound phage were eluted with 0.1 M triethylamine, neutralized with 1 M Tris–HCl, pH 7.4, and incubated with exponentially growing *E. coli* TG1 cells. After 30 min of incubation at 37°C, the cells were superinfected with M13KO7 helper phage for an additional 15 min and grown in 2xYT/ampicillin/kanamycin overnight at 37°C. Amplified phage were purified and panning was continued for three more rounds following the same conditions, except that phage-protease digestion conditions were changed as described above, the antigen concentration was reduced to 12.5, 10 and 10 μg/well and washing was increased to 7, 10 and 12× with PBST and 7, 10 and 12× with PBS for the second, third and fourth rounds of panning, respectively. After four rounds of panning, 24 randomly picked colonies were subjected to colony PCR and the PCR fragments were sequenced. A total of nine flagella-specific V_HHs (F1, F3, F7, F10, F13, F14, F20, F22 and F23) with one to four nucleotide point mutations were identified. The mutant clones along with the parental V1 clone were grown and subjected to phage enzyme-linked immunosorbent assay (ELISA) screening as described previously (Arbabi-Ghahroudi *et al.*, 2009*a*), except that 5 μg/ml of flagella was coated onto microtiter plates. Only the F7, F13, F20 and F23 clones showed positive signals and were selected for further characterization.

Expression and purification of soluble V_HHs

Genes encoding mutant V_HHs obtained from panning the error-prone PCR library against *C. jejuni* flagella were PCR-amplified from the pMED6 phagemid vector with *Bbs*I1-V_HH and *Bam*HI-V_HH primers (Table I). These PCR fragments were digested with BbsI and BamHI restriction endonucleases and ligated into the similarly digested pSJF2H expression vector (Arbabi-Ghahroudi *et al.*, 2009*a*). Upon ligation, all plasmids were transformed into electrocompetent *E. coli* TG1 and selected on LB agar plates containing ampicillin. Colonies were screened by colony PCR for inserts and the DNA sequenced. Small-scale expression was performed on individual clones and

Table I. Oligonucleotides used in this study.

Name	Sequence (5′→3′)	Purpose
MJ7BACK	CATGTGCATGGCCTAGACTCGCGGCCCAGCCGGCCATGGCC	Error-prone PCR
MJFOR 11	CATGTGTAGATTCTGCCTGGCCGGCCTGGCC	Error-prone PCR
*Bbs*I1-V_HH	TATGAAGACACCAGGCCCAGGTAAAGCTGGAGGAGTCT	Subcloning
*Bam*HI-V_HH	TTGTTCGGATCCTGAGGAGACGGTGACCTG	Subcloning
V1-DSB-for	TAGACAGTATTATCCAGATCCCGTGAAGGGCCGATTCACCTGCACCAGAGAC	DSB cloning
V1-DSB-rev	GGATAATACTGTCTATCTCTACTCCAGGAAATAGCGCACACTAC	DSB cloning
F23-DSB-for	TATGAAGACACCAGGCCCAGGTGCAGCTGGTGGAGTCT	DSB cloning

only clone F23 was found to have an expression level comparable with that of the parental V1 clone.

V_HHs V1 and F23 were expressed using a 5 day minimal media method (Baral and Arbabi-Ghahroudi, 2012). After induction of protein expression, cell cultures were harvested at 6000 × *g* (30 min, 4°C), the supernatant discarded and the periplasmic contents extracted from the cell pellet. Briefly, cell pellets containing V_HHs were resuspended in 20 ml of ice-cold TES (0.2 M Tris–HCl, pH 8.0, 20% (w/v) sucrose, 0.5 mM EDTA) and incubated on ice for 30 min. Next, 30 ml of ice-cold 1/8 TES was added, the mixture was incubated for an additional 30 min on ice, and the slurry centrifuged at 8000 × *g* (30 min, 4°C). The resulting supernatant containing V_HH was dialyzed overnight into buffer A (10 mM HEPES, pH 7.0, 500 mM NaCl) and V_HHs were purified by immobilized metal-ion affinity chromatography (IMAC) as described (Arbabi-Ghahroudi *et al.*, 2009*a*). Eluted fractions were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting (data not shown) before being dialyzed into PBS. V_HH concentrations were determined by absorbance measurements at 280 nm using theoretical molecular mass and extinction coefficients calculated with the ExPASy ProtParam Tool (http://expasy.org/tools/protparam.html) (Pace *et al.*, 1995).

Construction of disulfide-bond V_HH variants

Cysteine mutations were introduced at positions 54 and 78 of V1 and F23 V_HHs, for the generation of a non-canonical disulfide bond, using splice-overlap extension PCR essentially as described (Hussack *et al.*, 2011). To create the V1-DSB V_HH, which contains two disulfide bonds, the overlap PCR extension fragments were amplified using *BbsI*1-V_HH/V1-DSB-rev and V1-DSB-for/*BamHI*-V_HH primer sets (Table I). Likewise, the F23-DSB V_HH was created by amplifying the DNA encoding F23 with F23-DSB-for/V1-DSB-rev and V1-DSB-for/*BamHI*-V_HH primer sets (Table I). All DNA constructs were cloned into the pSJF2H expression vector, transformed into *E. coli* TG1 and expressed/purified as described for V1 and F23.

Size-exclusion chromatography and surface plasmon resonance analyses

Size-exclusion chromatography (SEC) was performed prior to surface plasmon resonance (SPR) analysis using a Superdex™ 75 column and 50 μM of V_HH, as described previously (Hussack *et al.*, 2011). Chromatograms were normalized as described previously (Kim *et al.*, 2012). Prior to SPR analysis on a Biacore 3000 (GE Healthcare, Baie-d'Urfé, QC, Canada), flagella from *C. jejuni* strain 81–176 was isolated (Riazi *et al.*, 2013), and biotinylated using a EZ-Link Sulfo-NHS-Biotinylation kit according to the manufacturer's instructions (Fisher Scientific, Ottawa, ON, Canada). Uncoupled biotin was removed by dialysis against 1000× volume of PBS for a total of eight times. For binding analysis, 2000 resonance units (RUs) of biotinylated flagella were immobilized onto a CAP sensor chip (GE Healthcare) previously loaded with the biotin CAPture reagent (50 μg/ml in HBS-EP buffer; GE Healthcare). Monomeric V_HHs eluted from the SEC column were injected over the immobilized flagella at concentrations ranging from 25–400 nM, using multiple injections of increasing concentrations and single-cycle kinetic analysis. The running buffer for all SPR experiments was HBS-EP (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, pH 7.4, 0.005% surfactant P20; GE Healthcare). The surface was stripped after each V_HH using regeneration buffers (8 M guanidine hydrochloride and 1 M NaOH) and a fresh biotinylated flagella surface was prepared as described above.

Thermal unfolding analysis of V_HHs

V_HH T_ms were determined by circular dichroism measurements at pH values 7.3 and 2.0 using a Jasco J-815 spectrometer (Jasco, Easton, MD) by following V_HH unfolding at 215 nm, essentially as described (Hussack *et al.*, 2011). The only exception to the previously described protocol (Hussack *et al.*, 2011) was that circular dichroism measurements were taken every 0.2°C from 25 to 96°C. In the case of V1 V_HH, 10 μg/ml was used for T_m determination at pH 7.3 because of aggregation at high temperatures using 50 μg/ml.

In vitro *protease digestions of V_HHs*

V_HHs were subjected to protease digestion assays with the major GI proteases pepsin (Sigma, Oakville, ON, Canada), sequencing-grade trypsin (Roche) and sequencing-grade chymotrypsin (Roche). V_HH digestions were performed and quantified exactly as described (Hussack *et al.*, 2011) and analyzed by SDS–PAGE thereafter. A total of three independent protease digestions were performed on each V_HH and each of these independent digestions was performed in duplicates.

In addition to individual protease digestions, a sequential digestion reaction was performed in which V_HHs (50 μg) were first digested with pepsin (10 μg/ml, 37°C, pH 2.0) for 15 or 30 min, followed by digestion with trypsin + chymotrypsin (10 μg/ml for each, 37°C, pH 7.4) for 15 or 30 min, in a total volume of 50 μl. After the pepsin digestion, the pH of the reaction was neutralized with 1 M NaOH to stop the reaction. After the trypsin + chymotrypsin digestion, protease inhibitor cocktail (Sigma) was added to stop the reaction. Sequentially digested V_HHs were compared with non-treated controls by SDS–PAGE and analyzed for functionality in a *C. jejuni* motility assay (see below).

Campylobacter jejuni *motility assay*

Campylobacter jejuni motility assays were performed as described previously (Kalmokoff *et al.*, 2006). V_HHs, at a final concentration of 1 μg/μl, were incubated with *C. jejuni* (5×10^4 colony-forming units) at room temperature for 30 min. The mixtures were plated, as described in Waseh *et al.* (2010), in the center of a Petri dish containing Müller–Hinton agar (0.4%) and incubated at 37°C under microaerobic conditions (5% O_2, 10% CO_2 and 85% N_2). Bacterial motility was determined by measuring the diameter of the circle produced by the growing bacteria at 24 h after plating the bacteria.

Results

Construction of the mutant V1 library by error-prone PCR and protease panning

The V_HH FlagV1M (referred to as 'V1' in this work) was isolated from a phage-displayed immune llama library against *C. jejuni* flagella (Riazi *et al.*, 2013). Before construction of a V1 error-prone PCR library to select for protease-resistant variants, we removed the His_6/HA tags from the pMED1 phagemid vector (Arbabi-Ghahroudi *et al.*, 2009*b*), generating pMED6 (Fig. 1A). The His_6/HA tags were previously shown

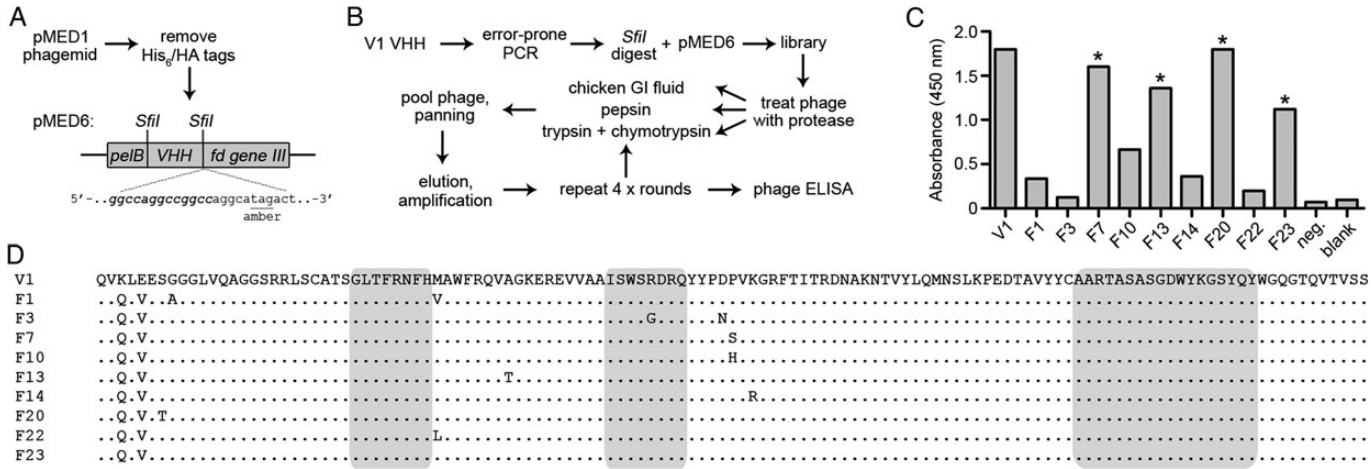

Fig. 1. Isolation of protease-resistant V_HH V1 variants. (**A**) Before constructing the error-prone PCR library based on *C. jejuni* flagella-specific V_HH V1, the phagemid vector pMED1 was modified to remove the His_6/HA tags that were previously shown to be susceptible to proteolytic cleavage. DNA encoding the His_6/HA tag was removed from the sequence between the 3′ SfiI site (bold and italics) and the amber stop codon (underlined). The multi-cloning site of the new phagemid vector, pMED6, is shown. (**B**) Work-flow diagram highlighting the construction of the V_HH V1 error-prone PCR library, protease treatment of phages and panning scheme. (**C**) Phage ELISA on nine V_HHs isolated from panning the V1 error-prone PCR library with protease treatment. *Campylobacter jejuni* strain 81–176 flagella was coated in microwells and binding of phage-displayed V_HHs was detected with anti-M13-IgG conjugated to HRP. Asterisks denote the four clones which showed comparable binding signals to the parent V_HH, V1. (**D**) Sequences of the nine V_HHs tested by phage ELISA in (**C**). Grayed areas highlight the hypervariable loops (IMGT numbering; www.imgt.org).

to be sensitive to proteolytic cleavage (data not shown). The new pMED6 phagemid vector contained an amber stop codon 4 nucleotides downstream of the 5′ SfiI restriction endonuclease site where the previous His_6/HA tag was located. We adopted an error-prone PCR method (Leung *et al.*, 1989; Stemmer, 1994) to introduce random mutations with the purpose of isolating protease-resistant mutants. A similar method has been successfully used for affinity maturation of conventional IgG antibodies as well as scFvs and V_{NAR}s (Miyazaki *et al.*, 1999; Daugherty *et al.*, 2000; Nuttall *et al.*, 2004; Fennell *et al.*, 2010). For library construction, random mutations were introduced into the V1 V_HH gene by error-prone PCR and the amplified PCR products were used to construct the mutant library with an approximate size of 2×10^9 transformants. Sequencing data from randomly selected colonies demonstrated the presence of point mutations within the V_HH amino acid sequence at the rate of three to five nucleotide substitutions per V_HH gene (data not shown). For panning, and in order to select V_HHs resisting the chicken GI tract environment, the rescued phage antibodies were pre-incubated with either chicken GI tract fluids harboring various proteases or the major GI proteases pepsin, trypsin and chymotrypsin (Fig. 1B). The filamentous phages (f1, fd and M13) are known to be resistant to most GI tract fluid proteases (Schwind *et al.*, 1992) and phage display, therefore, is a feasible display platform to select protease-resistant V_HHs. After panning, nine different V_HHs were isolated with two to four amino acid mutations in their framework region 1 (FR1), FR2, CDR2 and FR3 and around half of these clones (F7, F13, F20 and F23) turned out to be positive binders for *C. jejuni* flagella as determined by phage ELISA (Fig. 1C and D). All clones possessed a Lys3→Gln3 substitution and a Glu5→Val5 substitution (IMGT numbering system, http://www.imgt.org/). The CDRs of all variants were identical to the parent V1 V_HH with the exception of V_HH F3 which possessed an Arg→Gly substitution in CDR2. The consistent substitution of Lys with Gln and Arg with Gly is in agreement with the fact that trypsin digests peptide bonds after basic amino acids such as lysine and arginine. While several potential protease-resistant V_HHs were identified, the F23 V_HH had a comparable expression level to the parent V1 and showed the highest level of resistance to trypsin treatment among the V_HHs isolated in the screen. Therefore, the V_HH F23 clone was chosen for expression and further binding and stability analysis.

Construction of disulfide-engineered variant V_HHs and SEC analysis

In addition to the parental V1 V_HH and the F23 V_HH, which was isolated form protease panning of the error-prone PCR library, an engineered variant of each V_HH was produced with a second disulfide bond by mutating Ala54 and Ile78 to Cys54 and Cys78, giving V1-DSB and F23-DSB (Fig. 2A and B). All V_HHs were expressed and purified by IMAC and shown to be pure by SDS-PAGE (Fig. 2C). All ran at their expected molecular masses and there were no signs of higher-order multimers on the non-reducing SDS-PAGE gel. V_HH expression yields ranged from 9.5 mg (V1-DSB) to 21 mg (F23) per liter of bacterial culture (Table II), which is consistent with our previous findings that the Cys54-Cys78 disulfide bond compromises V_HH expression (Hussack *et al.*, 2011). SEC chromatograms suggested that all of the V_HHs were monomeric (Fig. 2E). Intact mass and fingerprinting analysis was not performed to confirm the formation of the Cys54-Cys78 disulfide bond within the engineered variants (Hussack *et al.*, 2011); however, the significant increase in V_HH T_ms (see below) and reduction in yields strongly suggest the introduced disulfide bond was successfully formed, as shown before for several other V_HH, V_H and V_L domains harboring the identical Cys54-Cys78 mutations (Hussack *et al.*, 2011; Kim *et al.*, 2012, 2013).

Binding and thermal unfolding analyses of V_HHs

The affinity of all 4 V_HHs was determined by SPR using single-cycle kinetics. The sensorgrams and affinities of V1, V1-DSB, F23 and F23-DSB are shown (Fig. 2D) and reported in Table II. All of the V_HH binding data fit a 1 : 1 binding model and V_HHs retained high affinity binding to *C. jejuni*

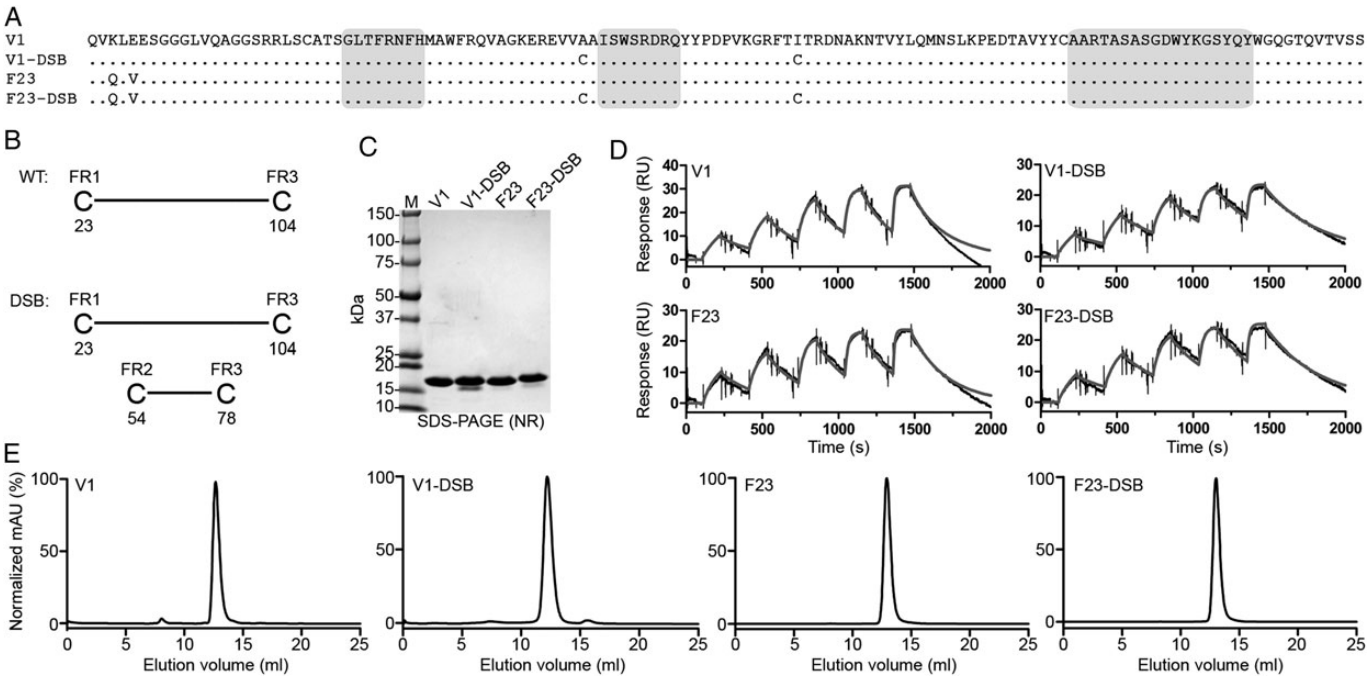

Fig. 2. Biophysical characterization of V1 and F23 V_HHs and their disulfide-bond engineered variants. (**A** and **B**) Introduction of a disulfide bond at position Cys54-Cys78 into parental V_HH V1 and the F23 variant gave V1-DSB and F23-DSB V_HHs, respectively. Grayed areas represent the hypervariable loops. (**C**) SDS–PAGE analysis of purified V_HHs. NR, non-reducing; M, molecular mass marker. (**D**) Single-cycle kinetic SPR sensorgrams illustrating the four V_HHs display high affinity binding to immobilized *C. jejuni* flagella. V_HHs were injected at concentrations ranging from 25 to 400 nM and affinities (K_Ds) were calculated using the BiaEvaluation 4.1 software updated with the single-cycle kinetics package. All data fit a 1 : 1 binding model. The K_Ds as well as on-rate and off-rate constants (k_a and k_d, respectively) for each V_HH are reported in Table II. (**E**) Superdex^{TM} 75 SEC profiles of the four V_HHs indicate that all are non-aggregating monomers, with all samples having 100% monomer peaks. V_HHs were injected at 50 μM concentrations.

Table II. Biophysical properties of *C. jejuni* flagella-specific V_HHs.

	V1	V1-DSB	F23	F23-DSB
Yield (mg/l)	17.0	9.5	21	16.5
k_a (M^{-1} s^{-1})	2.36×10^5	1.53×10^5	2.29×10^5	1.65×10^5
k_d (s^{-1})	5.63×10^{-3}	2.80×10^{-3}	4.26×10^{-3}	2.86×10^{-3}
K_D (nM)	23.9	18.2	18.6	17.3
T_m, pH 7.3 (°C)	61.7 ± 0.4[a]	79.1 ± 0.1	72.3 ± 0.2	80.2 ± 0.1
T_m, pH 2.0 (°C)	Nd	42.4 ± 0.1	30.5 ± 0.3	44.6 ± 0.1
Pepsin res. (%)[b]	22.3 ± 8.1	100.5 ± 6.7	6.8 ± 3.6	96.9 ± 15.8
Trypsin res. (%)[c]	84.4 ± 1.8	41.3 ± 2.7	101.1 ± 4.7	49.1 ± 15.4
Chymotrypsin res. (%)[c]	52.9 ± 6.5	49.5 ± 6.7	85.4 ± 3.3	90.9 ± 4.1

[a]10 μg/ml of V_HH used for circular dichroism due to the aggregation of V1 at 50 μg/ml.
[b]Digestions performed using 100 μg/ml of protease.
[c]Digestions performed using 10 μg/ml of protease.
Nd: not determined since the protein denatured at pH 2.0, 25°C; res., resistance.

flagella, with similar K_Ds, ranging from 23.9 nM (V1) to 17.3 nM (F23-DSB), demonstrating no significant changes in binding affinity upon introducing mutations in FR1 or addition of an extra disulfide bridge in the V_HH domain.

The T_m of all four V_HHs was determined by circular dichroism spectroscopy at pH values 7.3 and 2.0. Unfolding curves with a single-phase transition were obtained for all V_HHs at pH 7.3. At this pH, the T_ms of V_HHs containing the mutations in FR1 and/or the introduced disulfide bond are higher than V_HHs with a single, native disulfide bond. For example, the T_ms of F23, V1-DSB and F23-DSB were 72.3 ± 0.2, 79.1 ± 0.1 and 80.2 ± 0.1°C, respectively, compared with 61.7 ± 0.4°C for parental V1 V_HH (Fig. 3; Table II). It should be noted that V1 V_HH aggregated at temperatures >68°C when tested at 50 μg/ml (Fig. 3, inset); however, when the concentration was reduced to 10 μg/ml, no aggregation was detectable at higher temperatures and a single-phase unfolding curve was obtained. The T_ms of V_HHs were also determined at pH 2.0. Before performing unfolding experiments, V_HHs were incubated for 2 h at pH 2.0. At this pH, V1 was completely unfolded at the starting temperature (25°C) and a T_m could not be determined (Fig. 3). V1-DSB on the other hand was folded at 25°C at pH 2.0 and had a T_m of 42.4 ± 0.1°C, illustrating the significant impact the second disulfide bond has on V1-DSB stability. F23 was partially unfolded at 25°C at pH 2.0 and a T_m of 30.5 ± 0.3°C was determined, an improvement from the parental V1 V_HH which was completely unfolded under these conditions. This result underscores the success of the protease-panning strategy in selecting for more stable binders and with a higher T_m. Similar to pH 7.3, the clone with the highest T_m at pH 2.0 was F23-DSB, with a T_m of 44.6 ± 0.1°C, suggesting that the effects of the stabilizing mutations in F23 and the effects of the second disulfide bond were partially additive to create the hyperstabilized F23-DSB V_HH.

In vitro *protease digestion of V_HHs*

The resistance of V1, V1-DSB, F23 and F23-DSB to the major GI proteases was determined (Fig. 4; Table II). Both V1 and F23 V_HHs were susceptible to pepsin degradation, with 22.3 ± 8.1 and 6.8 ± 3.6% of V_HHs remaining intact after 60 min exposure to pepsin, respectively. The disulfide-engineered variants were considerably more resistant to pepsin, with V1-DSB

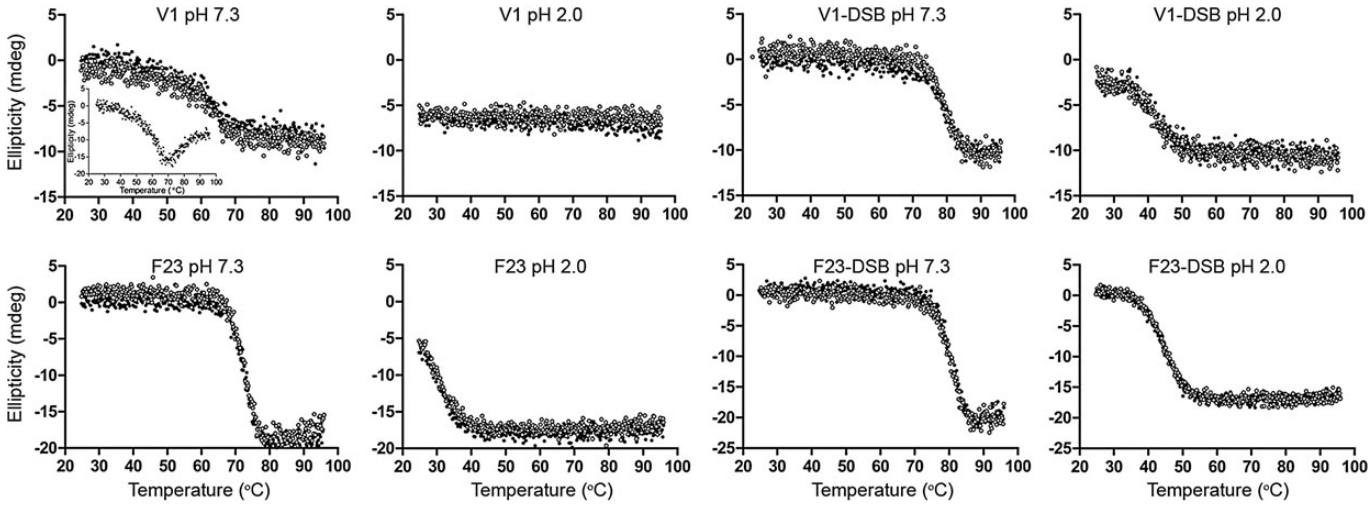

Fig. 3. Comparison of V_HH thermal unfolding temperatures at neutral and acidic pH. Thermal unfolding curves of V1 and F23 V_HHs and their disulfide-bond variants at neutral pH (7.3) and acidic pH (2.0) are shown. V_HH unfolding was measured at protein concentrations of 50 μg/ml by circular dichroism spectroscopy. At pH 7.3, V1 aggregated at higher temperatures (inset). When measured at 10 μg/ml, the protein did not aggregate and an unfolding curve was obtained. Measured T_ms are recorded in Table II. White circles = replicate 1, dark circles = replicate 2.

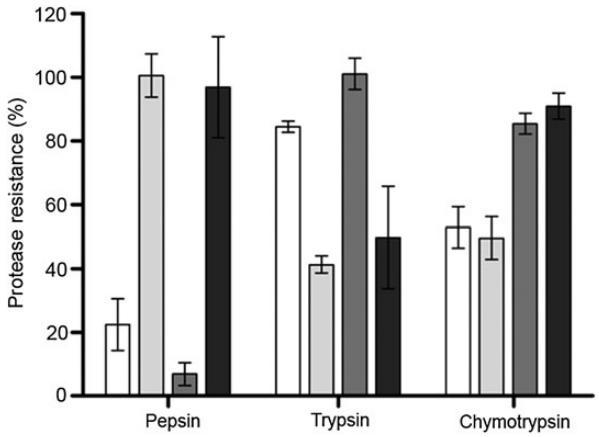

Fig. 4. GI protease resistance of V1 and F23 and their disulfide-bond variants. Digested V_HHs and controls were separated by SDS–PAGE. The bar graph summarizes the protease resistance profiles generated by densitometric analysis of SDS–PAGE gels. Three independent digestions were performed for each V_HH and each protease. Error bars represent mean protease resistance ± SEM. V1, White bars; V1-DSB, light gray bars; F23, dark gray bars; F23-DSB, black bars.

showing complete resistance (100.5 ± 6.7%) at a pepsin concentration of 100 μg/ml. F23-DSB was also very resistant to pepsin, with 96.9 ± 15.8% of the V_HH remaining intact after 60 min. When digested with trypsin for 60 min, F23 was completely resistant (101.1 ± 4.7%) followed by V1 (84.4 ± 1.8%), F23-DSB (49.1 ± 15.4%) and V1-DSB (41.3 ± 2.7%). It is obvious that the addition of the second disulfide bond between Cys54–Cys78 reduces trypsin resistance for both clones here, a result that is similar to previous findings for some *C. difficile* toxin A specific V_HHs (Hussack *et al.*, 2011). The significant reduction in trypsin resistance of F23-DSB suggests that structural changes in the V_HH domain may lead to the exposure of trypsin-sensitive sites (i.e. Lys and Arg) upon the addition of the second disulfide bridge. When digested with chymotrypsin for 60 min, F23-DSB displayed the highest resistance (90.9 ± 4.1%) followed by F23 (85.4 ± 3.3%), V1 (52.9 ± 6.5%) and V1-DSB (49.5 ± 6.7%). Therefore, in the case of these V_HHs, the second disulfide bond does not affect chymotrypsin resistance but does decrease trypsin resistance. Collectively, these data suggest that the F23 variant isolated from protease-panning was selected for very high trypsin and chymotrypsin resistance, and that the strongest combined resistance for all three proteases is the F23-DSB variant.

Sequential protease treatment of V_HHs

To determine whether the two strategies used to isolate robust, hyperstabilized V_HHs were effective in a biological setting, we exposed all four V_HHs to a sequential protease digestion scheme reminiscent of the GI tract (Fig. 5A, upper panel). Specifically, we treated V_HHs with pepsin at pH 2.0 followed by trypsin and chymotrypsin at pH 7.3 for 15 or 30 min each. Analysis of the sequential digestions and non-treated controls by SDS–PAGE is shown (Fig. 5A, lower panel). Near complete digestion of V1 is evident at 15 and 30 min relative to control V1 (compare 'V1' with 'V1(15)' and 'V1(30)'). F23 was more resistant than V1, with a strong band representing intact V_HH present after the sequential 15 min digest (compare 'F23' with 'F23(15)'). After 30 min, a near complete digestion of F23 was evident. Conversely, both disulfide-bond variants were strongly resistant to the sequential protease digestions, even after 30 min of treatment (compare 'V1-DSB' with 'V1-DSB(30)' and compare 'F23-DSB' with 'F23-DSB(30)'). Densitometry analysis of the bands revealed 74.5% of F23-DSB V_HH intact after the sequential 30 min digest compared with 47.6% of V1-DSB V_HH intact after the same treatment.

Campylobacter jejuni *motility assays*

Finally, we tested the *C. jejuni* motility inhibiting capacity of the V_HH variants. All V_HHs were effective in inhibiting the growth and spread of *C. jejuni* after incubation for 24 h, with essentially the same efficacy (Fig. 5B and C). We then used the sequential protease-treated V_HHs (Fig. 5A) in the same motility assay to confirm the disulfide-bond variant V_HHs retained their functionality. The protease-treated V1 and F23 V_HHs were almost entirely degraded after 15 or 30 min incubation conditions, and as expected, did not inhibit motility.

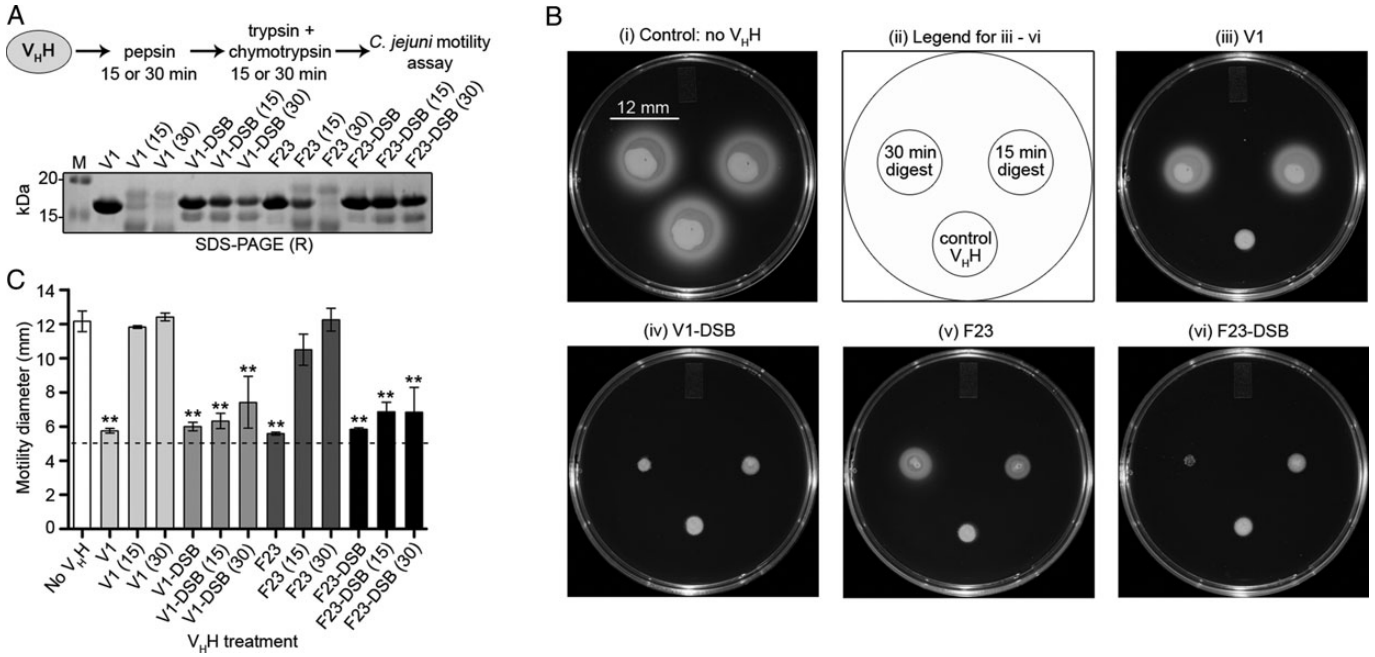

Fig. 5. Reduction of *C. jejuni* motility by GI protease-treated V_HHs. (**A**, top) Schematic diagram illustrating the sequential digestion of V_HHs with pepsin (10 μg/ml, pH 2.0) followed by trypsin and chymotrypsin (10 μg/ml, pH 7.3) for either 15 min ('15') or 30 min ('30') each. (**A**, bottom) Analysis of sequential digests by reducing SDS–PAGE. V1-DSB and F23-DSB show increased resistance to degradation compared with their respective parents, V1 and F23, especially after 30 min. M, molecular mass marker. (**B**) Representative *C. jejuni* motility assay. *Campylobacter jejuni* was applied to plates with control buffer or V_HH at 1 μg/μl. The diameter of the bacterial growth was measured after 24 h of plate inoculation and used to construct the graph in (**C**). (i) Control *C. jejuni* after 24 h incubation with control buffer (no V_HH) containing proteases, (ii) legend for images (iii)–(vi), (iii) V1 V_HH control, 15 and 30 min digests, (iv) V1-DSB V_HH control, 15 and 30 min digests, (V) F23 V_HH control, 15 and 30 min digests and (vi) F23-DSB V_HH control, 15 and 30 min digests. (**C**) Summary of motility assays. The bars represent the mean diameter of *C. jejuni* growth circles on plates treated with buffer control, V_HHs, or protease-digested V_HHs from three independent experiments ± SEM. The dashed line at 5 mm represents the spotting diameter of *C. jejuni* on the plates. Statistical analysis was performed by one-way ANOVA followed by Dunnett's multiple comparison test, all relative to the control with no V_HH (**$P < 0.01$).

On the other hand, the growth inhibiting activity of V1-DSB and F23-DSB mutants was basically unaffected by the protease treatment, further confirming that the protease-treated V_HHs remain largely intact (Fig. 5A) and active in settings reminiscent of the GI tract.

Discussion

In this study, we report the generation of a hyperstabilized V_HH against *C. jejuni* flagella with high thermal and proteolytic stability. This was achieved by combining an error-prone PCR approach and panning under protease pressure with the introduction of cysteine residues at two positions in V_HH FRs. The hyperstable V_HH mutant F23-DSB outperformed the parental V1 V_HH in terms of resistance to proteases, while it maintained the motility-inhibitory properties and affinity of the parent V_HH.

The evolutionary approach used in this study had dual beneficial effects: (i) it helped to isolate a V_HH with consistent mutations at residues at or near potential trypsin-sensitive sites (Lys3→Gln3; Glu5→Val5) and resulted in the F23 V_HH variant with complete resistance to trypsin up to 60 min; and (ii) the isolated F23 V_HH had an increased T_m (~11°C greater than V1), while its production level in *E. coli* and antigen-binding affinity were similar to the V1 V_HH. These findings show that certain amino acid combinations at positions 3 and 5 in the FR1 region may lead to a V_HH scaffold with increased stability and moderate-to-complete resistance to key GI tract proteases, in particular, to trypsin. Previous studies on the V_H domain of an anti-β-lactam murine scFv have shown that mutations in FR1, in particular at positions 6, 7 and 10, which may occur by the primer mixture used for the cloning of antibody gene, can have severe consequences on the folding yields and stabilities of antibodies (Jung *et al.*, 2001).

The introduction of cysteine residues at positions 54 and 78 formed a disulfide bridge that lead to increased stability, as previously shown for a panel of V_HHs, V_HS and V_LS (Hussack *et al.*, 2011; Kim *et al.*, 2012, 2013), and led to the generation of the hyperstable V_HH F23-DSB. However, the addition of a second disulfide bond leads to minor structural changes when introduced into the F23 V_HH scaffold, as evidenced by the lowered trypsin resistance of F23-DSB, leading to the speculation that one or more potential trypsin cleavage sites in FRs (namely, R18, R19, K43, R45, K64 and K86) are more accessible for proteolytic digestion. This is consistent with our previous work on other V_HHs (Hussack *et al.*, 2011). While it is obvious that the second disulfide bond leads to structural changes, these changes must be minor, and possibly restricted to influencing the framework regions only, because the antigen binding affinity of both V_HHs containing the second disulfide bond were nearly identical to that of the parental scaffolds. In the future, strategies to increase the trypsin resistant properties of F23-DSB could include the replacement of the framework residues noted above and other trypsin-sensitive residues (Frenken *et al.*, 1993) by site-specific mutations or re-applying the error-prone PCR approach followed by panning under protease pressure against the same target antigen (as shown in this study).

The antibody engineering approach used here produced robust binding agents with favorable biophysical properties. Currently, the protein-engineered F23-DSB V_HH is being

studied for the prevention or significant reduction of *C. jejuni* colonization in the chicken GI tract.

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