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Generation of panels of anti-idiotypic single-chain variable fragments mimicking Cry2Aa toxin using the chain shuffling technique

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ABSTRACT

Here, we firstly reported to generate panels of anti-idiotypic single-chain variable fragments (scFvs) of *Bacillus thuringiensis* Cry2Aa toxin by chain shuffling method. Light- and heavy-chain-shuffled libraries based on two parental clones were constructed. The signal of the light-chain-shuffled library was 4.2-fold higher than the heavy one. Solely after one round panning, eight mutants were rapidly isolated from the chain-shuffled libraries. The apparent affinity of the best clones was $2.65 \times 10^6 \text{ M}^{-1}$, a 2.59-fold improvement over the parental clone. The binding abilities between anti-idiotypic scFvs with brush border membrane vesicle of *Plutella xylostella* larvae were also determined. This work demonstrated that chain shuffling could rapidly generate panels of antibodies with similar binding abilities but of the distinct idiotype composition. The serials of mutants can be used to map the binding epitope of the receptors in target insects instead of Cry2Aa toxin.

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Introduction

Anti-idiotypic antibody is a useful tool for understanding the molecular mimics with many applications. It has been produced to different antigens and used widely to react with cellular receptors, including hormone receptors (Lan et al., 2017; Lan, Zheng, Khan, & Li, 2015), virus receptors (Huang & Campadelli-Fiume, 1996; Kiyohara et al., 2009), harpin receptor (Chen et al., 2008) and chimeric immune receptors (Cheung, Guo, Modak, & Cheung, 2003).

The standard method for the generation of anti-idiotypic antibodies is the conventional hybridoma techniques. However, due to the idiotype (the antigen-binding region) of an antibody which is used to produce anti-idiotypic antibodies was only a small part of the whole antibody. The positive rate of hybridoma-secreting anti-idiotypic antibodies is much less than common antibodies. In addition to hybridoma techniques, the selection of anti-idiotypic antibodies by phage display is an alternative way. This method could rapidly isolate antibodies even without immunization (Tu et al., 2012). However, for

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the same reason, the isolation of anti-idiotypic antibodies from naïve library often results in none or few positive clones (Goletz et al., 2002).

Chain shuffling is a mutagenesis method, which allows the recombination of a heavy or light chain of a specific antibody with a complementary variable domain library (Irving, Kortt, & Hudson, 1996). Since the shuffling approaches mimic somatic hypermutation and could avoid the deleterious mutagenesis in the framework regions of antibodies, they may be better than random or site-directed mutagenesis to maintain the functional antibodies (Ness et al., 2002). This will be beneficial when rapidly generating panels of antibodies with related specificity but distinct idiotypic composition from a limited parental clone, and it has many applications (Kang, Jones, & Burton, 1991).

Chain shuffling could also optimize the pairing of the heavy chain and light chain to improve the functions of antibodies. Depending on the antibodies, chain shuffling can result in the loss of binding affinity or generation of several new active combinations, some of which have improved affinity (Brockmann, 2010). The affinity improvement in some recent reports has been 1.4–15 folds for heavy-chain shuffling (Kramer, 2002; Marks et al., 1992; Rojas et al., 2004; Shi et al., 2010) and 2.1–185 folds for light-chain shuffling (Fitzgerald, Leonard, Darcy, Danaher, & O’Kennedy, 2011; Osbourn et al., 1996). In addition to the affinity, fine specificities and recognition of the epitope can also be changed by chain shuffling (Lu et al., 2003) or can overcome well-recognized protein-expression problems in bacteria (Rojas et al., 2004).

In previous work, we only isolated two anti-idiotypic scFvs (B10, F2) of *Bacillus thuringiensis* Cry2Aa toxin from Tomlinson I library (Wu, 2015). To obtain more functional antibodies with similar specificity and optimized pairing of heavy and light chains, these two parental clones were shuffled with variable heavy- and light-chain repertoires of the same un-panned library.

Material and methods

Chemical and reagents

Two anti-idiotypic scFv clones in *E. coli* TG1 (labeled B10 and F2) were isolated from Tomlinson I library by our laboratory. Polyclonal antibodies of Cry2Aa toxin were produced from New Zealand white rabbits by our laboratory. Tomlinson I library (a naïve scFv library constructed based on a human framework with side-chain diversity), pIT2 vector, *E. coli* TG1 were purchased from Source Bioscience (UK). T4 DNA ligase, *NotI* and *NcoI* were obtained from New England Biolabs (USA). 2× *Taq* PCR master mix and 2× *pfu* PCR master mix were purchased from DBI (Germany) and Dongsheng Biotechnology (China), respectively. DNA gel extraction spin kits, 6-well plate and 96-well plate were purchased from Corning (USA). Skim milk powder was obtained from Solabio (China). HRP-conjugated anti-M13 monoclonal antibodies were purchased from GE Healthcare (USA). All reagents were of analytical grade.

Construction of chain-shuffled libraries

The variable heavy region (VH) and light region (VL) of B10, F2 or un-panned Tomlinson I library were amplified by PCR with 2× *Taq* PCR master mix. For the amplification of V_H, primer 1 and primer 2 were used and for the case of V_L, primer 3 and primer 4 were used

Table 1. Sequence of the PCR primers used for construction of chain-shuffled libraries^a.

Primer	Sequence
Primer 1	CAG GAA ACA GCT ATG AC
Primer 2	CGA CCC GCC ACC GCC GCT G
Primer 3	C AGC GGC GGT GGC GGG TCG
Primer 4	CTA TGA GGC CCC ATT CA

^aComplementary sequences for SOE-PCR are in bold.

(Table 1). The PCR cycling conditions were as follows: 1 cycle, 5 min at 95°C; 30 cycles, 1 min at 94, 1 min at 55°, 1 min at 72°C; 1 cycle, 10 min at 72°C.

The purified VH and VL PCR products were connected to form a full-length scFv gene in a strand overlap extension (SOE)-PCR reaction by using 2× *pfu* PCR master mix. The SOE-PCR programme was 1 cycle, 5 min at 95°C; 7 cycles of 1 min, 94°C, 1 min at 55°C, 1 min at 72°C, and then primer 1 and primer 4 were added to the reaction vessel, the SOE-PCR was continued with the following programme: 30 cycles, 1 min at 94°C, 1 min at 55°C, 1 min at 72°C; 1 cycle, 10 min at 72°C.

The SOE-PCR products and pIT2 (vector) were digested with *Not* I and *Nco* I at 37°C for 4 h and were purified by using DNA gel extraction spin kits. The digested DNA and pIT2 were ligated together by T4 DNA ligase at 16°C for 18 h. Then, the ligation products were transformed into TG1. The transformed cells were cultured in 50-mL 2× TY (containing 100 µg/mL ampicillin and 1% glucose) at 37°C, 250 RPM until the mid-exponential growth was achieved and subsequently rescued by the addition of M13KO7 helper phage. The culture was incubated in a 37°C water bath for 30 min and was spinned at 3000 × *g*, 10 min. The pellet was suspended in 100 mL 2×TY (containing 100 µg/mL ampicillin, 50 µg/mL kanamycin and 0.1% glucose) and shaking at 30°C, 250 rpm overnight. The next day, the phage was precipitated according to the protocol of Tomlinson (I + J) library (de Wildt, Mundy, Gorick, & Tomlinson, 2000; Ossysek et al., 2015).

Panning of chain-shuffled libraries

For the panning steps, a six-well plate was coated with 1 mL of 100 µg/mL polyclonal antibodies of Cry2Aa toxin in a carbonate-bicarbonate buffer saline (CBS, 50 mmol/L, pH 9.6) at 4°C overnight. The plate was washed three times with phosphate-buffered saline (PBS, 50 mmol/L, pH 7.4) containing 0.05% v/v Tween-20 and blocked by completely filling with 2% skim milk powder in PBS (MPBS) and incubating the plates at room temperature for 2 h. After washing the plate three times, 1 mL of 10⁷ cfu/mL phage from the heavy- or light-chain-shuffled library in 2% MPBS were added to the well and incubated at room temperature for 2 h under gentle shaking. The subsequent washing steps, elution, multiplication of the phage, polyclonal and monoclonal phage ELISA were done according to the protocols of Tomlinson (I + J) library.

Ranking clones by competitive phage ELISA

The optimal working concentration of coating antigen (polyclonal antibodies of Cry2Aa toxin) and the dilution times of phage supernatant for each clone were determined by checkerboard titration (Liu et al., 2017). The 96-well plates were coated with the optimal working

concentration of polyclonal antibodies of Cry2Aa toxin in CBS. The next day, the plates were washed three times with PBS containing 0.05% v/v Tween-20 (PBST) by automated microplate washer and blocked by 2% MPBS (200 μ L/well) and incubated for 1 h at 37°C. Following the incubation, 50 μ L of varying concentrations of polyclonal antibodies of Cry2Aa toxins were incubated with 50 μ L of the optimal dilution of scFvs supernatants and added to each well. Following an incubation for 1 h at 37°C, the plates were washed, 100 μ L/well HRP-conjugated anti-M13 monoclonal antibodies (1:5000 diluted by PBS) were added and incubated for 1 h at 37°C. Following a washing step, 100 μ L/well of a tetramethylbenzidine solution (120 μ L of 10 mg/mL TMB-DMSO and 30 μ L of 0.65% (v/v) H₂O₂ diluted with 11.85 mL of citrate-sodium citrate buffer (CPBS, 100 mmol/L, pH5.5)) was added. The reaction was stopped after 15 min by adding 50 μ L/well of 2 mol/L H₂SO₄, and the absorbance was read by a microplate reader at 450 nm.

Dose-response curves were analysed by using software Sigmaplot version 12.0. The ratios of ELISA mean absorbance values ($n = 3$) of antibody-binding response in the presence of free antigen to the absence of antigen ($Y, B/B_0$) were plotted against the logarithm of free antigens concentrations (X) and fitted to the following four-parameter logistic equation (Liu et al., 2007).

$$Y = D + (A - D)/(1 + 10^{(C-X)B}),$$

where A is the high asymptote, B is the slope, C is the antigen concentration that was required to inhibit 50% maximal binding (IC_{50}), and D is the low asymptote.

Brush border membrane vesicle preparation and ELISA binding analysis

Brush border membrane vesicles (BBMVs) were prepared from midgut tissue of fourth instar *Plutella xylostella* larvae following the protocol described by Wolfersberger (1993). Binding ability of anti-idiotypic phage antibodies of Cry2Aa toxin with *Plutella xylostella* BBMV was determined by ELISA. First, 96-well plates were coated with 10 μ g/mL BBMV. Then, after blocking with 2% MPBS, 10⁶ phages (precipitated by PEG/NaCl and resolved in PBS) for each clones for each clones ($n = 3$) were added to the well. The bound phage antibodies were detected using HRP-conjugated anti-M13 monoclonal antibodies (1:5000 diluted by PBS). The subsequent steps were performed as a competitive phage ELISA. PBS and an irrelevant phage clone which recognize bovine serum albumin (BSA) from Tomlinson (I + J) library were used as negative controls.

Results and discussion

Constructions of chain-shuffled library

The amplified VL and VH genes of B10, F2 and Tomlinson I library were analysed by agarose gel electrophoresis (Figure 1(A)). The calculated sizes of VH and VL products were approximately 527 and 427 bp, respectively. The base length of SOE-PCR products was approximately 935 bp (Figure 1(B)). The sizes of constructed heavy- and light-chain-shuffled libraries were determined to be 1.4×10^6 cfu and 1.2×10^6 cfu.

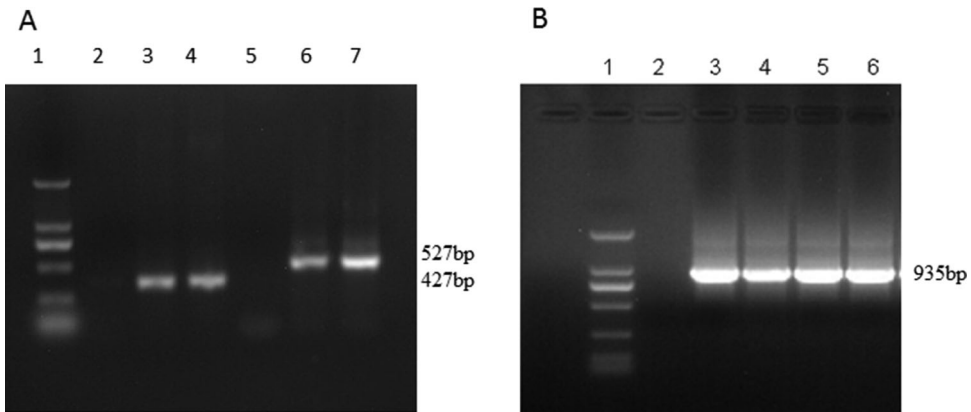


Figure 1. Analysis of amplified VH, VL and SOE-PCR products by agarose gel electrophoresis. (A) 1:DNA ladder, from top to bottom, 2000,1000,750, 500, 250, 100 bp; 3–4:VL (Tomlinson I); 6:VH (B10); 7: VH (F2). (B) 1: DNA ladder, from top to bottom, 2000, 1000, 750, 500, 250, 100 bp 3: VL (Tomlinson I)-VH (B10); 4: VL (Tomlinson I)-VH (F2); 5: VL (B10)-VH (Tomlinson I); 6: VL (F2)-VH (Tomlinson).

Isolation of mutants from chain-shuffled libraries

Before panning, the whole affinity of heavy- and light-chain-shuffled libraries was determined by polyclonal phage ELISA. The absorbance of light-chain-shuffled library was 4.2-fold higher than heavy-chain-shuffled library. It is thus demonstrated that in the present study, the heavy chains played a more important role than light chains for the binding ability.

After one round of panning, the output clone numbers for the heavy- and light-chain-shuffled libraries were 59 and 182, respectively. These clones were screened by monoclonal phage ELISA, in which 8 and 93 positive clones were isolated from the heavy- and light-chain-shuffled library. The positive rates for the two libraries were 13.6% and 51.1%. The high-positive rate may benefit the more functional antibodies from the chain-shuffled libraries than the libraries constructed by random mutagenesis.

After DNA sequencing, we finally obtained one heavy-chain-shuffled clones (2H1 originated from B10) and seven light-chain-shuffled clones (MTU10, MUT19, MUT21 originated from B10; 2F10, 3B1, 3F1 and 3H11 originated from F2) (Table 2). By comparing the sequence of mutants with their parental clones, we found that all the mutants have the same length of CDR loops as the parental clones, and seven clones (MUT10, MUT19,

Table 2. Protein sequences of parental clones and mutants derived from chain-shuffled libraries^a.

	CDR-H2	CDR-H3	CDR-L2	CDR-L3
B10 (parental clone)	IAYAGSNTN	KNSSGFD	YAASSL	CQQTSTTPY
2H1	ISSTGAYTSN	KADASFD	YAASSL	CQQTSTTPY
MUT10	IAYAGSNTN	KNSSGFD	YAASSL	CQQYSSNPS
MUT19	IAYAGSNTN	KNSSGFD	YAASDL	CQQTSTTPY
MUT21	IAYAGSNTN	KNSSGFD	YAASSL	CQQGYATPY
F2 (parental clone)	IDSYGTNTD	KAFNSFD	YAASAL	CQQYSSSPS
2F10	IDSYGTNTD	KAFNSFD	YAASDL	CQQYSSNPS
3B1	IDSYGTNTD	KAFNSFD	YAASDL	CQAAAAYS
3F1	CDRs of 3F1 were identical with F2. Mutation took place in K171E in framework and a missing G120 in linker			
3H11	CDRs of 3H11 were identical with F2. Mutation took place in K171E, Q221R, T234S in framework and a missing G120 in linker			

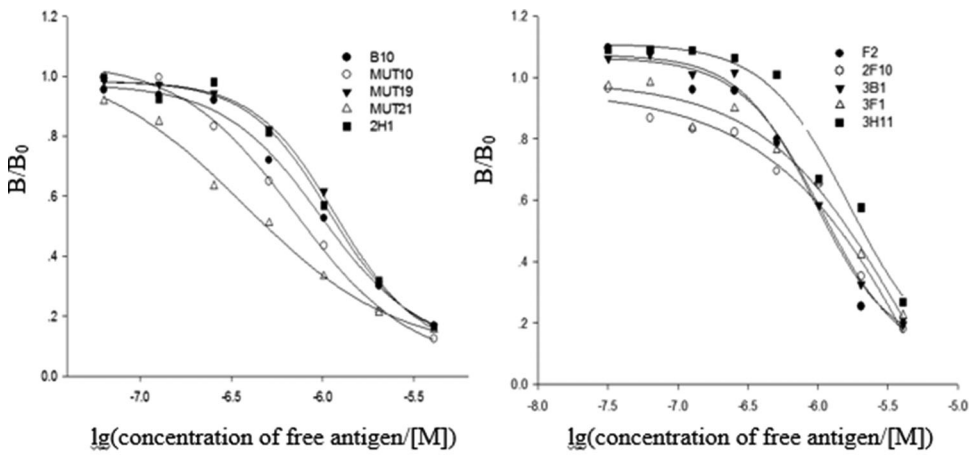
^aIn grey is shown the amino acid positions that were mutated during chain shuffling.

MUT21, 2F10, 3F1, 3H11 and 3B1) have one to five different amino acids with their parental clones, which probably arose by a somatic mutation from the same initial B-cell clones. The heavy-chain-shuffled clone (2H1) has 11 different amino acids in CDR regions, which may have derived from a different germline family.

Affinity determination

Affinity is an essential determinant of antibody function. The affinity of mutants could be rapidly ranked by a competitive phage ELISA even when the concentrations of scFvs are unknown (Clackson & Lowman, 2004). The ratios of the antigen concentration that required to inhibit 50% maximal binding (IC_{50}) of parental clones to mutants obtained from competitive phage ELISA were a close approximation to the ratio of affinities; it permitted the ranking of the binding activities of mutants.

IC_{50} and parameters of mutants originated from B10 and F2 are summarized in Figure 2. The IC_{50} of the best mutants (MUT21 from B10 and 3B1 from F2) were



	B10	MUT10	MUT19	MUT21	2H1	F2	2F10	3B1	3F1	3H11
A	0.8823	1.0140	0.8665	0.9785	-0.8772	-0.8385	0.9718	0.8985	1.2340	1.0081
B	-0.2679	-0.3158	-0.2194	-0.4108	0.2224	0.2401	-0.3288	-0.2697	-0.3763	-0.3186
C	-6.0105	-6.1512	-5.9677	-6.4239	-5.9617	-5.9734	-5.7398	-6.0203	-5.6015	-5.7614
D	0.0919	0.0365	0.1166	0.0776	0.1066	0.9378	-0.0687	0.1016	-0.2096	0.0214
$IC_{50}(\mu M)$	0.9761	0.7060	1.0772	0.3768	1.0922	1.0632	1.8205	0.9543	2.5000	1.7300

where *A* is the high asymptote, *B* is the slope, *C* is the antigen concentration that was required to inhibit 50% maximal binding (IC_{50}), and *D* is the low asymptote.

Figure 2. Determination of the apparent affinity of mutants and parental clones by competitive phage ELISA ($n = 3$). B/B_0 stands for the ratio ELISA mean absorbance values of antibody-binding response in the presence of free antigen to the absence of antigen. Various concentrations of free antigen inhibited the binding of phage antibodies with the coating antigen, the signal was detected by using HRP-conjugated anti-M13 monoclonal antibodies. The calibration curves were constructed by using a four-parameter equation by Sigmaplot 12.0.

0.3768 and 0.9543 μM . The ratios of $\text{IC}_{50(\text{B10})}/\text{IC}_{50(\text{MUT21})}$ and $\text{IC}_{50(\text{F2})}/\text{IC}_{50(\text{3B1})}$ were 2.59 and 1.11, MUT21 showed a moderately improved affinity by competitive phage ELISA. The apparent affinity is defined as the reciprocal of the concentration required to IC_{50} in a competitive ELISA. Hence, the apparent affinity for the best clone (MUT21) was $2.65 \times 10^6 \text{ M}^{-1}$.

In this study, we do not isolate highly improved clones from chain-shuffled libraries, which may be caused by the low diversity of the VH, VL genes repertoires from the same non-immunized library. The total diversity of Tomlinson I library is 1.47×10^8 . If the diversity of light chain and heavy chain was equal, the estimated diversity of light chain or heavy should have been around 1.21×10^4 members. This indicated that the diversity of the heavy- and light-chain-shuffled libraries was approximately 2.42×10^4 (two parental clones). This may be not sufficient to isolate highly improved clones.

Binding analysis of anti-idiotypic antibodies to BBMV of *Plutella xylostella*

Plutella xylostella is a target insect of Cry2Aa toxin. If these anti-idiotypic scFvs mimicked the toxin-receptor binding surface of Cry2Aa toxin, it could be used to map the toxin-binding site instead of Cry2Aa toxin. In Figure 3, the binding ability of these anti-idiotypic antibodies to BBMV of *Plutella xylostella* were determined. Generally, F2 and its mutants (2F10, 3B11, 3F1, 3H11) showed weaker binding ability than B10 and its mutants. MUT21 (a mutant of B10) was the strongest binder to BBMV of *Plutella xylostella* larvae by phage ELISA. The discrepant signal of phage antibodies may be due to the fact that they

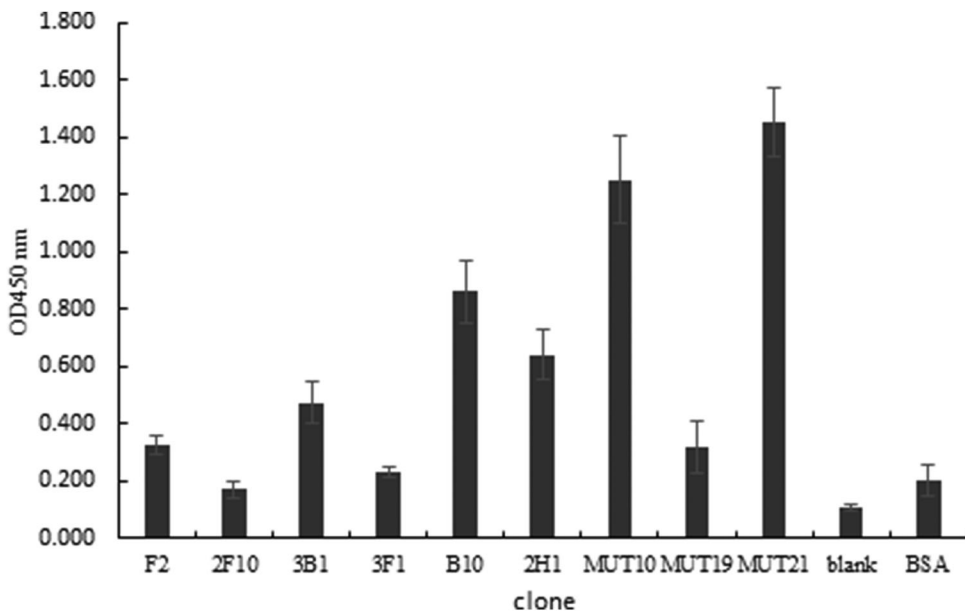


Figure 3. Determination of binding ability of anti-idiotypic phage antibodies with BBMV of *Plutella xylostella* larvae by ELISA ($n=3$). For each clones, 10^6 phages/well were added to ELISA plate coated with BBMV and detected by HRP-conjugated anti-M13 monoclonal antibodies. Blank is the mean signal of wells which had been added PBS instead of phage antibodies. BSA is an irrelevant phage antibody from Tomlinson (I + J) library which recognizes BSA.

mimicked the toxin epitopes, which have different distance or affinity to the binding site. The specific characters and application of these anti-idiotypic scFvs are under investigation by our group and will be discussed in the future publications.

Conclusion

The present work indicates that the chain shuffling could rapidly generate panels of antibodies with similar binding abilities but of distinct idiotypic compositions. Moreover, mutants with moderately improved affinities may also be obtained by the chain shuffling, even with the same naïve library where the antibody was originally isolated. The serials of mutants can be used to map the binding epitopes of the receptor in target insects instead of the Cry2Aa toxin or to be used as a cost-efficient surrogate molecule instead of the toxin in ELISA kit for toxin detection.

Disclosure statement

No potential conflict of interest was reported by the authors.

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